AUTOCRINE GROWTH CONTROL IN HUMAN CARCINOMA CELLS *IN VITRO*

A thesis submitted for the degree of Ph.D.

by

Bernard F. Gregory B.Sc. Hons.

The experimental work described in this thesis was carried out under the supervision of Professor Martin Clynes Ph.D. at the,

National Cell and Tissue Culture Centre,
School of Biological Sciences,
Dublin City University,
Glasnevin, Dublin 9,
Republic of Ireland.

ABSTRACT

This project examined methodologies used to establish primary cell cultures from non-small cell lung carcinoma (NSCLC) tumours. The objective was to enhance the *in vitro* proliferative potential of these clinically important tumours, particularly the squamous cell carcinoma (SCC) subtype. Existing techniques were successfully employed and refined here, permitting short-term maintenance *in vitro* and resulting in the establishment of a novel lung SCC cell line, DLRP, which was charaterised herein. The ability to generate long-term NSCLC cultures and cell lines on a routine basis however, remained elusive. The apparent defecit of relevant growth factors in currently available media for NSCLC was addressed. To this end, two likely sources of growth stimulators for malignant epithelial cells were explored.

The first approach pursued literature reports of growth-promoting activites in the malignant effusions (MEs) and preliminary evidence in this project detected the presence in these fluids, of both stimulatory and inhibitory activity for NSCLC growth (including DLRP) *in vitro*. However, the active stimulatory species did not appear to be tumour-type specific and was conceivably a serum-derived systemic species. The second option evolved from the first, with evidence for the production of 'self-stimulating' autocrine growth factors by the human epidermoid carcinoma cell line, HEp-2.

A bioassay was developed using HEp-2, which detected the presence of growth stimulators in MEs and HEp-2 conditioned medium (CM), based on their ability to stimulate the colony growth in monolayer of low density HEp-2 cells. Two separate fractions of HEp-2 CM, prepared by ultrafiltration (UF), i.e a 10-30 kDa and a >30 kDa fraction, both exhibited autocrine stimulatory activity (ASA). These fractions were distinguished by differential sensitivities to proteinase treatment. Production of several growth factors by HEp-2 is indicated by ASA elution profiles from gel-filtration, hydrophobic interaction, and heparin affinity chromatographic separations. Both heparin-binding and non-heparin-binding ASA are present in HEp-2 CM, but the heterologus elution profile of the heparin-binding ASA indicates that more than one high affinity heparin-binding mitogen is involved. Neutralising antibodies suggest that at least 60% of the ASA in the 10-30 MW fraction is attributable to a bFGF-like factor, and a similar species accounts for 40% of the >30 kDa activity. Antisense studies designed to inhibit bFGF gene expression in HEp-2 cells did not support these findings. The possibility remains that one or more of the less well characterised heparin-binding growth factors are involved in HEp-2 autocrine growth control.

I declare that the work described in this dissertation is entirely my own work, and has not been previously submitted for a degree at this, or at any other university.

Bernard F. Gregory

This thesis is dedicated to my parents

Elizabeth Sydney and Gerald Michael

TABLE OF CONTENTS

		Page
1.0	**INTRODUCTION**	**1**
1.1	**GROWTH CONTROL IN MULTICELLULAR ORGANISMS**	**1**
1.1.1	**Growth factor receptors and signal transduction**	**2**
1.1.2	**The role of oncogenes in aberrant growth control**	**4**
1.2	**AUTOCRINE GROWTH CONTROL**	**8**
1.3	**THE BIOLOGY OF POLYPEPTIDE GROWTH FACTORS**	**9**
1.3.1	**Epidermal growth factor-like polypeptides**	**9**
1.3.1.1	Epidermal growth factor	**10**
1.3.1.2	Transforming growth factor α	**10**
1.3.1.3	Heparin-binding epidermal growth factor	**10**
1.3.1.4	Amphiregulin	**11**
1.3.2	**Transforming growth factor β family of polypeptides**	**11**
1.3.3	**Platelet-derived growth factor family**	**13**
1.3.3.1	Vascular endothelial growth factor (VEGF)	**13**
1.3.4	**Insulin-like growth factors**	**15**
1.3.5	**Fibroblast growth factors**	**17**
1.3.5.1	Acidic and Basic FGF (FGF-1 and FGF-2)	**18**
1.3.5.2	Int-2 (FGF-3)	**19**
1.3.5.3	K-fgf/hst (FGF-4)	**19**
1.3.5.4	FGF-5	**20**
1.3.5.5	FGF-6	**20**
1.3.5.6	Keratinocyte growth factor (KGF/FGF-7)	**20**
1.3.5.7	FGF-8	**20**
1.3.5.8	FGF-9	**21**
1.3.5.9	FGF receptors	**21**
1.3.6	**Pleiotrophin**	**22**
1.3.7	**Interleukins**	**23**
1.3.7.1	Interleukin 1	**23**
1.3.7.2	Interleukin 3	**24**
1.3.7.3	Interleukin 4	**24**
1.3.7.4	Interleukin 6	**24**

Page

1.3.8 Hematopoietic growth factors 25
1.3.8.1 Colony-stimulating factors 25
1.3.8.2 Leukemia inhibitory factor and Oncostatin M 26

1.3.9 Chemokines 27
1.3.9.1 GROα/Melanoma growth-stimulating activity 28

1.4 MOTOGENIC CYTOKINES 28

1.4.1 Hepatocyte growth factor/Scatter factor 28

1.4.2 Autocrine motility factor 29

1.4.3 Migration stimulating factor 29

1.5 AUTOCRINE GROWTH FACTOR MECHANISMS 30

1.5.1 Platelet-derived growth factor as an autocrine growth regulator 31

1.5.2 Autocrine growth control with members of the epidermal growth factor family 32

1.5.3 Autocrine growth control by FGFs 35

1.5.4 Involvement of insulin-like growth factors in autocrine growth control 39

1.5.5 Interleukins and hematopoietic growth factors as autocrine growth regulators in hematopoietic neoplasias 41
1.5.5.1 Interleukin 3 in hematopoietic neoplasias 41
1.5.5.2 Interleukin 6 in hematopoietic neoplasias 41

1.5.6 Cytokines as autocrine growth regulators in nonhematopoietic neoplasias 42
1.5.6.1 Interleukin 6 in nonhematopoietic malignancies 42

1.5.7 Bombesin/GRP as an autocrine growth regulator 44

1.5.8 Autocrine motility factor as an autocrine growth factor 44

Page

1.6 PRIMARY CELL CULTURE OF LUNG CARCINOMAS 45

1.6.1 **Proliferation and differentiation of NSCLC tumour cells *in vitro*** 46

1.6.2 **The role of stromal cells in the growth of NSCLC cells *in vivo* and *in vitro* — paracrine interactions** 47

1.6.3 **Selective media for NSCLC** 53

1.7 THE PURPOSE OF THIS THESIS 56

1.7.1 **The use of malignant effusions in primary cell culture** 56

1.7.2 **The potential of the HEp-2 cell system as a source of growth factors for human lung squamous epithelial cells *in vitro*** 57

2.0 MATERIALS AND METHODS

2.1 PREPARATION OF CELL CULTURE MEDIA 60

2.1.1 Water quality 60
2.1.2 Glassware treatment 60
2.1.3 Sterilisation 60
2.1.4 Siliconisation 60
2.1.5 Basal growth media 61
2.1.5.1 Preparation of media from powder 61
2.1.5.2 Preparation of media from liquid concentrates 61
2.1.6 Supplements to basal media 62
2.1.7 Serum 62
2.1.8 Sterility checks 63
2.1.9 Preparation of growth media 63

2.2 ROUTINE MAINTENANCE OF ESTABILISHED CELL LINES 64

2.2.1 Cell lines used in this work 64
2.2.2 *In vitro* culture systems 65
2.2.3 Feeding cultures 65
2.2.4 Subculturing 66
2.2.5 Counting cell suspensions 67
2.2.5.1 Determination of viable cell numbers 67

		Page
2.3	**LONG TERM STORAGE OF CELL LINES**	**68**
2.3.1	Cell Freezing	**68**
2.3.2	Cell thawing	**68**
2.4	**CONTAMINATION CONTROL**	**69**
2.4.1	Microbial	**69**
2.4.1.1	Use of antibiotics	**69**
2.4.1.2	Anti-fungal agents	**69**
2.4.2	Mycoplasma	**70**
2.4.2.1	Detection of mycoplasma	**70**
2.4.2.2	Elimination of mycoplasma	**71**
2.4.3	Cross-contamination by other cell lines	**72**
2.4.3.1	Procedures employed to avoid cross-cell line contamination	**73**
2.5	**PRIMARY CELL CULTURE**	**74**
2.5.1	Collection of tumour tissue samples and transportation medium	**74**
2.5.2	Dissection of tumour tissue	**74**
2.5.3	Mechanical disaggregation	**74**
2.5.4	Explant technique	**74**
2.5.5	Enzymatic disaggregation	**75**
2.5.6	Ficoll density gradient	**75**
2.5.7	Harvesting cells from malignant effusions	**75**
2.5.8	Fibroblast elimination	**75**
2.5.8.1	Selective trypsinisation of fibroblasts and epithelial cells	**75**
2.5.8.2	Selective toxicity of fibroblasts to G418 sulphate	**76**
2.5.9	3T3 feeder layers	**76**
2.5.10	Collagen coating of tissue culture flasks	**76**
2.6	**CELL LINE CHARACTERISATION TECHNIQUES**	**77**
2.6.1	Immunohistochemical identification	**77**
2.6.1.1	Preparation of slides	**77**
2.6.1.2	Surface epithelium specific antibody	**77**
2.6.1.3	Anti-cytokeratin antibody	**77**
2.6.1.4	Non-small cell lung carcinoma specific antibody	**78**
2.1.6.5	Visualisation of primary antibody binding pattern with an FITC-labelled second antibody	**78**
2.6.2	Preparation for shipment of cell lines and tumour biopsy samples for DNA fingerprint analysis	**78**

		Page
2.7	**COLLECTION OF CONDITIONED MEDIUM (CM)**	**79**
2.7.1	75 cm^2 flasks	**79**
2.7.2	Roller bottle culture	**79**
2.7.3	Suspension cultures	**79**
2.7.4	Cell factory system for very large scale CM production	**80**
2.8	**PROCESSING Of CM**	**81**
2.8.1	Ultrafiltration	**81**
2.8.1.1	Fractionation of CM by ultrafiltration	**81**
2.8.2	Dialysis	**82**
2.8.3	Lyophilisation	**82**
2.9	**PHYSICOCHEMICAL TREATMENT CM**	**83**
2.9.1	Temperature treatment	**83**
2.9.2	Adjustment of CM pH	**83**
2.9.3	Measurement of Osmolarity	**83**
2.9.4	Exposure to proteinase	**83**
2.9.5	Addition of heparin	**84**
2.10	**PREPARATION OF CELL EXTRACTS**	**84**
2.10.1	Extraction buffers	**84**
2.10.2	Cell harvesting and extraction	**84**
2.11	**PURIFICATION PROTOCOLS**	**85**
2.11.1	Gel filtration chromatography	**85**
2.11.1.1	Desalting of chromatography samples/equilibration in running buffer	**85**
2.11.2	Heparin-Sepharose chromatography	**86**
2.11.2.1	Batch elution	**86**
2.11.2.2	Gradient elution	**86**
2.11.3	Cation-exchange chromatography	**87**
2.11.4	Hydrophobic interaction chromatography	**87**
2.11.5	Ammonium sulphate fractionation of biological fluid	**88**
2.11.6	BioRad protein assay	**88**

		Page
2.12	**BIOASSAYS**	**89**
2.12.1	Pretreatment of cells	**89**
2.12.2	Preparation of cell suspensions	**89**
2.12.3	Soft agar assays	**90**
2.12.3.1	Colony counting in soft agar	**90**
2.12.3.2	Transforming growth factor α-like assay	**91**
2.12.3.3	Clonogenic assay	**92**
2.12.4	Monolayer assays	**93**
2.12.4.1	Measurement of colony area and number by image analysis	**93**
2.12.4.2	HEp-2 autocrine assay in 24-well plates	**93**
2.12.4.3	Monolayer assays for human lung squamous cell carcinoma cell lines	**94**
2.12.5	Acid phosphatase measurement	**95**
2.12.5.1	HEp-2 autocrine assay in 96-well plates	**95**
2.12.6	^{125}I-EGF radioreceptor assay	**96**
2.13	**PURE GROWTH FACTORS**	**97**
2.13.1	Source	**97**
2.13.2	Preparation	**97**
2.14	**NEUTRALISING ANTIBODY EXPERIMENTS**	**99**
2.14.1	Source and specifications of anti-FGF neutralising antibodies	**99**
2.14.2	Preparation of antibodies	**100**
2.14.3	Preincubation of antibodies with CM and pure growth factors	**100**
2.15	**ANTISENSE OLIGODEOXYNUCLEOTIDES**	**101**
2.15.1	Source	**101**
2.15.2	Preparation	**101**
2.15.3	Spectrophotometric quantification of oligodeoxynucleotides	**101**
2.15.4	Calibration of multichannel micropipettes	**101**
2.15.5	Protocol for antisense experiments with HEp-2	**101**

Page

3.0 RESULTS

3.1 PRIMARY CULTURE OF HUMAN THORASIC TUMOUR TISSUE 103

3.1.1 Source and composition of tissue samples 103

3.1.2 Tissue processing techniques 104
3.1.2.1 Mechanical disaggregation of tissue samples 104
3.1.2.2 Enzymatic disaggregation of tissue samples 105
3.1.2.3 Malignant effusion as a source of epithelial cells for primary culture 105

3.1.3 Removal of blood cells 105

3.1.4 *In vitro* growth of tumour samples 108

3.1.5 Control of fibroblast proliferation 110
3.1.5.1 Differential trypsinisation of epithelial and fibroblast cell types 111
3.1.5.2 Differential toxicity of fibroblasts and epithelial cells to geneticin 114

3.1.6 Establishment of a continuous human lung squamous cell carcinoma cell line - DLRP 115
3.1.6.1 Clinical History of DLRP 115
3.1.6.2 Elimination of mycoplasma contamination in the cell line DLRP 117
3.1.6.3 Measurement of epidermal growth factor-like activity in medium conditioned by the cell line DLRP 119
3.1.6.4 Comparison of the percentage colony forming efficiencies of DLRP and another poorly differentiated human lung squamous cell carcinoma cell line DLKP in soft agar 121
3.1.6.5 Determination of serum batch preference for the cell lines DLRP, DLKP and HEp-2 123

3.1.7 The effect of purified growth factors on the proliferation of DLRP and DLKP 125
3.1.7.1 The effect of a selection of growth factors on DLRP proliferation 125
3.1.7.2 The proliferative response of DLKP to a selection of growth factors 128

3.1.8 Evidence for autocrine growth factor production by DLRP and DLKP 130
3.1.8.1 Preliminary autocrine assay development for DLRP 130
3.1.8.2 Preliminary autocrine assay development for DLKP 131

Page

3.1.9 Evidence for a species in malignant effusions with growth-promoting activity on human lung squamous cell carcinomas 132
3.1.9.1 The effect of ascites fluid on the growth of DLRP in monolayer **132**
3.1.9.2 The effect of ascites fluid on DLKP growth in monolayer **133**
3.1.9.3 The effect of ascites fluid on the growth in monolayer of SK-MES-1 **134**
3.1.9.4 The effect of ascites fluid on the growth of HEp-2 **135**

3.1.10 HEp-2 as an indicator cell line for the detection of growth-promoting activity in malignant effusions derived from carcinomas of different histological type 136
3.1.10.1 Ammonium sulphate fractionation of an ovarian malignant effusion **140**
3.1.10.2 Growth stimulatory acitivity in malignant effusions compared to that in serum and plasma from autologus donors **142**

3.1.11 Establishment of a continuous human breast adenocarcinoma cell line - BAC 144
3.1.11.1 Clinical History of BAC **144**

3.1.12 Characterisation of new human carcinoma cell lines 147
3.1.12.1 DNA fingerprint analysis of the cell lines DLRP and BAC **147**
3.1.12.2 Histopathology **151**
3.1.12.3 Immunohistochemical characterisation of the cell lines DLRP and BAC **152**

3.2 DEVELOPMENT OF THE HEp-2 AUTOCRINE GROWTH FACTOR ASSAY 164

3.2.1. Preliminary determination of cell plating density for HEp-2 in 24-well plates 164
3.2.1.1 Preliminary evidence for autocrine stimulation in the HEp-2 system **166**
3.2.1.2 Endpoint measurement in the HEp-2 autocrine assay: comparison of colony area and colony number **168**
3.2.1.3 Choice of basal medium type for the HEp-2 autocrine assay **171**
3.2.1.4 Growth of HEp-2 cells during conditioned medium production **172**
3.2.1.5 Reproducibility of the autocrine effect in HEp-2 CM **175**

3.2.2 Use of acid phosphatase as an endpoint for the measurement of autocrine growth stimulation in HEp-2 cells 176

		Page
3.2.3	**Ultrafiltration of HEp-2 CM**	**179**
3.2.3.1	Concentration of CM by ultrafiltration	**179**
3.2.3.2	Size fractionation of HEp-2 CM using ultrafiltration	**180**
3.2.3.3	Determinataion of autocrine activity in relation to protein concentration in CM fractioned and concentrated by ultrafiltration	**189**
3.2.3.4	Measurement of autocrine activity in the R10-30 fraction of HEp-2 CM using acid phosphatase as an endpoint	**191**
3.2.4	**Physicochemical stability properties of the HEp-2 autocrine activity**	**192**
3.2.4.1	pH stability	**192**
3.2.4.2	The effect of heparin on the pH stability of HEp-2 autocrine activity	**197**
3.2.4.3	Proteinase sensitivity of the HEp-2 autocrine activity	**199**
3.2.4.4	Comparative sensitivities to trypsin of autocrine activity in early versus late collections of HEp-2 CM	**204**
3.2.4.5	Heat stability of the HEp-2 autocrine activity	**205**
3.2.4.6	A comparison of the heat stability of autocrine activities in crude CM with that in the R10-30 and R30 ultrafiltration fractions	**209**
3.2.4.7	Trypsin sensitivity of heat resistant autocrine activity in early collection crude CM	**211**
3.2.5	**The effect of heparin on cell growth in the HEp-2 assay**	**213**
3.2.6	**Pure growth factors in the HEp-2 assay**	**214**
3.2.6.1	Epidermal growth factor and transforming growth factor α	**214**
3.2.6.2	Transforming growth factor β	**217**
3.2.6.3	Insulin and Insulin-like growth factors I & II	**218**
3.2.6.4	Acidic and Basic fibroblast growth factors	**219**
3.2.6.5	Interleukins 1α and 1β	**220**
3.2.6.6	Platelet-derived growth factor	**221**
3.2.6.7	Bombesin/Gastrin releasing peptide	**222**
3.2.7	**Determination of the toxicity of CHAPS to HEp-2 cells under autocrine assay conditions**	**223**
3.3	**PURIFICATION OF HEp-2 AUTOCRINE ACTIVITY**	
3.3.1	**Separation of concentrated HEp-2 CM on a BioGel P30 column**	**225**
3.3.2	**The use of Heparin-Sepharose affinity chromatography in the purification of HEp-2 autocrine activity**	**228**
3.3.2.1	Preliminary evidence for heparin-binding autocrine activity in concentrated HEp-2 CM: Batch elution in NaCl from Heparin-Sepharose	**228**
3.3.2.2	Separation of autocrine activity in HEp-2 CM by heparin-Sepharose chromatography using a 0-2.5 M continuous NaCl gradient	**229**

Page

3.3.3 Heparin-Sepharose chromatography of HEp-2 cell extracts 233
3.3.3.1 The separation of high salt HEp-2 cell extract on Heparin-Sepharose 233
3.3.3.2 The separation of low salt HEp-2 cell extract on Heparin-Sepharose 238

3.3.4 Cation exchange chromatography 245
3.3.4.1 The use of CM-Sepharose fastflow in the purification of autocrine activity in HEp-2 conditioned medium 245
3.3.4.2 Reconstitution of HEp-2 CM fractions separated by cation exchange chromatography 248

3.3.5 A two step purification of the HEp-2 autocrine activity 250

3.3.6 Hydrophobic interaction chromatography 253

3.4 INHIBITION OF THE HEp-2 AUTOCRINE EFFECT WITH ANTI-FGF NEUTRALISING ANTIBODIES AND ANTISENSE OLIGODEOXYNUCLEOTIDES

3.4.1 Anti-Fibroblast Growth Factor neutralising antibodies 256
3.4.1.1 Preliminary evidence for FGF-neutralisable activity in HEp-2 CM 256
3.4.1.2 Neutralisation of R10-30 autocrine activity with anti-FGF antibodies 259
3.4.1.3 Determination of the ND_{50} values for two commercially available anti-FGF neutralising antibodies in the HEp-2 system 261
3.4.1.4 Neutralisation of autocrine activity in HEp-2 CM with anti-FGF antibodies of known efficacy in the HEp-2 system 263
3.4.1.5 Neutralisation of autocrine activity in HEp-2 CM ultrafiltration fractions R10-30 and R30 with anti-FGF antibodies of known efficacy in the HEp-2 system 265

3.4.2 FGF antisense oligodeoxynucleotides 268
3.4.2.1 Determination of assay parameters for HEp-2 with 1% heat-inactivated serum in the 96-well plate system 269
3.4.2.2 Preliminary evidence for the arrest of HEp-2 proliferation using FGF antisense oligodeoxynucleotides 269

3.5 Measurement of Transforming growth factor α-like activity in HEp-2 CM 272
3.5.1 The NRK soft agar assay for TGFα 272

Page

4.0 DISCUSSION

4.1 PRIMARY CELL CULTURE 274

4.1.1 Cell line characterisation 276
4.1.1.1 DNA fingerprinting **276**
4.1.1.2 Cytogenetic and histological analysis **277**
4.1.1.3 Immunohistochemical analysis **277**
4.1.1.4 Mycoplasma status of new cell lines **278**
4.1.1.5 Clonogenicity in soft agar **279**
4.1.1.6 Growth factor responsiveness of DLRP and DLKP **279**

4.1.2 Negative regulators of NSCLC growth 281
4.1.2.1 Transforming growth factor β **281**
4.1.2.2 Antiproliferative effects of interleukins **281**
4.1.2.3 Growth regulatory peptides for NSCLCs **283**

4.1.3 Absence of positive autocrine/paracrine growth factors in NSCLC cultures *in vitro* 284

4.1.4 Prospects for improving *in vitro* growth performance with NSCLC -derived primary cultures 285

4.1.5 Growth factor species in malignant effusions 287

4.2 THE HEp-2 AUTOCRINE SYSTEM 294

4.2.1 Evidence for autocrine growth factor production by HEp-2 cells 294

4.2.2 Charactersation of the HEp-2 autocrine activity 295

4.2.3 Purification of HEp-2 autocrine activity 301
4.2.3.1 Heparin-Sepharose chromatography **303**
4.2.3.2 Purification of ASA using cation-exchange and heparin-Sepharose chromatography **304**

4.2.4 Inhibition of autocrine activity with anti-FGF antibodies 309

		Page
4.3	**HIGHER MOLECULAR WEIGHT FORMS OF BASIC FGF**	**311**
4.3.1	**Basic FGF binding species**	**312**
4.3.1.1	Miscelaneous binding species for bFGF	**312**
4.3.1.2	A regulatory role for the interaction of FGFs and other growth factors which bind heparan sulphate proteoglycans	**312**
4.3.1.3	Soluble forms of the FGF receptors	**315**
4.3.2	**Secretory mechanisms for the release of basic FGF from cells**	**316**
4.3.3	**Release of basic FGF from extracellular matrix storage sites**	**318**
4.3.4	**The role of basic FGF *in vivo* and *in vitro***	**320**
4.3.5	**Antisense FGFs - evidence for an autocrine role for bFGF in HEp-2**	**321**
4.3.6	**Effect of heparin on HEp-2 proliferation**	**322**
4.3.6.1	Mechanism of action for heparin	**322**
4.3.6.2	Possible differential effects of heparin on the mitogenic response to FGFs	**323**
5.0	**CONCLUSIONS AND FUTURE DIRECTIONS**	**325**
6.0	**ACKNOWLEDGEMENTS**	**328**
7.0	**REFERENCES**	**329**
8.0	**APPENDICES**	**365**

ABBREVIATIONS

A-431	Human cell line from vulval carcinoma
AIGF	Androgen-induced growth factor
AMF	Autocrine motility factor
AR	Amphiregulin
ASA	Autocrine stimulatory activity
ATCC	American Type Culture Collection
BAC	Human breast adenocarcinoma cell line
BSA	Bovine Serum Albumin
CaLu-6	Human lung adenocarcinoma cell line
CHAPS	3-[(3-Cholamidopropyl) dimethyl]-1-propane sulfonate
CM	Conditioned medium
CNTF	Cillary neurotrophic factor
DLKP	Human lung squamous cell carcinoma cell line
DLRP	Human lung squamous cell carcinoma cell line
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
Ep-16	Mouse hybrdioma
Epo	Erythropoietin
FGF	Fibroblast growth factor
GAF	Glia-activating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HB-EGF	Heparin-binding epidermal growth factor
HEPES	(4-[2-HydroxyEthyl]-Piperazine Ethane Sulphonic Acid
HEp-2	Human cell line from epidermoid carcinoma of the larynx
HGF/SF	Hepatocyte growth factor/Scatter factor
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IL-ra	Interleukin receptor antagonist
IL-Rt	Interleukin receptor
K-fgf	Kaposi's fibroblast growth factor
KGF	Keratinocyte growth factor

LIF	Leukemia inhibitory factor
ME	Malignant effusion
MEM	Minimal Essential Medium
MCF-7	Human breast carcinoma cell line
M-CSF	Macrophage colony-stimulating factor
MGSA	Melanoma growth stimulating activity
MIP	Macrophage inflammatory protein
MK	Midkine
MRC-5	Human foetal lung fibroblast cell line
NIH3T3	Mouse embryo fibroblasts
NRK	Normal rat kidney fibroblast cells
NSCLC	Non-small cell lung carcinoma
OLIGO	Oligodeoxynucleotide
OSM	Oncostatin
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PRG	Primary response gene
PTN	Pleiotrophin
rpm	Revolutions per minute
RPMI-1650	Roswell Park Memorial Institute culture medium 1650
RPMI-2650	Human squamous cell carcinoma of the nasal septum
RTK	Receptor tyrosine kinase
S.D	Standard deviation
S.E.M	Standard error of the mean
SCC-9	Human tongue squamous cell carcinoma
SCLC	Small cell lung carcinoma
SGF	Shope fibroma growth factor
SK-LU-1	Human lung squamous cell carcinoma cell line
SK-MES-1	Human lung squmoous cell carcinoma cell line
SSV	Simian sarcoma virus
TGF	Transforming growth factor
TNF	Tumour necrosis factor
U-937	Human monocytic lymphoma cell line
VEGF	Vascular endothelial growth factor
VGF	Vaccinia growth factor
v/v	volume to volume ratio
w/v	weight to volume ratio
703D4	Mouse hybridoma cell line
704A1	Mouse hybridoma cell line

1.0 INTRODUCTION

1.1 GROWTH CONTROL IN MULTICELLULAR ORGANISMS

Cell proliferation and differentiation are two highly coordinated mechanisms underlying the growth and development of multicellular organisms. The study of these fundamental processes at all stages of growth from embryo to adult, has revealed that the fate of an organism relies not merely upon its inherent genetic program, but also upon the outcome of its interaction with that organism's physical environment. This concept applies equally at the molecular level for all facets of growth from conception, through embryogenesis, to tissue maintenance and repair in the adult. Physiochemical parameters which influence developmental outcome include extracellular matrix components, membrane junctions between apposing cells, cell adhesion molecules, and also nutritional factors.

That these processes are highly ordered and controlled, is best exemplified by the observation that specific tissues are continuously replenished in the adult. In adult vertebrate tissues, such as the skin, intestine, and hematopoietic system, mature or differentiated cells are being replaced daily from a limited number of multipotent stem cells that were laid down during embryogenesis. In normal growth programs, these stem cells are expanded in number by clonal proliferation to produce progenitor cells. Such progenitor cells, already restricted by their commitment to a particular differentiation process, proliferate further in their respective lineages before they cease to divide, and begin to express the differentiated phenotype characteristic of that lineage. In this way cells lost through natural processes or through tissue injury are replaced.

In all of these tissues, a balance must be maintained between the renewal of stem cells, and the commitment to differentiation needed to maintain tissue integrity. Key players in this complex hierarchy of control processes are a diverse group of soluble factors produced by cells, called polypeptide growth factors. These molecules have been found to exert both positive and negative control over cell proliferation and differentiation. Initially most growth factors were named after the biological activity that lead to their isolation. However, later work revealed that many of these factors are multifunctional {Sporn & Roberts (1988)}. It was subsequently discovered that growth factors affect not only cell proliferation and differentiation during embryogenesis and wound repair, but they also mediate chemotactic responses seen during inflammation and are involved in modulating the immune response.

Factors originally identified as growth regulatory molecules for hematopoietic (blood) cells have

been called 'cytokines' but it has become increasingly clear in recent years that the action of cytokines is not restricted to specific cell lineages, or to stage-specific points during differentiation in hematopoietic cells, as initially thought. The terms 'polypeptide growth factor' and 'cytokine' therefore are used interchangeably in this thesis.

Polypeptide growth factors are distinguished from hormones in that growth factors are produced and act locally in tissues, while hormones are systemic endocrine products, active mainly as metabolic regulators. The actions of polypeptide growth factors are effected through binding to specific transmembrane receptors on the plasma membrane of target cells. This interaction triggers a cascade of intracellular biochemical pathways which ultimately translates the signal to the cell nucleus.

1.1.1 GROWTH FACTOR RECEPTORS AND SIGNAL TRANSDUCTION

Several classes of transmembrane growth factor receptors have been identified. The best understood are receptors with tyrosine kinase activities. These contain an extracellular domain where the ligand binds, followed by a hydrophobic transmembrane region, and then a cytoplasmic domain which contains a tyrosine kinase activity. This kinase activity distinguishes this class of receptor from others, and many of the known growth factors including the FGFs, the EGF family, and PDGF, all transduce their signals by binding to transmembrane receptors tyrosine kinases RTK(s).

Dimerisation of RTKs is associated with ligand binding and accompanies the activation of the tyrosine kinase activity {Ullrich *et al.*, (1990)}. This leads to molecular interactions between adjacent cytoplasmic receptor domains, and results in the activation of kinase functions in these receptors. Autophsophorylation of the occupied receptors is often the first observation of kinase activity. However, the phosphorylation of the receptor itself modulates its ability to interact with other substrates in the signal transduction pathway {Kock *et al.*, (1991)}.

Many of the intracellular events elicited after binding of growth factors to their receptors involve protein phosphorylataion, and some of the substrates for RTKs are themselves kinases. These include those which phosphorylate on serine and threonine {Morrison *et al.*, (1988)}, as well as tyrosine residues {Ralston & Bishop (1985)}. Phosphorylation therefore, appears to be one of the major biochemical mechanisms involved in the transmission and modulation of the mitogenic signal for many growth factors.

Another second messenger event is the breakdown of the inositol lipid, phosphatidylinositol bisphosphate. This produces diacylglycerol (DAG) and inositol triphosphate (IP_3). The former activates a kinase called protein kinase C (PKC) {Nishizuka (1988)}, and the latter mobilises intracellular Ca^{2+} concentrations {Berridge & Irvine (1984)}. A 21 kDa protein called Ras is also activated following ligand binding in some receptor complexes. Ras is phosphorylated by a guanosine triphosphatase (GTPase) which is a receptor-associated kinase {McCormick (1989)}. The activation of the Na^{+}/H^{+} antiport also follows receptor activation and the resulting ion fluxes across the plasma membrane lead to cytoplasmic alkalinisation.

The biochemical events linking the primary substrates of growth factor receptors to the changes in the nucleus are only beginning to be defined. However, transient changes in the expression of a set of genes called 'primary response genes' (PRGs), or 'cellular immediate early genes', follow the cascade of signals induced by ligand binding. These PRGs appear to translate the growth factor signal into subsequent 'secondary' changes in gene expression. A common feature of the transcriptional induction of PRGs is that *de novo* protein synthesis is not required {reviewed by McMahon & Monroe (1992); Herschman (1991)}. The proteins encoded by PRGs include structural proteins such as actin and tropomyosin, but an important role in the proliferative response is played by a subset of PRGs called transcription factors. The critical role played by transcription factors such as c-*fos*, c-*jun* and c-*myc*, is related to their ability to activate and/or repress the expression of the specific expression of 'secondary genes'.

Non-tyrosine kinase receptors

A separate class of growth factor receptors do not signal through tyrosine kinase, and most of the hematopoietic growth factor receptors cloned to date fall into this category. These include erythropoietin, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), as well as interleukins 2, 3, 4, 5, 6, and 7 {Nicola & Metcalf (1991)}. Sub-families within this large group of receptor types have been characterised {for review see Taga & Kishimoto (1992)}. A distinguishing feature for many of the hematopoietic growth factor (cytokine) receptors is that in addition to the specific ligand-binding receptor, auxilary components are required to generate functional high affinity complexes capable of signal transduction. These so-called 'transducer' proteins are frequently shared by different ligand-binding receptor molecules, and this mechanism provides a basis for understanding the pleiotrophic and redundant effects exhibited by cytokines.

Another feature which characterises these receptors are their relatively large extracellular domains and short cytoplasmic domains. The spacing of cysteine residues in the extracellular domains of some hematopoietic growth factor/cytokine receptors defines a sub-group with immunoglobulin-like structure. In addition, the post-receptor binding events associated with hematopoietic growth factor/cytokine receptor signalling are not yet well understood. However, some of the sub-families are known to possess a serine/threonine kinase domain (e.g. type II TGF-β receptor). Others (e.g IL-3, IL-6) have no associated enzymatic activities, but preliminary studies with these indicate that they may interact with intracellular tyrosine kinases as a first step in generating their signal {Taga & Kishimoto (1992)}.

Another distinct subfamily of cytokine receptors (e.g. IL-8-R) belong to a larger group of guanine nucleotide-binding protein (G-protein)-coupled receptors. This superfamily also includes the receptor for bombesin/GRP, and are characterised by seven hydrophobic membrane-spanning domains. The three subunit G-proteins associated with these 'seven-pass' receptors couple ligand-binding to the effectors adenyl cyclase and phospholipase C {Spiegel (1992)}.

1.1.2 THE ROLE OF ONCOGENES IN ABERRANT GROWTH CONTROL

The basis of a mechanistic understanding of the control of cell growth and differentiation has been provided by the integration of previously distinct fields of research. These include the study of the biology of growth factors and their receptors, viral oncogenes, and the role of steroids and retinoids.

It was found that several retroviral oncogene products had protein kinase activity which functioned to phosphorylate tyrosine residues, as opposed to the more common serine and threonine protein kinases identified previously. The study of abnormal cell growth, in the carcinogenic process leading to malignancy, identified growth factors as the mediators of cell growth and differentiation. Purification and sequencing of receptors for these growth factors, such as the epidermal growth factor receptor (EGF-R), demonstrated that similar tyrosine kinase activities resided in the cytoplasmic domains of these receptor proteins. It was also found that activation of kinase activity, as well as autophosphorylation of the receptors, followed ligand binding. Viral oncogene products were found to encode aberrant growth factor receptors, such as the truncated EGF-R encoded by the v-*erb* B oncogene {Downward *et al.*, (1984)}. A connection between oncogenes and growth factors had also been established by the finding that the B-chain of the dimeric platelet-derived growth factor had significant sequence holomogy

with the predicted product of the transforming v-*sis* oncogene that was expressed in cells 'transformed' by the simian sarcoma virus {Doolittle *et al.*, (1983)}.

The study of oncogenes has demonstrated the potential of normal cellular genes to become deregulated. Alterations in the DNA coding sequences such as point mutations, or more gross aberrations affecting the regulatory sequences, can lead to the expression of dysfunctional products which have lost important regulatory constraints on their acitivty. Apart from point mutations and proviral insertions, other less subtle lesions in chromosomal DNA can lead to the activation or loss of specific gene expression. These include, gene amplifications, and alterations in chromosome structure such as deletions, and the translocation of entire chromosome segments. Normal genes with critical roles in the control of cell growth, proliferation and differentiation, which have the potential to become deregulated, are called proto-oncogenes. When the expression of these genes produces protein products with sufficiently impaired regulatory constraints to result in deregulated cell growth, they are called oncogenes, and their products are referred to as 'oncoproteins'.

A growing number of 'altered' versions of cellular genes have been identified and designated as oncogenes. These appear to be important lesions in the carcinogenic process. Investigations into the mechanisms of malignant transformation have implicated growth factors, growth inhibitors, and their receptors, as proto-oncogenes {Heldin & Westermark (1984)}. Other classes include the genes for proteins involved in growth factor-induced signal cascades, and also the nuclear transcription factors {Rollins & Stiles (1988)}.

Studies in mouse cells have shown that the signal for cells to divide is coordinated by two sets of complementary growth factors. 'Competence factors' induce resting or quiescent cells in the G_0 phase of the cell cycle to enter the G_1 phase. Sustained action of competence factors for several hours is needed, if stimulated cells are not to revert to the resting state before a second group of factors, called 'progression factors', can induce their transition through the G_1 phase, and ensure commitment to DNA synthesis. There exists a critical period at the end of G_1 when the simultaneous action of both sets of factors is required, but thereafter only the action of progression factors is needed to enable cells to complete the cell cycle. Examples of competence factors include, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). Insulin and insulin-like growth factor-I (IGF-I) are examples of progression factors. The ubiquitous polypeptide growth regulator, TGF-β, appears

to exert its inhibitory effects in the G_1 phase {Aaronson (1991)}. Some oncogenes appear to be capable of substituting for competence factors in the cell cycle if their expression becomes activated. Such oncogenes are classified as 'dominant' oncogenes. A schematic representation of the coordinated actions of competance and progression factors in the cell cycle is depicted in figure 1.1.1.

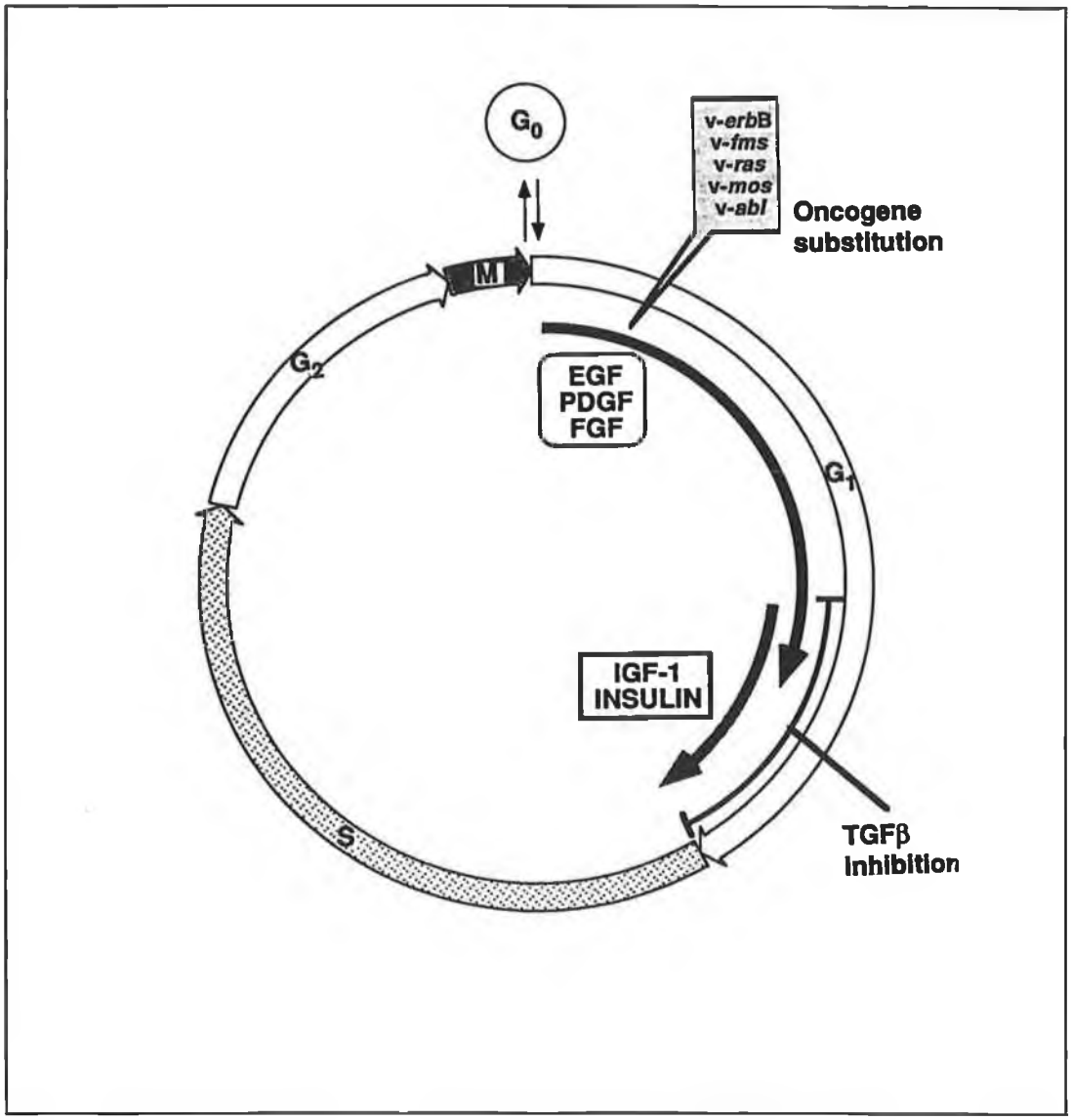


Figure 1.1.1 A schematic representation of the requirements for growth factors during the cell cycle of mouse keratinocyte cells (taken from Aaroson, 1991).

In the context of aberrant expression of proto-oncogenes a number of mechanisms might prevail to contribute to uncontrolled cell proliferation. The inappropriate production and/or overexpression of a growth factor, in cells already expressing the cognate receptors, or conversely, the acquisition of a functional receptor for which cells already expresses the ligand, may shift the balance of growth control in favour of proliferation. Another mechanism whereby cells escape the normal restrictions on their growth, is through loss of effective negative regulation. The loss of functional negative regulation may arise due to the inability of cells to express functional inhibitor molecules and/or their receptors. A similar dysfunction in negative regulation may arise if an inhibitor is not efficiently released or activated by cells, or if a defect exists in the post-receptor signalling pathway.

Studies using cell fusions between normal and immortalised cell lines have concluded that cellular senescence is a dominant trait, and that cell immortalisation is a consequence of one or more gene deletions. It has been hypothesised that senescence in normal cells is accompanied by the production of a protein inhibitor which blocks the entry of cells into the S phase of the cell cycle. The cellular response to this negative regulator makes the cell insensitive to mitogenic stimuli. Immortalisation of normal cells might occur, as outlined above, by the effective loss of function of such an inhibitor. This can be the consequence of an inability to produce an effective protein, or failure to respond to it {Periera-Smith & Smith (1988)}. The existence of normal genes called 'tumour suppressor genes', which function to supress tumourigenicity, is now well established, and the failure to induce cellular senescence therefore, is one mechanism by which tumour suppressor genes can contribute to tumourigenesis {O'Brien *et al.*, (1986)}.

The nuclear phosphoprotein, p53, is just one exanple of a tumour suppressor gene. Its normal function is to block the entry of cells at the G_1/S boundary {Levine *et al.*, (1991)}. Normal p53 functions are lost by a variety of genetic mechanisms including allelic loss, chromosomal rearrangements and deletions. However, point mutations are the most commonly observed genetic change associated with this gene, and are a frequent event in many types of human cancer {Hollstein *et al.*, (1991)}.

Tumourigenesis requires the concerted action of a number of molecular aberrations in cellular growth control mechanisms before cells can progress from being merely hyperproliferative, to acquiring a malignant phenotype — characterised by the ability to invade surrounding tissues,

enter the blood stream, and form colonies in distant organs. The spontaneous activation of mitogenic signalling pathways, due to inappropriate growth factor production, is now definitively established as one of the critical steps in this process. One mechanism of growth factor control which is prone to perturbation is the production of autocrine growth factors {Aaronson (1991)}.

1.2 AUTOCRINE GROWTH CONTROL

The concept of autocrine growth control was first proposed by Sporn & Todaro (1980) in the context of malignant transformation. It was postulated as a mechanism to explain how tumour cells acquired growth autonomy. In this scenario, it was proposed that tumour cells could stimulate their own proliferation by secreting a growth factor(s) for which the same cells concurrently expressed the cognate receptor(s). Growth factors produced by one cell type, but active on another, also in the immediate microenvironment, are called 'paracrine' growth factors. The original autocrine hypothesis was broadened by Sporn & Roberts (1985) to include the regulation of normal cell growth, and to account for the loss cell sensitivity to negative regulatory signals. This loss of sensitivity to negative growth regulators may be a consequence of the loss of receptor function or the inability of cells to produce and/or activate negative growth factors.

In the introduction to this dissertation I present a brief overview of the biological characteristics of polypeptide growth factors from the major classes identified to date. Evidence is also presented for the involvment of particular polypeptide growth factors in the formation of autocrine loops, and the contribution that such growth modulatory pathways may have in the genesis of malignant cells. In particular, the potential contribution of autocrine mechanisms to the development and progression of non-small cell lung carcinomas *in vivo*, and their maintenance as *in vitro* cultures, is also addressed.

1.3 THE BIOLOGY OF POLYPEPTIDE GROWTH FACTORS

1.3.1 EPIDERMAL GROWTH FACTOR-LIKE POLYPEPTIDES

The best characterised members of this family of growth factors are epidermal growth factor (EGF), and transforming growth factor α (TGFα). Both growth factors are homologous monomeric polypeptides, and like all of the members in this family, they bind to the same high affinity receptor — the epidermal growth factor receptor (EGF-R). This is a single-chain 175 kDa transmembrane glycoprotein. The ligand-binding domain is located on the 621 amino acid (aa) extracellular segment, and is separated by a 23 aa hydrophobic membrane-spanning region from the 524 aa intracelllular domain which incorporates a tyrosine kinase activity {Ullrich *et al.*, (1984)}.

EGF and TGFα do not have an affinity for immobilised heparin {Besner & Klagsbrun (1988); Besner *et al.* (1990); Higashiyama *et al.* (1991)}, but two other members of the EGF family, heparin-binding epidermal growth factor (HB-EGF), and amphiregulin (AR), both have strong affinities for this glycosaminoglycan.

The EGF family also includes three virally encoded polypeptide growth factors, which are all encoded by members of the poxvirus family. These include vaccinia growth factor (VGF){Stroobant *et al.*, (1985)}, myxomavirus growth factor (MGF) {Upton *et al.*, (1987)}, and Shope fibroma growth factor (SFGF) {Chang *et al.*, (1987)}. The structural relationship between the EGF family members is based on the so-called 'EGF unit' — a 45-50 aa sequence containing six cysteine residues located in characteristic positions. Fully processed EGF, TGFα and VGF share a sequence identity of about 35%, constituted by one such EGF unit {Derynck *et al.*, (1992)}.

EGF, TGFα, HB-EGF, AR, and VGF are all synthesised as larger precursors that incorporate a hydrophobic domain {Gray *et al.*, (1983); Lee *et al.*, (1985); Higashiyama *et al.*, (1992); Plowman *et al.*, (1990); Venkatesan *et al.*, (1982)}. This hydrophobic sequence anchors the precursor growth factors in the plasma membrane, before they are released by appropriate stimuli. Release of precursor TGFα has been shown to be a highly regulated process, effected by an elastase-like enzyme {Pandiella & Massagué (1991a & 1991b)}. Moreover, membrane-bound TGFα can also interact with EGF receptors on adjacent cells to transduce its mitogenic signal {Wong *et al.*, (1989); Brachmann *et al.*, (1989)}.

The EGF unit for each growth factor is found in the extracellular domain of these transmembrane precursors. The suggestion that such membrane-anchored growth factors might act as receptors for unknown ligands {Pfeffer & Ullrich (1985)} has been substantiated by the demonstration that the HB-EGF precursor is identical to the diptheria toxin receptor {Naglich *et al.*, (1992)}.

1.3.1.1 Epidermal growth factor

Epidermal growth factor is a 53 aa polypeptide {Carpenter & Cohen (1979)} with six cysteine residues which are involved in the formation of three intramolecular disulphide bridges {Savage *et al.*, (1972 & 1973)}. The mature 6 kDa EGF is cleaved from the C-terminus the 130 kDa EGF precursor {Gray *et al.*, (1983)}.

1.3.1.2 Transforming growth factor α

Transforming growth factor α is a 50 aa protein with a mature molecular weight of 5.6 kDa {Derynck *et al.*, (1984)}. It shares 40% homology with EGF and conserves the 6 cysteine residues {Marquardt *et al.*, (1984)}. The overall three-dimensional fold structure of TGFα has been shown to be similar to EGF {Harvey *et al.*, (1991)}. Post-translational glycosylation and incomplete proteolytic processing, generates TGFα molecules with varying molecular weights {Texidó *et al.*, (1988)}.

The target cells for EGF and TGFα include epithelial, mesenchymal and glial cells. Many *in vitro* and *in vivo* biological activities have been attributed to EGF, but perhaps the most documented is its ability to stimulate the proliferation and differentiation of epithelial, and mesenchymal cells {Carpenter & Wahl (1990)}. Both EGF and TGFα are angiogenic factors *in vivo*, but TGFα is reported to be more potent than EGF in this respect {Barrandon & Green (1987)}. In addition, overexpression of TGFα, but not EGF, in several carcinomas implies a role for TGFα in the progression of these malignancies.

1.3.1.3 Heparin-binding epidermal growth factor

Heparin-binding EGF-like growth factor (HB-EGF) was purified originally from the medium conditioned by the histiocytic lymphoma cell line U-937, and its N-terminal amino acid sequence was determined. The mature protein has an apparent MW of 22 kDa, is heat and acid stable, and elutes from immobilised heparin in 1.0-2.0 M NaCl. Complementary DNA cloning of HB-EGF from a U-937 cDNA library revealed that the unique HB-EGF sequence shared 40-

53% homology with the other members of the EGF-family, and that the mature 86 amino acid (aa) glycosylated protein is synthesised initially as a large precursor of 208 aa. HB-EGF was found to stimulate the proliferation of fibroblasts and smooth muscle cells, but not endothelial cells {Higashiyama *et al.*, (1991, 1992)}. The mitogenic effect of HB-EGF on smooth muscle cells is more potent than that of EGF or TGFα and this appears to be due to its interaction with heparan sulphate proteoglycan (HSPG) on the cell surface {Higashiyama *et al.*, (1993)}.

1.3.1.4 Amphiregulin

Amphiregulin is an 88 amino acid glycoprotein. It was first isolated from the conditioned medium of a phorbol ester-treated mammary carcinoma cell line, MCF-7. It was also shown to be a bifunctional growth regulator in this system {Shoyab *et al* (1988)}. AR has been shown to interact with the EGF receptor {Shoyab *et al.*, (1989)}, and inhibits tumour growth {Plowman *et al.*, (1990)}. However, it is mitogenic for normal human keratinocytes {Cook *et al.*, (1991a)}.

1.3.2 TRANSFORMING GROWTH FACTOR β FAMILY OF POLYPEPTIDES

Transforming growth factor β (TGFβ) is the prototype of a family of homlogous polypetides. Receptors for TGFβ have been found on virtually all cell types examined, and most cell types studied were also found to produce it. The name of this pleiotrophic protein, 'transforming growth factor β', belies the fact that TGFβ does not cause oncogenic transformation of normal cells. The response to TGFβ depends on both the cell type and/or its cellular context. In general however, TGFβ has an inhibitory effect on the proliferation of normal epithelial cells, whereas its effect on cells of mesenchymal origin tends to be stimulatory. TGF-β also affects the expression of differentiated functions in many cells, as for example in the maintenance of the extracellular matrix, where it can stimulate both the genes for extracellular matrix (ECM) structural proteins and those for ECM-degrading enzymes.

The multifunctional nature of TGFβ-like polypeptides, and their ubiquitous distribution in many tissues from man to *drosophila*, indicate the important role this molecule plays in the regulation of growth and differentiation. This observation is underscored by the copious volumes of literature published in this field since the initial isolation of TGFβ from human platelets {Assoian *et al.*, (1983)}, and its subsequent cloning {Derynck *et al.*, (1985)}. Several reviews covering all aspects of the biology of these proteins are available {Barnard *et al.*, (1990);

Massagué (1990); Sporn & Roberts (1993);}. The following therefore, is a cursory overview of the major biochemical characteristics and biological functions of TGFβ-like polypeptides.

The known TGFβ family of polypeptides now numbers five. The original TGFβ has since been designated TGF-β1 {Cheifetz *et al.*, (1987)}, and all the subesquently identified members are classified numerically thereafter as, TGF-β2, TGF-β3, TGF-β4, and TGF-β5 respectively. These dimeric proteins share approximately 70-80% sequence homology, including nine conserved cysteine residues. Only the first three TGFβ isoforms have been detected in mammals as yet, but the sequence homology between species for these three is closer to 100%. TGF-β1, TGF-β2 and TGF-β3 have also been identified in humans {ten Dijke *et al.*, (1988)}. TGF-β4 was isolated from chick embryo chondrocyte cDNA library {Jakowlew *et al.*, (1988b)}, and TGF-β5 was identified in a *Xenopus laevis* cDNA library {Kondaiah *et al.*, (1990)}.

All TGFβ forms are synthesised as inactive precursors (320 amino acids long) which are subsequently processed to yield the 25 kDa active dimers {Derynck *et al.*, (1985 and 1988); ten Dijke *et al.*, (1988); Jakowlew *et al.*, (1988a and b); Kondaiah *et al.*, (1990)}. The mature molecule is proteolytically cleaved by the action of a subtilisin-like proteinase which produces two 12.5 kDa monomers from the C-termial half of the precursor {Barr *et al.*, (1991)}. Both monomers are disulphide-linked, but remain non-covalently bound to the remaining fragment of their precursor molecule during secretion {Gentry & Nash (1990)}. This precursor fragment is also a dimer and has been called the 'latency associated peptide', (LAP). The latent form of TGF-β1 secreted by cultured cells consists of the mature protein non-covalently bound to LAP {Lyons *et al.*, (1990)}. However, a large molecular weight 'binding protein' (TGF-β-BP) is also found associated with the TGF-β1/LAP complex in platelets {Wakefield *et al.* (1989); Miyazono *et al.*, (1988)}.

All normal cell types, and the majority of malignant cells, have receptors for TGFβ on their cell surface {Tucker *et al.*, (1984); Frolik *et al.*, (1984)}. Three types of high affinity receptors for TGFβ have been identified. These fall into three classes on the basis of size; type I (53 kDa), type II (70-85 kDa), and type III (250-250 kDa) {Cheifetz *et al.*, (1986); Massagué *et al.*, (1990); Segarini (1990, 1991) and Cheifetz *et al.*, (1988)}.

Several related families of inhibitory polypeptides have been identified which share 25-40% sequence homology with TGFβ, and have at least seven of the nine cysteine residues conserved.

Among these are the inhibins and activins {Mason *et al.*, (1985)}; müllerian inhibiting substance {Cate *et al.*, (1986)}; the decapentaplegic gene from *drosophila* {Padgett *et al.*, (1987)}; BMP-2A, BMP-2B and BMP-3 {Wozney *et al.*, (1988)}; the Vg-1 protein product from *Xenopus* {Weeks *et al.*, (1987)}; and Bg-1, the mammalian homolog of Vg-1 {Lyons *et al.*, (1989)}.

1.3.3 PLATELET-DERIVED GROWTH FACTOR FAMILY

Platelet-derived growth factor (PDGF) is a heat-stable, cationic, disulphide-linked dimeric protein, with an affinity for immobilised heparin {Shing *et al.* (1984)}. The constituent polypeptide monomer subunits are called the A- and B-chains respectively, and are the products of separate genes. Mature PDGF can therefore be a homodimer of the A or B chain (PDGF-AA, PDGF-BB) or alternatively, a heterodimer of both (PDGF-AB). Molecular weights for these isoforms are in the range 28-31 kDa. A 56% homology between amino acid sequences of the A and B chains, as well as the conservation of eight cysteine residues in both, suggest a similar tertiary structure for both hetero- and homodimeric isoforms {Ross *et al.*, (1986)}.

Two high affinity cell surface receptors have been identified for PDGF and designated type α and type β respectively. Both receptors are structurally related transmembrane proteins, with extracellular portions containing five immunoglobulin-like domains separated from an intracellular tyrosine kinase domain by a transmembrane region {Yarden *et al.*, (1986); Matsui *et al.*, (1986); Claesson-Welch *et al.*, (1988, 1989)}. Type α receptors bind PDGF isoforms comprising both A- and B-chains, but the type β receptor binds only isoforms containing the PDGF B-chain. Binding of PDGF to its receptor induces receptor dimerisation and autophosphorylation {Bishayee *et al.*, (1989); Heldin *et al.*, (1989); Seifert *et al.*, (1989); Kanakaraj *et al.*, (1991); and Eriksson *et al.*, (1992)}.

PDGF has potent mitogenic activity for cells of mesenchymal origin (e.g. fibroblasts). However, normal endothelial and epithelial cells are largely unresponsive to this mitogen {Ross *et al.*, (1986)}.

1.3.3.1 Vascular endothelial growth factor (VEGF)

Another member of the PDGF family of growth factors is vascular endothelial growth factor (VEGF). VEGF is a heparin-binding, disulphide-linked, homodimeric glycoprotein. It has a molecular mass between 32-42 kDa, and is mitogenic for endothelial cells {Gospodarowicz *et al* (1989)}. Synonymous with VEGF are the vascular permeability factor described by Senger

et al., (1983)} and vasculotropin {Plouet *et al*, (1989)}.

Alternative splicing of the human VEGF gene produces mature protein with individual chains of different length. These include proteins with 121, 165, 189, and 206 aa residues respectively {Leung *et al.*, (1989); Tischer *et al.* ,(1991); Charnock-Jones *et al.*, (1993)}. All forms are secreted, but the most abundant form *in vivo* is the $VEGF_{165}$ which has an apparent molecular mass of 42 kDa. The larger species are cell-surface- or extracellular matrix-bound, due to the inclusion of basic sequences which confer higher affinity for polyanions such as heparin. Partial proteolysis by plasmin is known to release the angiogenic VEGF protein from the ECM, and as such, may act as another control mechanism for neovascularisation {Houck *et al.*, (1992)}.

Two putative receptors for VEGF have been identified, and these share substantial sequence homolgy with each other {de Vries *et al.*, (1992); Terman *et al.*, (1992)}. They also share homology with the tyrosine kinase family to which they belong. Their tyrosine kinase domains contain a 70 aa insert, analagous to the receptor for macrophage colony-stimulating factor (M-CSF), the protooncogene c-*fms*. The extracellular portions contain seven immunoglobulin-like domains.

Endothelial cells and monocytes express receptors for VEGF and are currently believed to be among the few target cells for VEGF (Terman *et al.*, (1991); Millauer *et al.*, (1993); Shweiki *et al.*, (1993)}. VEGF expression has been detected in keratinocytes found at wound edges {Brown *et al.*, (1992)}, but VEGF mRNA expression in the non-endothelial cell component of many tumours has also been documented {Dvorak *et al.*, (1991)}. These include two carcinoma cell lines from which VEGF has also been purified; the human epidermoid carcinoma cell line A-431, which also expresses VEGF receptors {Myoken *et al.*, (1991)}, and a rat glioma cell line {Conn *et al.*, (1990)}.

1.3.4 INSULIN-LIKE GROWTH FACTORS

Insulin and the structurally related insulin-like growth factors -I and -II, are homologous proteins which constitute a family of growth-promoting peptides. They were originally isolated from human serum, and called somatomedins on the basis of their insulin-like effects on muscle and adipose tissue (i.e glucose transport and metabolism) {Blundell *et al.*, (1978)}. Growth hormone is known to regulate the expression of IGF-I, but it has a lesser effect on IGF-II gene expression. IGFs are present in relatively high concentrations (20-80 nM) in blood plasma, and at lower concentrations in the majority, and probably all tissues {reviewed in Humbel (1990)}. The large body of literature published in this field permits only some of the biology of these proteins to be overviewed here.

Insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) are non-glycosylated, single chain peptides, with 70 and 68 aa residues, respectively. They share approximately 76% sequence homology with each other, and 50% homology with proinsulin. In the mature IGF proteins there are four domains. The N-terminal or B domain, preceeds the C, A, and D domains, which are structurally homologus to proinsulin. The A and B domains are believed to be important for the binding of IGF-I to its receptor {Rotwein (1991)}.

IGF-I and IGF-II are the products of single genes which have been assigned to chromosomes 12 and 11 respectively {Tricoli *et al.*, (1984)}, with the IGF-I gene located 1.3 kb 3′ to the insulin gene {Scholfield (1991)}. Analysis of the gene structures revealed the existence of alternative promotors, regulating multiple transcription start sites, as well as variable polyadenylation sites. Differential splicing also gives rise to multiple mRNA transcripts {Kajamoto & Rotwein (1991); Rotwein & Hall (1990)}.

The greater part of the IGFs found in serum (>95%) exist in association with carrier proteins {Zapf *et al.*, (1975)}. IGF binding proteins (IGFBPs) serve as transport molecules for these growth factors in serum, but they also regulate their interaction with IGF cell surface receptors. Six forms IGFBPs, ranging in size from 24-45 kDa, have been isolated and sequenced, and at least four serum proteinases specifically cleave IGFBPs {Clemmons *et al.*, (1993); Bach *et al.*, (1993)}. Most of the serum IGF is bound to IGFBP-3 in a 150 kDa complex which includes IGF-I or IGF-II, and an 80 kDa acid labile component. IGF-I and IGF-II demonstrate equal afinities for IGFBP-3 {Baxter & Martin (1989)}.

Two types of transmembrane receptors have been identified for IGFs {reviewed Kasuga *et al.*, (1991); Nissely & Lopaczynski (1981)}. The type I receptor is a disulphide-linked heterotetramer, composed of two α subunits (115 kDa) that form the ligand binding site, and two transmembrane β subunits (90 kDa) with tyrosine kinase activity in their cytoplasmic domain.

A structurally unrelated type II receptor does not display tyrosine kinase activity. It is characterised by a large extracellular domain, connected to a relatively short 164 aa cytoplasmic domain by a single-spaning transmembrane region. The cytoplasmic domain of the type II IGF receptor is unique {Lobel *et al.*, (1988)}. The human cDNA for mannose 6-phosphate receptor is 99.8% identical to that of the type II IGF receptor {Oshima *et al.*, (1988)}, and a soluble form consisting of the extracellular domain of this receptor (type II IGF/Mannose 6-phosphate) has been isolated {Causin *et al.*, (1988)}. Differential binding patterns are displayed by IGF-I and IGF-II for the respective receptors. Higher affinity for the type I receptor is seen with IGF-I compared to IGF-II, but IGF-II displays a higher affinity for the type II receptor than IGF-I {Massagué & Czech (1982)}.

1.3.5 FIBROBLAST GROWTH FACTORS

Until recently, fibroblast growth factors (FGF) have constituted a family of seven polypeptide growth factors displaying significant sequence and structural homologies. In the last year however, the purification and cDNA cloning of two new heparin-binding mitogens, now brings to nine the number of FGF family members. The number of reviews which have appeared recently in the literature, covering all apsects of FGF biology, reflect the widespread interest in, and the extent of this field {refer to; Brem & Klagsbrun (1993); Basilico & Moscatelli (1992); Gospodarowicz (1990); Klagsbrun (1989)}. A 3 intron/2 exon gene structure and the conservation of two cysteine residues is common to all FGFs. An important functional property shared by these growth factors is their ability to bind heparan sulphate proteoglycans in the extracellular matrix. The high affinity of FGFs for the related glysocaminoglycan heparin, also provided the basis for the initial purification to homogeneity of the first FGFs described, acidic and basic FGF (aFGF, bFGF) {Shing *et al.*, (1983); Gospodarowicz *et al.*, (1984)}. The absence of a classical signal sequence in the genes for aFGF and bFGF can no longer be considered unique among the FGFs, since sequencing of the complementary DNA for FGF-9 revealed that it too does not encode a signal for secretion {Miyamoto *et al.*, (1993)}.

The profusion of names for the different FGFS reflects the orignial source and/or biological activity that lead to their identification. The term 'FGF' has been proposed as a unifying system of nomenclature, and although this is being adopted for new members, some of the earlier FGFs are commonly referred to by the original designations. In this thesis, the first two members of this family described, acidic and basic FGF respectively, are referred to by the original nomenclature, rather than by the lesser used designations FGF-1 and FGF-2. Other FGFs include *int*-2 (FGF-3), *hst*/K-fgf (FGF-4), FGF-5, FGF-6, keratinocyte growth factor (KGF/or FGF-7), andorgen-induced growth factor (AIGF/ or FGF-8) and glia-activating factor (GAF/ or FGF-9).

Four of the FGFs were initally identified as oncogenes in transformation assays. Complementary DNA cloning revealed that these genes encoded proteins which shared 40-50% homologies with the prototype acidic and basic FGFs, and indicated that the homologies were greatest in the internal regions of the proteins. These four 'oncogene' members are rarely found in adult tissues {Rifkin & Moscatelli (1989)}. Basic FGF is widely distributed in normal and malignant tissues, but acidic FGF is found mainly in brain and neural tissues {Gospodarowicz (1990)}.

Important roles for FGFs in development, wound repair, angiogenesis and tumourigenesis have been described. The variety of biological roles can be attributed to the fact that cells representative of meso- ecto- and endodermal embryonic germ cell layers are the targets of FGFs. The principal biological properties of each of the FGFs is described here.

1.3.5.1 Acidic and Basic FGF (FGF-1 and FGF-2)

Acidic and basic FGF are homologous proteins thought to have arisen from a single ancestral gene. The degree of homology between these multifunctional growth factors is 53% {Esch *et al.*, (1985)}. An important biological activity shared by both of these growth factors is their potent angiogenic effect *in vivo* {Folkman & Klagsbrun (1987)}. This can be attributed to the ability of these factors to stimulate the proliferation of the endothelial cells lining blood vessels {Schweigerer *et al.*, (1987)}, but also because they can modulate the secretion of enzymes capable of degrading the extracellular-matrix {Mignati *et al.*, (1989)}, and promote endothelial cell migration {Stokes *et al.*, (1990)}.

Acidic and basic FGF are both single-chain polypeptides of 154 aa with mature molecular weights ~18 kDa {Ueno *et al.*, (1986); Thomas *et al.*, (1984)}. However, the isoelectric points of the respective proteins are very different. Basic FGF is a cationic protein with a p*I* ~9.6 {Gospodarowicz (1989)}, and acidic FGF has a p*I* ~5.6 {Thomas *et al.*, (1984)}. Human bFGF has four cysteine residues, including two which are conserved among the FGFs (except FGF-8). By comparison acidic FGF contains three cysteines, but unlike bFGF, none of these are required for biological activity {Seno *et al.*, (1988)}. However, site-directed mutagenesis studies with bFGF indicate that intrachain disulphide bond formation does not seem to be required for biological activity {Klagsbrun (1989)}. Higher MW forms of basic FGF exist. These arise from the use of alternative transcription start sites in the bFGF gene (see discussion Section 4.3).

Among the plethora of cells whose *in vitro* proliferation is stimulated by acidic and basic FGF are, human keratinocytes and fibroblasts {Shipley *et al.*, (1989)}; vascular smooth muscle cells {Weich *et al.*, (1990)}; osteoblasts {Rodan *et al.*, (1987)} and ovarian epithelial cells {Gospodarowicz *et al.*, (1989)}.

1.3.5.2 INT-2 (FGF-3)

The *int*-2 gene was identified originally in mouse mammary tumours. This normal cellular gene became activated following the integration of mouse mammary tumour virus (MMTV) into the mouse genome, resulting in tumour formation {Dickson *et al.*, (1984)}. The *int*-2 gene encodes a 231 aa protein which is 46% homologous to bFGF {Dickson & Peters (1987)}. A short signal sequence accounts for the detection of *int*-2 protein in the golgi-endoplasmic recticulum of transfected cells, but the protein appears to be inefficiently secreted {Dixon *et al.*, (1989)}. A 28.5 kDa primary translation product is processed by glycosylation, giving rise to 30.5 and 31.5 kDa forms {Dixon *et al.*, (1989)}. In humans, a larger 271 aa form of *int*-2 is produced from transcripts initiated at an alternative CUG start site, and like the bFGF N-terminal extended forms, these are localised in the nucleus {Acland *et al.*, (1990)}.

Expression of *int*-2 is developmentally regulated, being restricted to several specific stages in embryogenesis {Jakobovits *et al.*, (1986)}. It is not expressed in normal adult tissues, but expression has been detected in a variety of solid tumours where it is associated with amplification of the gene. These include squamous cell carcinomas of the head and neck {Merritt *et al.*, (1990)}, and breast carcinomas {Zhou *et al.*, (1988)}.

1.3.5.3 K-fgf/hst (FGF-4)

The gene for FGF-4 was identified by two groups independently as a transforming gene in mouse NIH3T3 fibroblasts transfected with human stomach tumour DNA (thus the name *hst*) {Yoshida *et al.*, (1987); Koda *et al.*, (1987)}, and also DNA from Kaposi's sarcoma {Delli-Bovi *et al.*, (1987 & 1988)}. The gene encodes for a 206 aa protein containing a signal sequence. The first 80 aa of the N-terminal are unique but the remaining sequence shares 38% homology with human bFGF. There exists one potential N-linked glycosylation site, and glycoslylation brings the MW of the mature secreted product to approximately 23 kDa {Delli-Bovi *et al.*, (1988)}.

Like the other oncogenic FGFs, expression of FGF-4 appears to be developmentally restricted. It is expressed mainly during embryogenesis {Paterno *et al.*, (1989)}, but its has been detected in some solid tumours including, oesophageal {Tsuda *et al.*, (1991)}, gastric {Sakamoto *et al.*, (19886)}, and breast tumours {Theillet *et al.*, (1991)}. Like aFGF, heparin enhances the mitogenic activity of FGF-4 {Delli-Bovi *et al.*, (1988)}.

1.3.5.4 FGF-5

The FGF-5 gene, initially isolated from human bladder tumour DNA in the NIH3T3 transformation assay, was found to be secreted by endometrial carcinoma, bladder carcinoma and human hepatoma cell lines {Zhan *et al.*, (1988)}. The gene encodes a single chain 267 aa residue protein with an extended N-terminal hydrophobic sequence that enables it to be efficiently secreted. A 29.5 kDa protein is predicted from the primary translation product, but cleavage of the signal peptide, and glycosylation on at least one residue, yield molecules of 32.5-38.5 kDa {Bates *et al.*, (1991)}.

The biological functions of FGF-5 are not very clear, but its prolonged expression during embryogenesis {Hérbert *et al.*, (1990)}, and in exponentially growing normal adult human fibroblasts {Werner *et al.*, (1991)} suggests an important role for this growth factor in rapidly dividing cells. Moreover, the fact that FGF-5 expression in quiescent fibroblasts can be induced in response to growth factors such as PDGF, EGF, and TGFα, without the requirement for *de novo* protein synthesis, implicated it as a member of the family of primary response genes.

1.3.5.5 FGF-6

FGF-6 is the least characterised member of the FGF family. It was isolated from a mouse cosmid library using a probe to the K-fgf/hst gene, and displays 70% homology to the *hst* product at the C-terminus {Marics *et al.*, (1989)}. It has also been shown to be secreted {deLapeyriere *et al.*, (1990)}

1.3.5.6 Keratinocyte growth factor (KGF/FGF-7)

Keratinocyte growth factor (KGF) was isolated from medium conditioned by a human embryonic lung fibroblast cell line. It is an acid and heat labile, epithelial-specific paracrine-acting mitogen {Rubin *et al.*, (1989)}. The complementary DNA sequence of KGF identified it as the seventh member of the FGF family {Finch *et al.*, (1989)}. The KGF gene encodes a 194 aa protein with a unique N-terminal sequence, but the remainder of the sequence shares 39% homology with bFGF. The mature 28 kDa protein is glycosylated and secreted.

1.3.5.7 FGF-8

The heparin-binding 'androgen-induced growth factor' (AIGF), detected in the conditioned medium of the mouse mammary carcinoma cell line, SC-3 {Nonomura *et al.*, (1988)}, has recently been purified. Partial amino acid sequence has identified it as the eight member of the

FGF family. A cDNA clone of FGF-8 encodes a unique 215 amino acid protein with a putative signal sequence. Sequence analysis revealed that FGF-8 shares 30-40% sequence homology with basic FGF, KGF, FGF-5, FGF-6, FGF-3 (int-2) and FGF-4 (hst-1/K-fgf) but not with aFGF. In addition only one of the two highly conserved cycteine residues in the mouse FGF family is conserved in AIGF (FGF-8) {Tanaka *et al.*, (1992)}.

1.3.5.8 FGF-9

A heparin-binding glia-activating factor (GAF), isolated from the culture supernatant of a human glioma cell line {Naruo *et al.*, (1993)}, has recently been cloned. Sequence analysis revealed that it shares 30% homology with other FGFs, as well as conserving two of the cysteines common to all FGFs. Like acidic and basic FGF, FGF-9 does not encode a typical signal sequence. The degree of homology with rat FGF-9 (also cloned by these workers), suggests that FGF-9 is a highly conserved gene {Miyamoto *et al.*, (1993)}.

1.3.5.9 FGF receptors

Both high and low affinity receptors have been described for FGFs (for review see Klagsbrun & Baird (1991)}. Most of the information available to date stems from studies with basic FGF (see discussion Section 4.3). Briefly, heparan sulphate proteoglycans comprise the low affinity receptors, and the high affinity FGF receptors are transmembrane proteins. Five distinct high affinity receptors have now been cloned and sequenced, and are designated FGFR-1, -2, -3, -4, and -5 respectively. All of these transmembrane receptors belong to a tyrosine kinase gene family and their extracellular domains are characterised by three immunoglobulin-like regions (3-Ig). A number of isoforms of FGFR-1 and -2 exist in certain cell types as a result of alternative exon splicing, some of which lack the first immunoglobulin-like region generating a two-immunoglobulin form (2-Ig) {Jaye *et al.*, (1992)}. Two structural elements determining the ligand specificity of mouse FGF receptors 1 & 2 have been localised in the C-terminal half of the third Ig-like domain, as well as a region in the second Ig domain. This region is encoded by two distinct exons, and the alternative splicing of FGFR mRNA or 'exon switching' results in changes in ligand specificity, and is responsible for the observed redundancy among FGF receptors {Zimmer *et al.*, (1993)}. 'Exon switching' has more recently been found to generate a novel form of FGFR-3 {Avivi *et al.*, (1993)}.

1.3.6 Pleiotrophin

Pleiotrophin (PTN) is a developmentally regulated heparin-binding, neurotrophic factor, which is synthesised as a 168 aa precursor, and processed by the removal of a 32 aa signal sequence to give a 136 aa polypeptide with a molecular mass of 18 kDa. It is found mainly in the brain and acts as a growth and differentiation factor for cultured perinatal neurons. It is highly conserved among mammalian species {Li *et al.*, (1990)}. Courty *et al.*, (1991) reported the loss of PTN mitogenic activity following its storage in acid conditions. In the same study PTN was found to be mitogenic for bovine brain capillary endothelial cells and was angiogenic in the rabbit corneal-pocket assay. The acid sensitivity of PTN would appear to account for earlier conflicting reports concerning the mitogenic potential of PTN. Synonyms for PTN are heparin-binding growth associated molecule (HB-GAM), heparin-affinity regulatory peptide (HARP), heparin-binding neurotrophic factor (HBNF), p18 and osteoblast-specific factor (OSF-1) {Böhlen & Kovesdi (1991)}. A high affinity receptor for PTN was recently identified on NIH3T3 cells {Neame *et al.*, (1993)}.

PTN shares 55% sequence homology with a highly basic, 13.2 kDa heparin-binding protein, called midkine (MK), and these polypeptides have been proposed to constitute a family of developmentally regulated genes {Li *et al.*, (1992)}. Midkine is a retinoic acid-induced differentiation factor which is expressed during the mid-gestation period of mouse embryogenesis {Kadomatsu *et al.*, (1988)}.

A role in tumourigenesis was suggested for PTN by the detection of PTN mRNA in meningioma cells {Mailleaux *et al.*, (1992)}. In another study, PTN was expressed in over half of the primary breast cancer samples screened {Fang *et al.*, (1992)}. High levels of PTN are also expressed in melanomas {Wellstein *et al.*, (1992)}. Further evidence to support an important role for PTN in growth control was provided in a recent publication by Chauhan *et al.*, (1993). These authors demonstrated that overexpression of PTN in NIH3T3 fibroblasts resulted in phenotypic transformation of these cells. This was manifested by increased cell number of HIH3T3 at confluence, as well as focus formation, anchorage-independent growth, and tumour formation in nude mice. In another recent publication, comparing the expression in resected normal and malignant lung tissues (non-small cell carcinomas), a reciprocal pattern of expression for PTN and the homologous midkine (MK) gene was noted. PTN was expressed in all of the normal samples (n=17), but only two expressed MK. Conversely, only 1/20 malignant lung samples expressed PTN, but all expressed MK {Garver *et al.*, (1993)}. The

biolgical significance of these findings remain to be determined.

1.3.7 INTERLEUKINS

Interleukins (ILs) are the principal mediators of interactions between the immune and inflammatory cells. Twelve members of the interleukin family have been identified to date, and their genes have been cloned. These cytokines function as regulators of cell proliferation and differentiation in white blood cells {reviewed by Mizel (1989)}. Some interleukins have recently been seen to have a role in the regulation of nonhematopoietic cell growth. These include interleukins 1, 3, 4 and 6. For this reason, a brief summary of the biological properties of these cytokines is included.

1.3.7.1 Interleukin 1

Interleukin 1 (IL-1) exists in two forms, IL-1α and IL-1β. These cytokines are the products of separate genes and share 25% amino acid sequence homology {Oppenheim *et al.*, (1986)}. They are produced in response to stimulation of macrophages by inflammatory agents, but some cells in healthy individuals (such as skin keratinocytes) also produce these cytokines. A comprehensive review of the biological properties of IL-1 was published recently by Dinarello & Wolff (1993). IL-1 plays a critical role in the normal immune response to injury and infection, affecting not only the induction of specific cell types, but also entire cell systems. In this respect it mediates the induction of its own production, as well as that of other cytokines including IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) {Herrmann *et al.* (1988)}. Production of IL-6 and tumour necrosis factor (TNF) are also induced by IL-1 {Levett *et al.*, (1986); Navarro *et al.* (1988)}. The mature IL-1α and IL-1β have molecular weights of approximately 17 kDa, but these are the products of 31 kDa precursors {Giri, *et al.*, (1985); Hazudu *et al.*, (1988)}.

There are two distinct receptors which bind both forms of IL-1. The first, an 80 kDa, type I receptor (IL-1RtI) is found on T cells, fibroblasts, keratinocytes, endothelial cells and hepatocytes {Dinarello (1991); Urdal *et al.* (1988)}. IL-1RtI is a member of the Immunoglobulin (Ig) superfamily, and has been cloned from human and mouse cells {Sims *et al.* (1988)}. The second receptor, IL-1RtII, with an apparent MW of 68 kDa, is also a member of the Ig superfamily, and is found in bone marrow cells, neutrophils, and B cells {Dinarello (1991)}. A third ligand exists in the IL-1 family which functions as a receptor antagonist (IL-1ra) for IL-1 {Dinarello & Thompson (1991)}. Receptor-bound IL-1ra does not transduce a

signal.

1.3.7.2 Interleukin 3

Interleukin 3 (IL-3) is a 28 kDa pleiotrophic glycoprotein, which is expressed mainly by activated T cells. It is capable of stimulating proliferation and differentiation in several committed (lineage specific progenitor cells) and non-committed (stem cells) hematopoietic cells {Ihle *et al.*, (1981)}. IL-3 production by nonhematopoietic cells has also been reported. These include human thymic epithelial cells {Dalloul *et al.*, (1991)} and murine keratinocytes {Peterseim *et al.*, (1993)}.

Two receptor subunits are required to generate a functional signal-transducing IL-3 receptor. These have been designated IL-3Rα (70 kDa) and IL-3Rβ (120-140 kDa), and both have been cloned in humans {Kitamura, *et al.*, (1991)}. Only low affinity binding of IL-3 is possible in the absence of the IL-3Rβ subunit, which IL-3Rα shares in common with the cytokines interleukin-5 (IL-5), and granulocyte-macrophage colony-stimulating factor (GM-CSF), for the generation of a high affinity receptor complex {Goodall *et al.*, (1993)}.

1.3.7.3 Interleukin 4

Interleukin 4 (IL-4) is a pleiotrophic cytokine, produced mainly by activated T cells. Murine IL-4 is a glycoprotein of approximately 19 kDa {Grabstein (1986)}. Human and murine IL-4 cDNAs encode signal sequences which are cleaved to produce mature proteins with 129 and 120 aa residues respectively {Carr *et al.*, (1991)}. The receptor for IL-4 has been detected on hematopoietic and non-hematopoietic cells {Lowenthal *et al.* (1988)}. The mouse and human receptor genes have been cloned and these encode a high affinity transmembrane protein with a MW of 140 kDa {Mosley *et al.* (1989); Idzerda *et al.* (1990)}.

The paracrine production of IL-4 in tissues has been shown to inhibit the growth of both breast and colon carcinoma cells {Toi *et al.*, (1992)}.

1.3.7.4 Interleukin 6

The multifunctional cytokine interleukin-6 is produced by normal and transformed cells of lymphoid and non-lymphoid origin. A 212 aa precursor is predicted from the human IL-6 cDNA sequence with two potential N glycosylation sites. After cleavage of the signal sequence, a 184 aa mature protein is predicted, with a molecular weight of 21 kDa {Haegeman *et al.*,

(1986); Hirano, *et al.*, (1986); May *et al.*, (1986); Zilberstein *et al.*, (1986)}. One of the major functions of IL-6 is the induction of acute phase protein synthesis by liver hepatocytes following infection {Bauman *et al.*, (1984)}, but this cytokine has also been implicated in the pathology of a variety of proliferative diseases including psoriasis, plasmacytomas, and melanomas, as well as the autoimmune disease, rheumatoid arthritis {Gastl *et al.*, (1993)}.

The IL-6 receptor consists of two glycoprotein subunits. An 80 kDa subunit acts as a low affinity receptor (IL-6R) in the absence of the signal transducing protein gp130. Both subunits have been sequenced and cloned {Hibi *et al.*, (1990); Baumann *et al.*, (1990); Yamasaki *et al.*, (1988)}. The gp130 subunit is also involved in the formation of signal complexes for other cytokines including interleukin 11, leukemia inhibitory factor (LIF), oncostatin M, and cillary neurotrophic factor (CNTF) {Kishimoto *et al.*, (1992); Yin *et al.*, (1993)}.

1.3.8 HEMATOPOIETIC GROWTH FACTORS

Non-interleukin cytokines include erythropoietin (Epo), three colony stimulating factors, the interferons (IFN) α, β, and γ, tumour necrosis factor (TNF) -α and -β, as well as two recently identified inhibitory cytokines, leukemia inhibitory factor (LIF) and oncostatin M (OSM). Some of these cytokines with nonhematopoietic activities are discussed below.

1.3.8.1 Colony-stimulating factors

Colony stimulating functions derive their name from the biological activity used to characterise them initially. These cytokines were found to stimulate the colony formation of hematopoietic progenitor cells grown *in vitro* in soft agar. Some CSFs have been reassigned as interleukins since their original discovery, but three are still classified as colony stimulating factors. These include granulocyte-macrophage CSF (GM-CSF), macrophage CSF (M-CSF), and granulocyte CSF (G-CSF). GM-CSF is mentioned here because of its potential involvment as a modulator of cell growth in some solid human tumours, and derived cell lines {Foulke *et al.*, (1990)}.

Human GM-CSF cDNA encodes a 141 aa residue precursor which is processed by removal of a 17 aa signal sequence. Multiple glycosylation sites exist in this protein, as well as two intramolecular disulphide bonds essential for biological function {Burgess *et al.*, (1990)}. Apart from hematopoietic cells, fibroblasts and endothelial cells in the bone marrow stroma have been shown to secrete GM-CSF {Adreef & Welte (1979)}.

Both high and low affinity receptors for GM-CSF have been characterised in humans and mouse {Goodall *et al.*, (1993)}. In a situation reminiscent of the interleukin 3 receptor system, the α subunit of the GM-CSF receptor binds the ligand with low affinity, but cannot transduce a signal without the β subunit (which is also the 'convertor' used by the IL-3 receptor to generate a functional IL-3 receptor).

1.3.8.2 Leukemia inhibitory factor and Oncostatin M

Leukenia ihibitory factor (LIF), and oncostatin M (OSM), were originally identified by their ability to inhibit the proliferation of macrophages and melanoma cells respectively {Kurzrock *et al.*, (1991); Zarling *et al.*, (1986); Brown *et al.*, (1987)}. Both cytokines were subsequently found to be pleiotrophic, with activities on both hematopoietic, and nonhematopoietic cells.

Among the inhibitory actions of oncostatin M, was its tumouristatic effect on the growth of a neuroblastoma cell line (HTB 10), and a similar effect was demonstrated with the breast carcinoma cell line MCF-7. However, OSM also exhibits a positive regulatory role, as demonstrated by its mitogenic effects on the growth of normal fibroblasts and Karposi's sarcoma cells {Horn *et al.*, (1990); Miles *et al.*, (1992); Nair *et al.*, (1992)}. LIF is expressed in a variety of cell lines and primary tissues, including thymic epithelial cells, liver fibroblasts, embryonic stem cells, brain glial cells, as well as activated T lymphocytes and monocytes {Metcalf (1992); Bhatt *et al.*, (1991)}. In nonhematopoietic cells, LIF appears to modulate differentiation functions such as enhancing the synthesis of acute phase proteins in hepatocytes. A more direct growth regulatory role for LIF in bone osteoblasts has been shown {Vlasselar *et al.*, (1992)}.

Human cDNA for LIF encodes a 180 aa protein with a predicted molecular mass of 20 kDa. In the mouse an alternate mRNA transcript encodes for a protein with a variant signal sequence which directs the mature protein into the extracellular matrix {Rathjen., *et al.*, (1990)}.

OSM is a heat and acid stable, single chain glycoprotein {Zarling *et al.*, (1986); Rose & Bruce (1991)}. Human cDNA from the U937 lymphoma line predicts a 252 aa peptide with a 25 aa signal sequence. Cleavage of the signal peptide produces a protein that is then further processed by cleavage at the C-terminal to form the 195-196 aa mature peptide {Linsley *et al.*, (1990)}. The human gene shares 22% sequence homology with human LIF, and displays 19% homology with human IL-6. At the protein level OSM shares a common structural motif with IL-6, IL-11,

and cillary neurotrophic factor (CNTF). These cytokines are thought to be descended from a common ancestral gene {Bazan (1991); Bruce *et al.*, (1992)}.

LIF exerts its biological effects though a receptor complex which include a low affinity receptor (LIF-Rβ), and the gp130 transducer common to the IL-6 receptor system {Gearing *et al.*, (1991)}. The LIF-Rβ/gp130 complex constitutes a high affinity binding site for LIF, but it also binds OSM {Davis *et al.*, (1993)}. A separate low affinity receptor specific to OSM has not yet been identified, but recent evidence in the lung cancer cell line H2981 suggests that one may yet be found {Liu *et al.*, (1992)}.

1.3.9 CHEMOKINES

Chemokines are members of a superfamily of small (8-10 kDa) pro-inflammatory, inducible cytokines, which act mainly as chemoattractants and activators of specific leukocytes. They share 20-50% sequence homology at the amino acid level and are sub-divided into two groups on the basis of a difference in a conserved aa sequence motif consisting of four cysteines. In one group, the α subfamily, the two cysteines are separated by another amino acid (C-X-C), but in the second or β subfamily, these cysteines are contiguous.

The α group includes interleukin-8 (IL-8), GROα/melanoma growth stimulating activity (MGSA), platelet factor-4 (PF-4), IP-10, ENA-78, as well as platelet basic protein and its derivatives (from proteolytic processing) - CTAP III and β thromboglobulin. The β subfamily of chemokines includes macrophage inflammatory protein-1α (MIP-1α) and -1β (MIP-1β), RANTES, MCP-1/MCAF, MCP-2, MCP-3 and I-309 {reviewed in Oppenheim *et al.*, (1991)}.

Chemokines bind to heparin and glycosaminoglycans, a factor that may facilitate their chemoattractant functions by allowing them to bind to endothelial cells and/or extracellular matrices {Tanaka *et al.*, (1993)}. Evidence for growth regulatory effects on keratinocytes, fibroblasts and melanoma cell lines is also beginning to emerge, and suggests that chemokines play a role in wound healing.

Receptors for most chemokines of both families have been found on red blood cells {Neote *et al.* (1993)}.

1.3.9.1 GROα/Melanoma growth-stimulating activity

Melanoma growth-stimulating activity (MGSA) was originally isolated and cloned from the human melanoma cell line Hs294T {Richmond and Thomas (1986)}, but an identical gene (designated GROα) was discovered in Chinese hamster embryo cells using subtractive hybridization techniques in normal and tumorogenic fibroblasts. Moreover, in many of these cell lines, production of GROα was associated with its constitutive overexpression {Sager *et al.*, (1991)}. Several stimuli including serum, PDGF and the inflammatory mediators IL-1 and TNF, induce GROα expression in fibroblasts, mammary epithelial cells, umbilical vein endothelial cells, and monocytes {Oppenheim *et al.*, (1991); Sager *et al.*, (1991)}.

The cDNA for GROα encodes a 107 aa precursor which includes a 34 aa residue signal sequence. The mature 73 aa peptide does not appear to be post-translationally modified. GROα binds to one of two receptors for IL-8 {Lee *et al.*, (1992)}. This receptor is a member of the rhodopsin-like 'seven-pass' superfamily. These transmembrane receptors are characterised by seven hydrophobic transmembrane domains coupled to G-proteins, as described above (Section 1.1.1). In addition, the recently described multispecific chemokine receptor is also reported to bind GROα {Neote *et al.*, (1993)}.

1.4 MOTOGENIC CYTOKINES

Several cytokines have been described to date which function as stimulators of cell motility *in vitro*. These proteins have been implicated in the spread of cancer cells from their primary sites *in vivo* by a process called metastasis. Some of these motogenic cytokines are also mitogenic, and may be involved in autocrine and paracrine mechanisms in the producer tissues.

1.4.1 Hepatocyte growth factor/Scatter factor

Hepatocyte growth factor (also known as Hepatopoietin A) and Scatter factor (HGF/SF) are synoymns for a distinct 82 kDa, 674 aa heterodimeric glycoprotein. It was isolated originally on the basis of different biological functions, firstly from rat platelets as a mitogen for hepatocytes, and secondly, as an activity secreted by human embryo fibroblsts which caused the scattering of cells from compact epithelial colonies. The mature HGF/SF protein is derived from a 728 aa single-chain precursor, and this is proteolytically processed to yield a heterodimer with 69 kDa and 34 kDa subunits, linked by a disulphide bond {Furlong *et al.*, (1992)}. The receptor for HGF/SF was identified as the protooncogene c-*met*. The HGF/SF receptor

comprises a 190 kDa heterodimeric transmembrane protein possessing tyrosine kinase activity, disulphide-linked to an α subunit of 45 kDa. The kinase activity is located on the cytoplasmic side of a 145 kDa β subunit that spans the cell membrane {Bottaro *et al.*, (1991)}. Considerable post-translational modifications, particulary on the eleven potential N-linked glycosylation sites results in the production of different isoforms {Giordano *et al.*, (1988)}.

HGF/SF has been shown to be secreted by a variety of cell types, including fibroblasts, smooth muscle cells, and also by a non-differentiated keratinocyte cell line {Adams *et al.*, (1991)}. The scatter factor purified by Rosen *et al.*, (1989) from bovine smooth muscle cells was found to be a heparin-binding factor, eluting from heparin-Sepharose between 1.3 and 1.4 M NaCl.

HGF/SF is mitogenic for a variety of different cell types including epithelial cells, melanocytes and endothelial cells, but it is not mitogenic for fibroblasts {Rubin *et al.*, (1991)}. It has also demonstrated affects on the motility of epithelial {Stoker (1989)}, and endothelial cells {Morimoto *et al.*, (1991)}. In some epidermoid and sarcoma cell lines, HGF/SF acts as an inhibitor of cell proliferation {Higashio *et al.*, (1990); Shima *et al.*, (1991)}.

1.4.2 Autocrine Motility Factor

Another factor also displaying motogenic activity for tumour cells is autocrine motility factor (AMF). This 55 kDa protein was initally purified from serum-free medium conditioned by a melanoma cell line {Liotta *et al.*, (1986)}. It was subsequently purified from the serum-free conditioned medium of a fibrosarcoma cell line HT-1080. A 78 kDa glycoprotein identified as the AMF receptor, was cloned from a HT-1080 cDNA library {Watanabe *et al.*, (1991)}.

1.4.3 Migration stimulating factor

A migration-stimulating factor purified from the conditioned medium of foetal and breast cancer patient fibroblasts has also been described {Grey *et al.*, (1989)}. This species was purified to homogeneity by a sequential fractionation scheme employing ammonium sulphate precipitation, heparin-affinity, gel filtration, and reverse-phase chromatography. The 70 kDa protein purified in this way, had a moderate affinity for heparin-agarose, eluting at 0.3 and 0.6 M NaCl. It was also found to be distinct from AMF.

1.5 AUTOCRINE GROWTH FACTOR MECHANISMS

Private versus public autocrine loops

As originally proposed, the autocrine hypothesis suggested that self-stimulatory polypeptides were secreted by cells, and interacted with their cognate receptors on the surface of the same producer cell, or on cells of the same type in the immediate microenvironemnt. However, for several growth factors, there is now evidence that the ligand may induce its mitogenic signal by interacting with its receptor intracellularly. This mechanism, called a 'private autocrine loop', avoids the requirement for growth factor secretion, and may provide one explanation for the failure of neutralising antibodies to act as effective inhibitors of cell proliferation in some cell systems where coexpression of receptors and their cognate ligands have been detected {Browder *et al.*, (1989)}.

Evidence for operative autocrine loops

Two approaches have been taken to meet the rigorous evidence required to establish the presence of an operative *in vitro* autocrine growth stimulatory mechanism. Both involve the use of an antagonist, and require the demonstration of growth inhibition as a direct consequence of blocking the action of the putative autocrine growth factor loop. To this end, anti-receptor and anti-growth factor antibodies have been used to inhibit ligand/receptor interactions. A more specific approach has been the use of antisense RNA oligodeoxynucleotide sequences. The latter technique permits the expression of specific genes to be inhibited.

Several growth factors have been implicated in autocrine growth control mechanisms and examples of these are discussed below.

1.5.1 PLATELET-DERIVED GROWTH FACTOR AS AN AUTOCRINE GROWTH REGULATOR

Platelet-derived growth factor (PDGF) was one of the first growth factors to be implicated as a positive autocrine growth regulator in malignantly transformed cells. The discovery that the gene for the B-chain of this growth factor was the normal cellular counterpart of the virally encoded v-*sis* oncogene (of the simian sarcoma virus, SSV) provided the first direct link between oncogenes, and the normal cellular genes involved in cell proliferation and differentiation {Devare *et al.*, (1983); Doolittle *et al.*, (1983); Waterfield (1983)}. Much work has since been done in the elucidation of the PDGF signal transduction mechanism.

An autocrine role for PDGF in a wide variety of malignancies has been implied by the observed coexpression of this growth factor and its receptors, in sarcomas {Leveen *et al.*, (1990); Smits *et al.*, (1992)}, mesotheliomas {Versnel *et al.*, (1991)}, gliomas {Nistér *et al.*, (1988); Hermansson *et al.*, (1992)} and lung carcinomas {Antoniades *et al.*, (1992)}. However, there have been conflicting results concerning the mechanism of autocrine PDGF stimulation. Studies with the simian sarcoma virus (SSV), have shown that the cell transformation induced by this virus, is mediated by the activation of cell surface receptors {Hannink & Donoghue (1988)}. However, other reports present evidence for a private autocrine loop, with the initiation of the ligand-induced receptor signal arising intracellularly {Keating & Williams (1989)}. In support of a public PDGF autocrine mechanism, Johnsson *et al* (1985) were able to block the PDGF B-chain/SSV transformation with anti-PDGF antibodies. Similar results were obtained by Huang *et al.*, (1984) using suramin, a drug known to displace several growth factors, including PDGF, from their receptors {Hosang (1985); Coffey *et al.*, (1987); Pollak & Richard (1990)}.

Despite a report demonstrating the endogenous activation of PDGF receptors in sarcoma cells {Fleming *et al.*, (1992)}, clear evidence that PDGF-dependent autocrine pathways mediate the proliferation of human tumour cells has only emerged recently. Vassbotn *et al.*, (1994) have now presented evidence for a direct link between the activation of the PDGF autocrine growth factor pathway and the transformed phenotype in a spontaneous human glioblastoma cell line, A172. Concomitant expression of PDGF-BB and its receptor (PDGF-R type β) were reported in this cell line, and constitutive autophosphorylation of the receptor was apparent in the absence of exogenously supplied ligand. Moreover, endogenous tyrosine kinase activity could be blocked using a neutralising antibody against PDGF. The same antibody was also capable of inhibiting DNA synthesis in these cells, as well as their ability to form colonies in soft agar (a

characteristic of malignantly transformed cells). Moreover, the anti-PDGF antibody also inhibited the growth of A172 cells in monolayer, and reversed their transformed phenotype morphology in this system.

1.5.2 AUTOCRINE GROWTH CONTROL WITH MEMBERS OF THE EPIDERMAL GROWTH FACTOR FAMILY

EGF-like growth factors in autocrine growth control of normal cells

Transforming growth factor α has been shown to act as an autocrine stimulator in human keratinocytes where it was found to regulate its own production. This is achieved by inducing TGFα mRNA in response to TGFα stimulation {Coffey *et al.*, (1987)}. Further evidence for autocrine growth control by TGFα in keratinocytes has been presented by Cook *et al.*, (1991) who were able to inhibit the autonomous growth of human keratinocytes with an antibody that bound to the EGF receptor, thereby blocking the binding of TGFα.

EGF-like growth factors in malignantly transformed cells

TGFα and its receptor appear to be expressed by a majority of malignant cells derived from solid tumours, particularly carcinomas, but not by hematopoietic tumour cell lines {Derynck *et al.*, (1987)}. However, Madtes *et al.*, (1988) have shown that activated macrophages produce TGFα.

TGFα in Lung Cancer

Imanishi *et al.*, (1988) implicated TGFα as a possible autocrine growth factor for human lung adenocarcinoma cells, and the same group subsequently inhibited the growth of two human lung adenocarcinoma cell lines (A549 and PC-9) using anti-TGFα neutralising antibodies {Imanishi *et al.*, (1989)}.

More recently Burton & Knight (1992) demonstrated that an autocrine TGFα loop may indirectly stimulate the growth of the human lung cancer cell line BEN-57 by inducing the secretion of parathyroid hormone-related peptide (PTHrP). Both proteins were stimulatory for BEN-57 cells, but anti-PTHrP polyclonal antisera, and a PThrP receptor antagonist, inhibited cell growth in this system.

TGFα in Ovarian Cancer

In a recent study of 17 ovarian carcinoma cell lines of epithelial origin, 16 were found to secrete TGFα, and all expressed the EGF receptor. In the same, study 8/13 of the cell lines examined demonstrated a mitogenic response to exogenous TGFα, and of these, five were capable of serum-free propagation. The growth of four of the cell lines capable of serum-free propagation could be inhibited by the addition of an anti-TGFα neutralising monoclonal antibody to the culture medium of these cell lines. Taken together these findings suggest that TGFα may act as an autocrine growth stimulator in ovarian carcinomas {Stromberg *et al.*, (1992)}.

TGFα in Pancreatic Cancer

TGFα was implicated as one of two autocrine growth stimulators in the human pancreatic carcinoma cell line MIA-PaCa 2. These cells were capable of serum-free proliferation, and elaborated multiple immunoreactice forms of TGFα. In addition, MIA-PaCa 2 cells were growth stimulated by 1.5-fold in the presence of authentic TGFα {Ohmura *et al.*, (1990)}.

TGFα in Prostate Cancer

Liu *et al.*, (1994) recently presented evidence that a TGFα autocrine loop may act as a mediator of the androgen-induced cell proliferation in the androgen-dependent prostate cancer cell line ALVA101. These workers found that testosterone, (T) and α-dihydrotestosterone (DHT), both induced the mRNA for TGFα and its receptor (TGFα/EGF receptor) in serum-free culture conditions. Both androgens, as well as TGFα and EGF, stimulated cell growth and in either case the mitogenic response could be inhibited by an anti-TGFα/EGF receptor antibody.

TGFα in Breast Cancer

Estrogen-dependent epithelial breast cancer cell lines secrete high levels of TGFα-like activity into their culture medium {Dickson *et al.*, (1986)}, and detection of the EGF receptor has also been reported on such cell lines {Davidson *et al.*, (1987)}. An autocrine role for TGFα in these tumours is supported by the demonstration that an anti-EGF receptor antibody was capable of inhibiting the growth of the MDA-468 breast carcinoma cell line {Ennis *et al.*, (1989)}.

Amphiregulin as an autocrine growth factor

The previously idenitifed 25 kDa, EGF receptor-binding autocrine growth factor, CRDGF (Colorectum Cell-Derived Growth Factor), produced by the human colon adenocarcinoma cell line HT29 {Colouscou *et al.*, (1988)}, has subsequently been identified as amphiregulin after immunological and partial N-terminal amino acid sequence analysis revealed its identity with this member of the EGF family {Colouscou *et al.*, (1992)}.

Amphiregulin has been shown to act as an autocrine growth stimulator in normal human epidermal keratinocytes {Cook *et al.*, (1991a)}. However, in the context of the production of other positive autocrine growth factors, then AR, acting in its capacity as a negative regulator of cell proliferation, might contribute to the hyperproliferative state if its expression were reduced or functionally inactivated.

1.5.3 AUTOCRINE GROWTH CONTROL BY FGFs

Basic FGF in autocrine growth control

While evidence supporting a role for basic FGF as an autocrine growth stimulator continues to emerge, the mechanism by which its action is effected is still uncertain. In some cell systems, studies support the existence of a 'private' autocrine loop (also called an 'intracrine' loop), but other findings are consistent with an external or 'public' autocrine pathway.

Basic FGF in Melanoma

Strong evidence has emerged in recent years to implicate basic FGF as an autocrine growth factor for melanoma cells {reviewed by Rodeck *et al.*, (1991)}. The development of a serum-free medium for normal melanocytes provided an ideal model with which to study growth factor requirements, and other events associated with the progression from the benign to the malignant phenotype in this system. In humans, at least three distinct stages of the disease can be distinguished histopathologically, and clinically. Initially the growth of benign proliferative lesions in stage 1 results in the formation of 'acquired' or 'dysplastic' nevi. This is followed in stage 2 by the development of primary melanoma — characterised by an increased capacity to disseminate *in vitro*. Rapid metastasis characterises the development of malignant melanoma in stage 3.

Four components are essential for long-term proliferation of normal melanocytes *in vitro*. These include IGF-I, a phorbol ester {e.g. 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)}, an inducer of cAMP formation, such as α-melanocyte-stimulating hormone, and basic FGF {Herlyn *et al.*, (1987)}.

Basic FGF is not produced by normal melanocytes, but constitutive production of this growth factor is an early event in melanoma progression. The acquisition of an endogenous source of bFGF is accompanied by a concommitant loss in the requirement for exogenous bFGF by these cells in culture {Rodeck *et al.*, (1991)}. The most convincing evidence for the role of bFGF as an autocrine regulator in melanoma however, comes from experiments with anti-basic FGF neutralising antibodies, and antisense oligodeoxynucleotides. Antisense oligonucleotides directed against the AUG codon, and the donor/splice acceptor sites of bFGF mRNA, were found to inhibit *in vitro* growth of primary and metastatic melanoma cells by 70-90% {Becker *et al.*, (1989)}. By comparison, only anti-bFGF neutralising antibodies injected into melanoma cells, but not those added to their culture medium, inhibited melanoma growth (by approximately

60%). The fact that anti-bFGF antibodies added to the culture medium were incapable of inhibiting melanoma cell proliferation, as opposed to those delivered intracellularly, suggested that an intracrine bFGF mechanism was operative in these cells {Halaban *et al.*, (1988)}.

Basic FGF in Gliomas

The involvment of bFGF as an autocrine growth factor in gliomas was suggested by a report that the C6 rat glioma cell line produced a bFGF-like immunoreactive species, and that bFGF stimulated the proliferation of these cells in a serum-free system. An intracrine stimulatory mechanism was implied by the finding that C6 cells did not secrete this FGF {Okumura *et al.*, (1989)}.

In another study Morrison *et al.*, (1990) demonstrated that the human glioma cell line SNB-19 expressed bFGF mRNA and synthesised a non-secreted bFGF-immunoreactive species. SNB-19 cells also expressed high affinity FGF receptors and were stimulated to produce colonies in soft agar by the addition of exogenous bFGF. A subsequent publication by Morrison (1991) demonstrated 80% inhibition of SNB-19 monolayer growth, using a specific antisense oligodeoxynucleotide directed against the AUG initiation site, or against the first splice/donor acceptor site of bFGF mRNA.

Further evidence to support the autocrine role of bFGF in human gliomas was presented in a recent publication by Takahashi *et al.*, (1992). These workers detected the presence of bFGF in 18 of 19 human gliomas, and correlated a proportionate increase in the expression of bFGF protein with an increase in the number of nuclear organiser regions (a marker for the degree of malignant progression) in these tumour cells. In this same study, the degree of vascularisation, and the relatively high levels of bFGF expression detected in these glioma tumours, suggested a paracrine role for bFGF.

Basic FGF in Colon Cancer

The role of bFGF as an autocrine growth stimulator in colon carcinoma was suggested by a report demonstrating the expression of FGF receptors and a bFGF-immunoreactive species in five colon carcinoma cell lines {New & Yeoman (1992)}.

AIGF/FGF-8 — an autocrine mediator in Mammary Carcinoma

The androgen-dependent mouse mammary cell line SC-3, was shown to secrete a growth-promoting activity in response to testosterone. This species, called androgen-induced growth factor (AIGF), was found to have a high affinity for immobilised heparin {Nonomura *et al.*, (1988)}. Yamanishi *et al.*, (1991) subsequently demonstrated that androgen-stimulated growth of SC-3 cells, as well as their mitogenic response to partially purified AIGF, was inhibited by an anti-basic FGF polyclonal antibodies. This suggested that an autocrine loop involving a bFGF-like growth factor mediated the mitogenic effect of testosterone in these cells. The subsequent purification and cloning of AIGF revealed that it was distinct from bFGF and could be considered as an eight member of the FGF family {Tanaka *et al.*, (1992)}

Acidic FGF-like growth factor in oesophageal carcinoma

A murine model for the study of malignant progression in immortalised oesophageal epithelial cells was described by Katayama & Kan (1991), and presents evidence for the involvement of an aFGF-like growth factor in the autocrine growth control of these cells. Using a serum-free medium containing neural extract, these authors were able to select rapidly proliferating immortalised premalignant epithelial cells from normal cultures. When neural extract was removed, these premalignant oesohpageal cells were most sensitive to exogenously added EGF and aFGF. Moreover, extracts of actively proliferating premalignant cells were found to contain an aFGF-like activity. While premalignant cells formed only small keratinized tumours in syngenic hosts, a clone was selected from this population on the basis of its ability to grow in the absence of neural extract. This clone displayed constitutive cytosolic aFGF production at all stages of the growth cycle, and formed large invasive tumours in syngenic hosts.

Acidic FGF in the control of parathyroid cell growth

Evidence that an autocrine acidic FGF loop mediates the calcium-regulated growth of parathyroid cells was presented by Sakaguchi (1992). Hormone secretion and growth stimulation are negatively regulated by extracellular calcium in the rat cell line used in this study, and these cells have also previously been reported to express two high affinity receptors for aFGF {Sakaguchi *et al.*, (1991)}. More aFGF mRNA and protein were detected at low Ca^{2+} concentrations, and the FGF receptors for this ligand were translocated from intracellular sites to the plasma membrane under these low Ca^{2+} conditions. Significantly, anti-aFGF antibody only inhibited cell growth at low Ca^{2+} concentrations.

Evidence for the involvment of the oncogenic FGF members in autocrine growth control

The oncogenic FGFs *int*-2 (FGF-3), *hst*/K-fgf (FGF-4), FGF-5 and FGF-6, all have the potential to be involved in public autocrine growth loops because they posses signal sequences, and are therefore capable of being secreted. However, although the expression of these genes have been detected in tumours, there has been little direct evidence for their involvement in autocrine growth stimulation in these systems.

A role for FGF receptors in autocrine growth control

A recent study by Kobrin *et al.*, (1993) compared mRNA expression levels for the 2-Ig and 3-Ig isoforms of FGFR-1 in normal and malignant human pancreas tissues using PCR and an RNAse protection assay. Both forms were expressed in relatively equal proportions in normal tissue, with a tendancy for predominance of the 3-Ig form. However, in the cancerous tissues, the 2-Ig FGFR-1 form predominated, and was overexpressed (by 4-fold) relative to that in normal tissue. This group had previously reported the overexpression of aFGF and bFGF mRNA by human pancreatic cancer cells {Korc *et al.*, (1992)}, and although both acidic and basic FGF were known to bind the 3-Ig and 2-Ig isoforms of FGFR-1 {Jaye *et al.*, (1992)}, only bFGF expression in pancreatic cancer cells correlated with a shorter patient post-operative survial time (unpublished observations). This led Kobrin *et al.*, (1993) to suggest that bFGF might preferentially activate the overexpressed 2-Ig FGFR-1 isoform in pancreatic cancer cells.

FGFR-1 gene amplification has also been reported recently in ovarian carcinoma {Theillet *et al.*, (1993)}.

1.5.4 INVOLVEMENT OF INSULIN-LIKE GROWTH FACTORS IN AUTOCRINE GROWTH CONTROL

In addition to their endocrine functions, it is now widely accepted that IGF I and IGF II can act locally as paracrine and autocrine growth factors {Humbel (1990)}.

IGFs in Normal Lung

Evidence for an IGF-I autocrine stimulatory loop in the human embryonic lung fibroblast cell line, WI-38, was presented recently. In this report, the presence of 2 μM of a 15-base pair antisense oligodeoxynucleotide directed against the start site for translation of IGF-I mRNA, reduced levels of the growth factor in WI-38 CM by 83%. The same oligonucleotide caused a dose dependent reduction in [^{3}H] thymidine uptake into DNA, ranging from 77% at 2 μM to 95% at 20 μM. {Moats-Staats *et al.*, (1993)}.

IGFs in Lung Cancer

The human lung adenocarcinoma CaLu-6 was shown to secrete increasing amounts of immunoreactive IGF-I into its culture medium as a function of time, and these cells were also found to respond mitogenically to the exogenously supplied growth factor. An autocrine loop was suggested by the finding that anti-IGF-I monoclonal antibody could completely abolish the mitogenic effect of autocrine and exogenous IGF-I {Minuto *et al.*, (1988)}. Evidence for an IGF-I autocrine loop has also been presented for small cell lung cancer cell lines *in vitro* {Jaques *et al.*, (1988); Nakanishi *et al.*, (1988)}.

IGFs in Breast Cancer

The MCF-7 breast carcinoma cell line secretes immunoreactive IGF-I, expresses mRNA for IGF-I, and is stimulated to proliferate by exogenous IGF-I {Huff *et al.*, (1986)}. Moreover, in serum-free conditions, the growth of MCF-7 was inhibited by an anti-IGF-I monoclonal antibody {Freed & Herington (1989)}.

An anti-IGF-I receptor monoclonal antibody (αIR_3) also inhibited the mitogenic effect of IGF-II on two other breast carcinoma cell lines, MCF-7L and MDA-231 implying that this receptor mediates the effects of IGF-II {Kent Osborne *et al.*, (1989)}. However αIR_3 was not physiologically inert because binding to the receptor resulted in receptor phosphorylation {Steele-Perkins *et al.*, (1988)}. A role for IGF-I in breast cancer is also supported by the fact that *in vitro* these cells secrete and are responsive to this growth factor {Perroteau *et al.*,

(1986); Huff *et al.*, (1986)}.

IGFs in Pancreatic Cancer

Besides the production of a TGFα-like factor, Ohumura *et al.*, (1990) also reported the detection of immunoreactive IGF-I in the serum-free medium conditioned by the human pancreatic carcinoma cell line MIA-PaCa 2, mentioned above. Evidence for an autocrine loop involving IGF-I in these cells was implicit in the discovery that authentic IGF-I stimulated MIA-PaCa 2 cell growth by 1.45 fold, and an anti-IGF-I receptor monoclonal antibody inhibited their prolifertion. It appears therefore that both IGF-I, and TGFα, are involved in autocrine stimulation of pancreatic carcinoma cell proliferation.

IGFs in other cancers

A teratoma-derived cell line, 1246-3A, produces an insulin-like protein, and its growth was inhibited by an anti-insulin antibody, suggesting autocrine growth control in these cells by an insulin-like protein {Yamada *et al.*, (1988)}.

The possibility of an IGF-I autocrine loop in ovarina cancer cells is suggested by the report of IGF-I and IGF-I receptor coexpression in these cells {Yee *et al.*, (1991)}.

Similarly the growth and differentiation of human colon cancer cells has also been reported to be regulated by IGF-I {Baghdiguian *et al.*, (1992)}.

1.5.5 INTERLEUKINS AND HEMATOPOIETIC GROWTH FACTORS AS AUTOCRINE GROWTH REGULTORS IN HAEMATOPOIETIC NEOPLASIAS

1.5.5.1 Interleukin 3 in Hematopoietic Neoplasias

IL-3 in Leukemias

A common translocation event in pre-B-Cell leukemias (chromosome 5 → 14) which results in the juxtaposition of the IL-3 gene behind an IgH enhancer, results in overexpression of the translocated IL-3 gene. This creates a functional autocrine loop in these cells which is thought to favour leukemogenesis {Grimaldi *et al.*, (1989)}.

Further evidence for the involvement of IL-3 in autocrine transformation was presented recently by Algate & McCubrey (1993). These workers isolated IL-3 independent hematopoietic cell lines from an IL-3 dependent cell line, and found that autocrine transformation was associated with increased IL-3 mRNA stability. This was due to the insertion of an intracisternal A particle provirus into the 3′ untranslated region of the IL-3 gene. The sequence at the site of insertion is associated with cytokine and oncogene mRNA stability. An increase in IL-3 cytokine levels resulting from a more stable transcript, may explain the mechanism of autocrine transformation in these cells.

1.5.5.2 Interleukin 6 in Hematopoietic Neoplasias

IL-6 in Myelomas

Interleukin 6 has been implicated as an autocrine growth factor in human mulitple myelomas {Kawano *et al.*, (1988)}, but evidence for a clear role for IL-6 as an autocrine stimulator in myeloma, as opposed to a paracrine effector, had been lacking {Klien *et al.*, (1989)}. More recent work supports an autocrine mechanism for the action of IL-6 in these malignancies. Takahashi *et al.*, (1993) demonstrated that a non-IL-6 producing myeloma cell line (S6B45) transfected with IL-6 cDNA produced cells that proliferated *in vitro*, and *in vivo*, with the requirement for exogenous IL-6. These cells were shown to express IL-6 mRNA and protein, and could be inhibited by anti-IL-6, and anti-Il-6 receptor monoclonal antibodies.

In another study, the separation of human myeloma cells from a pleural fluid, and bone marrow samples of multiple myeloma patients, was achieved using an immunomagnetic separation technique. This provided cultures of myeloma cells which were 99% pure. Using these cultures,

Borset *et al.*, (1993) found that myeloma cells were not directly responsible for the overproduction of IL-1 and IL-6 which is associated with these disorders. This was because the pure myeloma cultures isolated produced no IL-1 and only small amounts of IL-6.

However, more recently, a cell surface antigen CD40 expressed on the surface of freshly isolated myeloma cells and myeloma cell lines was found to mediate the induction of autocrine IL-6 production in the IL-6-dependent myeloma cell line ANBL-6 {Westendorf *et al.*, (1994)}.

IL-6 in Lymphomas and Plasmacytomas

An autocrine role for IL-6 in plasmacytomas was proposed by Van Damme *et al.*, (1987), and others have presented evidence for a similar involvment in lymphomas {Yee *et al.*, (1989); Shimizu *et al.*, (1988)}.

1.5.6 CYTOKINES AS AUTOCRINE GROWTH REGULATORS IN NONHEMATOPOIETIC NEOPLASIAS

In the last number of years, evidence has emerged for the involvment of hematopoietic growth factors in the growth control of nonhematopoietic malignant cell lines {Berdel *et al.*, (1989); Foulke, *et al.*, (1990); Serve, *et al.*, (1991)}. Some examples of hematopoietic growth factor autocrine loops in nonhematopoietic cancers are discussed below.

1.5.6.1 Interleukin 6 in nonhematopoietic Malignancies

IL-6 in Renal Carcinoma

Interleukin-6 (IL-6) has been reported as a positive autocrine growth factor in human renal cell carcinoma {Miki *et al.*, (1989)}. A more recent publication demonstrated a functional IL-6 autocrine loop in the human renal carcinoma cell line, ACHN {Gruss *et al.*, (1992)}. ACHN cells constitutively expressed IL-6 ligand and its receptor, and were growth arrested by exposure to a neutralising monoclonal antibody against human recombinant IL-6. The same study demonstrated that the expression of IL-6, and its induction of ACHN proliferation, could be inhibited in a specific manner by interferon-γ, and that this inhibition was reversible by recombinant human IL-6.

IL-6 in Melanoma

Evidence has emerged from a series of investigations into melanoma progression to support an autocrine role for IL-6 in this process. Cornil *et al.*, (1991) found that an activity identical to IL-6, which was elaborated by normal dermal fibroblasts, inhibited the growth of early stage melanoma lesions, but not advanced stage melanomas. In a subsequent publication from the same laboratory, purified recombinant IL-6 demonstrated the same stage-specific inhibition, even though melanomas at all stages expressed IL-6 receptor and its signal transducer gp130. Measurement of IL-6 mRNA, and its secreted protein product, revealed that 5/8 melanoma cell lines, resistant to IL-6 induced inhibition, also expressed the transcript of this gene and the IL-6 protein could also be measured in the respective CM. Moreover, antisense oligonucleotides directed against IL-6 were inhibitory in endogenous producer cell lines only, whereas neutralising antibodies against this cytokine were ineffective in reducing growth. Taken together, these results suggested a switch from a paracrine inhibitory role for IL-6, to that of a 'private' autocrine stimulator during the malignant progression in melanomas {Lu *et al.*, (1993)}.

IL-6 in Ovarian Cancer

An autocrine stimulatory role for IL-6 in ovarian cancers was suggested by Watson *et al.*, (1990) who reported the constitutive expession of production of this cytokine in ovarian cells. Using antisense oligonucleotides directed against IL-6, the same group of workers were able to inhibit the proliferation of the three ovarian carcinoma cell lines, OVCAR-3, CAOV-3 and OC-436, by 80-85% with 1-5 μM of antisense oligonucleotide {Watson *et al.*, (1993)}. In the same report, IL-6 neutralising antibodies did not consistently inhibit proliferation, suggesting that the induction of cell proliferation by IL-6 in these cells was effected indirectly. A previous publication from this group using OVCAR-3 and CAOV-3, as well as primary ovarian tumour cultures, found that these cells constitutively produce biologically active IL-6, but their proliferation was not inhibited by anti-IL-6 neutralising antibodies. Proliferation in the same cultures was not affected by exogenously supplied IL-6 {Watson *et al.*, (1990)}.

IL-6 in Lung Cancer

Takizawa *et al.*, (1993) have recently implicated IL-6 as an autocrine growth inhibitor in human lung cancer cell lines *in vitro* (see discussion, Section 4.1.2.2).

1.5.7 BOMBESIN/GRP AS AN AUTOCRINE GROWTH REGULATOR

Gastrin-releasing peptide (GRP), the mammalian equivalent of the amphibian peptide bombesin, was shown to act as an autocrine growth factor for small cell lung cancer *in vivo* {Cuttitta *et al.*, (1985)}. However, it seems that not all SCLCs are dependent on this autocrine loop because Layton *et al.*, (1988) demonstrated that the inhibitory effect of bombesin antagonists on SCLC growth was not mediated through the bombesin receptor. Moreover, not all SCLC cell lines were dependent on bombesin *in vitro.*

1.5.8 AUTOCRINE MOTILITY FACTOR AS AN AUTOCRINE GROWTH FACTOR

It was initially believed that the biological function of autocrine motility factor (AMF) was solely motogenic. However, the first evidence that this factor also has mitogenic activity, as a paracrine factor in normal tissue regeneration, and in tumour dissemination, emerged recently {Silletti & Raz (1993)}. Internalisation of AMF as a complex with its receptor (gp78), following cell surface binding, was shown to be involved in the control of cell motility during metastasis. AMF is also reported to stimulate the lung colonising ability of tumours *in vivo* {Watanabe *et al.*, (1993).

1.6 PRIMARY CELL CULTURE OF LUNG CARCINOMAS

The cellular origin of lung cancers is still a contentious issue. However, there is biological and histological evidence for the existence of a common progenitor cell type {Carney & De Leij (1988)}. Histologically, four major types of lung cancer are defined, namely, squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and small cell carcinoma (SCLC). For clinical and therapeutic purposes lung cancers are divided into two groups, Small Cell and Non-Small Cell Lung Carcinomas. Small cell lung cancers (SCLC), which account for approximately 25-30% of all lung cancers, are distinguished from non-small cell lung carcinomas (NSCLC) by their etiology, pathology and clinical features. SCLC has traditionally been divided into two 'classic' and 'variant' subtypes {Carney *et al.*, (1985); Gazdar *et al.*, (1985)}. The small cell or classic type of SCLC accounts for approximately 70% of all SCLCs, and is distinguished from the variant form by its relatively more differentiated status, and by expression of high levels of neuroendocrine markers (e.g the enzymes neuron specific enolase, creatine kinase, and L-dopa decarboxylase, as well as neuropeptides such as bombesin and neurotensin). Classic SCLCs also demonstrate slower proliferation rates *in vitro* in comparison to the variant subgroup. A recently revised histological classification system {Cook *et al.*, (1993)} takes into account the existence of a 'transitional' type of SCLC which exhibit some of the properties of classic and variant subgropus.

Non-small cell lung carcinomas exhibit more phenotypic heterogeneity than SCLC {Gazdar *et al.*, (1985)} and are less sensitive to cytotoxic therapies. The degree of differentiation varies from being primarily undifferentiated in large cell carcinoma, through poor, to moderate, to well differentiated in the case of adeno- and squamous cell carcinomas. Due to their propensity to undergo terminal keratinisation, squamous cell carcinomas have been particularly difficult to establish as continuous cell lines.

The very low frequency of establishment of permanent NSCLC tumours *in vitro* from primary tumours has already been documented {Sherwin & Richters (1975)}. Successful primary cultures of these tumours would have many important applications in the study of the biology of NSCLC. An application of particular interest concerns the possibility of using individual tumours as a source of cells for *in vitro* cultures. These cultures might then be used to predict drug sensitivity profiles for the donor patient *in vivo*. If this procedure could be performed routinely then it would enable clinicians to design more rational chemotherapy regimen for this otherwise clinically resistant class of lung cancer. Recent studies examining such an approach

with NSCLC tumours have used dye exclusion-based assays to assess drug sensitivity profiles {Wilbur *et al.*, (1992)}. However, difficulty in the routine establishment of NSCLC tumours *in vitro* has hampered progress in this direction, especially when the initial numbers of tumour cells obtained at biopsy are limited {Shaw *et al.*, (1993)}. In general, the relevant epithelial component of NSCLC tumours tends to undergo terminal differentiation, thereby losing its capacity to proliferate *in vitro*.

1.6.1 Proliferation and differentiation of NSCLC tumour cells *in vitro*

The reasons for the loss of *in vitro* proliferative potential by epithelial-derived tumour cells is as yet poorly understood. However, given the heterogeneous composition of tumour tissue, a complex series of growth factor interactions, operating via autocrine and paracrine systems, can be hypothesised *in vivo*. Functional loss of one or more pathways, as a consequence of *in vitro* isolation, may explain the reduced proliferative capacity of NSCLCs in culture. The associated induction of growth-inhibitory or differentiation-promoting pathways may therefore be a response to, or a consequence of, this loss in proliferative potential.

In normal human bronchial epithelial (NHBE) cells grown in serum-containing medium, transforming growth factor β1 (TGF-β1) was implicated as the major growth inhibitor present in serum. Growth inhibition by TGF-β1 was correlated with the initiation of terminal squamous differentiation of NHBE cells {Masui *et al.*, (1986)}. These workers used the 'immortalised' normal human bronchial epithelial cell line, BEAS-2B, as a model for lung carcinogenesis, and demonstrated that these non-tumourigenic cells were growth inhibited, and induced to differentiate terminally, in response to TGF-β1. However, Masui *et al.*, (1988) and other investigators {Coffey *et al.*, (1988)} demonstrated that lung carcinomas are relatively insensitive to differentiation-inducing and inhibitory effects of serum and TGF-β1. Subsequent studies have also found that cells which had been transfected with oncogenes, and became tumourigenic, also developed a resistance to the inhibitory effects of TGF-β1 {Pfeifer *et al.*, (1989)}.

Transfection studies in normal human bronchial cells with mutants of the p53 tumour suppressor gene, also support a connection between the loss of sensitivity to TGF-β1, and the tumourigenic conversion induced by this genetic lesion {Gerwin *et al.*, (1992)}. Using the same model cell line as Masui *et al.*, (1986), these investigators found that transfected wild-type (WT) p53 suppressed the colony forming efficiency of BEAS-2B cells in monolayer culture, while mutant (MT) p53 enhanced it. Clones of BEAS-2B transfected with MT p53 were less responsive to

the induction of terminal squamous differentiation by TGF-β1 and serum than the parent BEAS-2B cells, and these TGF-β1-resistant clones also formed tumours in athymic mice.

The role of polypeptide growth factors as positive and negative regulators in the development of lung carcinomas may be viewed in the context of cellular senescence where the default program for cell growth is to stop dividing and differentiate terminally.

Another facet of the *in vitro* culture of primary tissue in serum-supplemented medium, is the presence of stromal cells. Fibroblasts are the main type of stromal cells associated with tumour tissue, and these tend to outgrow their epithelial counterparts in *in vitro* cultures. Careful preparatory techniques are the only satisfactory alternative to selective media in avoiding excessive numbers of fibroblasts. The disruptive techniques required to liberate epithelial cells from tumours can significantly influence the yield of stromal cells obtained, and the presence in serum of the powerful fibroblast mitogen and chemoattractant, platelet-derived growth factor {Ross *et al.*, (1986)}, is probably one of the principal contributers to excessive fibroblast proliferation.

1.6.2 The role of stromal cells in the growth of NSCLC cells *in vivo* and *in vitro* — paracrine interactions

The function of stromal cells in the generation of tumour stroma is similar to that seen in the normal wound healing process. An understanding of the respective processes may help to explain the role of stromal cells in tumourigenesis and lead to new intervention therapies. Dvorak (1986) has compared the two processes in detail and described tumours as 'wounds that do not heal'. In this hypothesis, he suggests that the generation of a temporary extravascular fibrin-fibrinogen gel — an important process in the initial stage of normal wound healing, is pre-empted and subverted in successful tumours, by means of their ability to continuously deposit a fibrin-fibrinogen matrix. This matrix facilitates progressive tumour growth, principally by encouraging neoangiogenesis. A supply of nutrients and oxygen via the blood is essential if any tumour is to grow beyond 1-2 mm^3. The fibrin-fibrinogen gel may also block the host's immune response to the malignantly transformed cells by inhibiting the infiltration of macrophages and lymophocytes into the tumour. In support of this hypothesis, Seitz *et al.*, (1993) have measured higher plasma levels of a number of markers for coagulation and fibrinolysis in patients with small and non-small cell carcinomas. These included the thrombin-antithrombin III complex, prothrombin activation fragment F1 and F2, plasmin-α2-antiplasmin

complex, and D-dimer. No difference was reported in the levels of activation markers between the SCLC and NSCLC patients, but those with limited disease had lower levels than those with extensive disease.

The main difference between the deposition of the fibrin-fibrinogen gel in the normal process versus that seen in tumour tissue, is the trigger initiating matrix and/or clot formation. In the normal process, initiation is induced by wounding, and the associated increase in vascular permeability is temporal. In tumours however, the ability of these cells to constitutively secrete a vascular permeability factor (VPF), {also known as vascular endothelial growth factor, (VEGF)}, results in continuous leakage of fibrinogen, plasminogen and clotting factors into the extravascular space by inducing increased permeability of the microvasculature. This leads to continuous matrix deposition {Senger *et al.*, (1983)}.

In the normal wound healing process, the movement and proliferation of fibroblasts into the wound is stimulated by the release of PDGF from platelets at the site of injury. Given that platelets are largely absent in tumour tissues, the role of a mitogen and chemoattractant for fibroblasts was previously ascribed to other growth factors such as TGFα. However, recent studies examining the expression of genes for the c-sis/platelet-derived growth factor (PDGF)-2 protooncogene (i.e. PDGF-BB) and its receptor, the type β form of the platelet-derived growth factor receptor (PDGF-Rβ), have provided a molecular basis to connect chronic injury of epithelial cells with the induction of the genes for c-*sis*/PDGF-2 and the PDGF-Rβ in such cells, leading to the development of proliferative disorders. For example, reversible co-expression of mRNA for the growth factor c-sis/PDGF-2 and its receptor, PDGF-Rβ, was demonstrated *in vivo* in swine skin epithelial cells, in response to chronic injury {Antoniades *et al.*, (1991)}.

Chronic injury has also been implicated in the development of proliferative disorders such as idiopathic pulmonary fibrosis (IPF) and lung cancer in humans {Antoniades (1991); Thornton *et al.*, (1992)}. *In vivo* expression of c-sis/PDGF-2 mRNA and protein, but not its receptor in IPF, has been demonstrated by Antoniades *et al.*, (1990). The same group went on to show that the mRNAs for both c-sis/PDGF-2 and its receptor, as well as the specified proteins, were expressed in lung cancer epithelial cells *in vivo*, but not in their nonmalignant counterparts {Antoniades *et al.*, (1992)}. In a more recent publication, Forberg *et al.*, (1993) have demonstrated the expression of functional type β PDGF receptors in the large cell lung carcinoma cell line H-157.

Taken together, these studies provide evidence to suggest that IPF may be a stage in the malignant progression in lung epithelial cells. In this scenario, paracrine production of PDGF by epithelial cells in response to injury would lead to fibrosis in cases of chronic injury. The growth factor would become an autocrine stimulator for these cells only after they had acquired the ability to express functional PDGF type β receptors, either in the process, or as a consequence of malignant progression. If such a mechanism is operative in lung cancer it supports the notion that a normal physiologic wound healing process may be subverted in cancer cells resulting in unregulated autonomous growth.

Given that lung epithelial cells can secrete PDGF, it seems plausible that the excessive proliferation of fibroblasts in primary cultures of malignant lung epithelia may be due in part to paracrine action of PDGF produced by malignant epithelial cells.

In contrast to the results obtained in this project, some workers have reported that the presence of fibroblasts were beneficial to the establishment of primary epithelial cell cultures {Dietal *et al.*, (1987)}. In support of a paracrine function for stromal cells in lung epithelial cell growth, Stuart Aaronson's group isolated two paracrine-acting mitogens for epithelial cells in medium conditioned by a human embryonic lung fibroblast cell line. The first such factor characterised was keratinocyte growth factor (KGF) {Rubin *et al.*, (1989)}. Unlike other members of the FGF family, KGF is not mitogenic for fibroblasts, but it does stimulate the growth of tracheobronchial epithelial cells {Finch *et al* (1989)}. In normal skin epithelial cells, KGF has been implicated as the major player in mesenchyme-driven epithelial cell proliferation, from the observation that KGF mRNA expression was induced 160-fold, one day after injury {Werner *et al.*, (1992)}. The induction of KGF mRNA was accompanied by increased expression of its receptor, and secretion of the growth factor preceeded epithelial cell proliferation. The corresponding induction of aFGF, bFGF and FGF-5 mRNAs in the same cells, was in the order of 2-10 fold, and no expression of mRNA for FGFs -3, -4 or -6 were detected.

The second paracrine mitogen for epithelial cells in the lung was found to be a variant form of hepatocyte growth factor/scatter factor (HGF/SF) and this was expressed by stromal fibroblasts derived from adult lung, skin, gastrointestinal tract and prostate {Rubin *et al.*, (1991)}.

A more recent publication from this group reported the purification of KGF and HGF/SF from medium conditioned by human adult lung fibroblasts. The KGF and HGF/SF proteins isolated

from this fibroblast-conditioned medium (by heparin-Sepharose chromatography), were found to be the principal heparin-binding mitogens for primary adult rat alveolar type II cells, produced by these lung fibroblasts. Whether such fibroblast-derived growth factors have a paracrine function in malignant lung tissues remains to be established, but a role in lung injury was proposed by the authors {Panos *et al.*, (1993)}. Changes in the level of expression of KGF by fibroblasts in adult lung, in response to injury, might contribute to the neoplastic progression in lung epithelial cells by stimulating the proliferation of epithelial cells. This expanded population of epithelial cells, might then give rise to a more malignant subpopulation, by the acquisition of the ability of a single cell (or cells) to coexpresses the KGF ligand; thereby generating an autocrine loop in this system.

Yoshinaga *et al.*, (1992) detected a protein with indistinguishable immunological and biological properties to HGF/SF which was produced by two human lung cancer cell lines, namely PC-1 and PC-13. However, these authors were not able to detect the HGF/SF receptor (the c-met proto-oncogene) by western analysis in either cell line, and neither responded to exogenous HGF/SF. More recent evidence has implicated HGF/SF as an autocrine growth regulator for normal human bronchial epithelial (NHBE) cells and NSCLC cell lines. In this case, the investigators were able to detect HGF/SF mRNA in NHBE and NSCLC cells, and they detected immunoreactive HGF-like protein in the cytoplasm of these cells in culture using an anti-recombinant human (rh) HGF/SF antiserum. The level of phosphorylation of the c-met/HGF/SF receptor, which was constitutively phosphorylated in these cultured cells, was decreased in the presence of anti-rhHGF/SF antiserum. Interestingly, while the growth of the NSCLC lines studied was inhibited by 45% using the anti-rhHGF/SF antiserum, the proliferation of NHBE cells were stimulated with this antibody. The authors suggest that this might be explained if the antiserum neutralises a mitogenically incative variant of the HGF/SF gene encoded by an alternately spliced 1.3 kb transcript of the gene. The 1.3 kb HGF/SF transcript, which encodes a 28 kDa protein, is present at a ten-fold higher concentration in NHBE cells than the 6 kb transcript which encodes the active 63 kDa form. However, the smaller HGF/SF acts as a competitor for the HGF receptor and may effectively neutralise the mitogenic effects of the larger HGF/SF species in NHBE cells. The anti-rhHGF/SF may be acting to unmask the mitogenic effects the co-expressed larger HGF/SF isoform in NHBE cells {Tsao *et al.*, (1993)}. Such a shift in growth factor expression patterns resulting in the acquisition of a functional HGF/SF autocrine loop may represent another example of the changes in growth regulation pathways that occur in the malignant progression of NSCLC. This idea is supported by reports

that the majority of primary NSCLCs examined demonstrate expression of the c-*met*/HGF/SF receptor {Prat *et al.*, (1991)}. In contrast to these results, other investigators have reported that HGF/SF inhibited the *in vitro* growth of selected SCLCs {Rygaard *et al.*, (1993)}. This observation underlines the potential for different growth regulatory pathways in these two types of lung cancer.

Other recent work supporting this hypothesis was aimed at defining the factors governing the ability of NSCLC primary tumours to be established as monolayer cell lines *in vitro* {Liu & Tsao (1993)}. These authors found that samples from which cell lines arose, had higher mean mRNA levels for TGFα and the c-met-protooncogene, than tumours which did not. They also found it easier to establish cell lines from poorly differentiated tumours, especially adenocarcinomas. In the same study, 8/29 unselected human NSCLCs were successfully established as cell lines with heterogeneous nutritional requirements.

Aggressive cancer cells often display characteristics typical of mesenchymal cells such as fibroblasts. These features include the production of growth factors which are normally elaborated by stromal cells, for example HGF/SF {Yoshinga *et al.*, (1992)}. Release of tumour cells from stromal dependency appears to be a recurrent theme in the malignant progression of a number of different types of cancer. An example was outlined above, with acquisition of a functional IL-6 autocrine loop in the malignant progression of melanoma. In yet another example of this mechanism of malignant progression, the influence of heparin-binding FGFs and stromal cells on the development of growth autonomy in prostate epithelial cells was examined. Using a slow growing, nonmalignant, rat prostate tumour model grown in syngenic hosts, the authors found that a change in the ligand specificity of the FGF-2 receptor from recognising the stromally elaborated FGF-7 (also called KGF) to an isoform which recognised FGF-2 (i.e. bFGF) accompanied growth factor independence from stromal cells. The change in ligand specificity was effected at the level of RNA processing by an 'exon-switching' mechanism, and this was also followed by the activation of the gene for its cognate ligand, bFGF. Activation of embryonic FGF-3 and FGF-5 ligand genes, along with the normally stromal FGF-R1 gene, also coincided with malignant progression in this model system {Yan *et al.*, (1993)}.

The clonal proliferation of normal respiratory rat tracheal epithelial (RTE) cells has been optimised in Ham's F12 basal medium supplemented with bovine serum albumin (BSA), bovine pituitary extract (BPE), insulin, hydrocortisone, epidermal growth factor and cholera toxin

{Nettesheim *et al.*, (1987)}. Using a chemically-induced carcinogenesis model in this serum-free system, Thomassen (1993) studied the *in vitro* growth factor requirements in the malignant progression of RTE cells. By measuring monolayer colony forming efficiencies of RTE cultures initiated from cells at sequential stages of the malignant progression, it was found that these cells displayed reductions in the requirement for supplements concomitant with malignant progression. Preneoplastic cells, capable of enhanced proliferation with respect to their normal counterparts, no longer required EGF and cholera toxin. However, in previous studies, such cells were found to be incapable of forming tumours when injected into nude mice, but these populations did contain cells which could progress to adeno- and squamous cell carcinomas *in vivo* {Pai *et al.*, (1983)}. In the study of Thomassen (1993), it was demonstrated that neoplastically transformed RTE cells could proliferate with only BSA and BPE.

Although these studies did not lead to a complete definition of the growth requirements of RTE cells, they did support the hypothesis that preneoplastic transformation of RTE cells was associated with a reduction in the number of exogenously added growth factors required for their clonal proliferation, and not as a consequence of their acquired resistance to inhibitory factors in selective media which contained serum. This conclusion was supported by the results of Terzaghi-Howe (1989) who found that serum added to serum-free culutres of RTE cells did not affect the long-term proliferative potential of these cells.

Excessive proliferation of fibroblasts may be expected to cause depletion of essential media nutrients which are deleterious to primary epithelial cells in *in vitro* culture. However, in the light of recent evidence for the elaboration of paracrine-acting growth factors by lung fibroblasts, the production of paracrine inhibitory factors, or differentiation-inducing factors, by stromal cells, may also account for the negative regulation of NSCLC cells *in vitro*.

In order to separate the complex processes involved in the regulation of growth and differentiation in normal and neoplastic lung tissue, the unique nutritional requirements and the contribution of cell-secreted factors to the overall biology of malignant lung must be established. This can best be achieved by isolating the constitutive cell types that make up these tissues. In this way the complex interactions within tissues can begin to be deciphered. The physical separation of interactive cell types in tissues, and the selective nature of the *in vitro* culture technique, together combine to highlight the deficiency of appropriate growth requirements for particular cell types, and as such, can be helpful in the identification of these factors.

1.6.3 Selective media for NSCLC

The search for selective media for particular cell types has been a long and painstaking process. To date, the number of cell types for which selective media have been developed are relatively few. Most notable in terms of their selectivity is the MCDB series which were devised to meet the different requirements of non-transformed cells such as normal skin keratinocytes and fibroblasts {Ham & McKeehan (1979)}. A significant finding in the development of these media was that low Ca^{2+} levels avoided the induction of differentiation in skin epitlelial cells. This is manifested in kerationocytes by the expression of keratins and the formation of cornified envelopes. The MCDB series of media have since formed the basis of many other defined media including a modification of MCDB 151 for normal human bronchial epithelial cells {Lechner & LaVeck (1985)}. However, the inclusion of cholera toxin in such media appears to be of limited benefit, given that this agent only partially antagonised the differentiating effects of TGF-β in the studies of Masui *et al.*, (1986). Several approaches have been outlined for the development of serum-free media {Maurer (1992)}.

Simms *et al.*, (1980) developed a selective medium for SCLC based on RPMI-1640 basal nutrient medium supplemented with hydrocortisone, insulin, transferrin, 17β-estradiol and selenium. However, this medium, designated 'HITES', did not support the growth of NSCLC {Gazdar & Oie (1986)}. It was subsequently found that substitution of HITES medium with 2.5% foetal calf serum was beneficial for the initiation of some SCLCs {Carney *et al.*, (1985)} and some workers routinely split tissue samples between supplemented and non-supplemented HITES medium in an effort to improve success rates with SCLC {Baillie-Johnson *et al.*, (1985)}. The fact that serum supplementation may still be necessary in some cases of small cell carcinoma underlines the variability in growth factor requirements, even for tissues of the same cell types (as they are currently classified). In any event, the majority of SCLCs grow as floating aggregates {Carney *et al.*, (1985)} — a factor which facilitates the removal of adherent fibroblasts. In addition, the principal source of tissue for *in vitro* culture of SCLC (bone marrow aspirates) ensures that a significantly lower percentage of stromal cells are harvested in samples of this type of lung cancer.

Another modification of RPMI-1640 basal medium was developed as a selective medium for human lung adenocarcinomas using collagen/fibronectin precoated flasks and called ACL-3 {Brower *et al.*, (1986)}. The supplements used in the ACL-3 formulation were, insulin, transferrin, hydrocortisone, selenium, epidermal growth factor, bovine serum albumin, sodium

pyruvate, glutamine and triiodothyronine. Of these supplements, only insulin and transferrrin were essential for optimal growth. Further modification of ACL-3 by Gazdar & Oie (1986b) involved reducing the quantities of EGF and BSA. This made ACL-4 a cheaper and more useful formulation for the primary culture of human lung adenocarcinoma cells, but the resulting formulation, designated ACL-4, like the original ACL-3, did not support the growth of squamous cell carcinomas or small cell carcinomas.

Although RPMI-1640, the basal medium used to prepare the ACL-4 formulation, could be substituted with a 1:1 mixture of Ham's F12 and Dulbecco's modified essential medium (DMEM), serum supplementation was still required in the initial stages for the optimal growth of some tumours. This was because neither ACL-3 nor ACL-4 contained high molecular weight attachment factors and therefore tended to select for non-adherent cell strains. Attachment factors appear to be particularly important for the *in vitro* growth of squamous cell carcinomas and these are present in serum. Using a serum supplemented partially defined medium, based on Rheinwald's basal medium formulation, Gazdar & Oie (1986a) were able to achieve a 20% success rate in the establishment of human squamous cell lung carcinomas *in vitro*.

One hypothesis which might explain the restricted *in vitro* growth of lung squamous cell carcinomas is the differentiation status of the lesion at the time of resection. In the normal bronchial tree and alveoli, any one of a number of proliferating epithelial cells, may in theory give rise to a precancerous lesion. These include type II pneumonocytes, Clara cells, mucus cells, and basal cells. It is noteworthy too that in histological terms, squamous metaplasia has not yet been considered a true malignant state {Brambilla *et al.*, (1993)}. If this hypothesis can be translated into biological terms to mean that a greater part of a tumour cell population may not yet have acquired an appropriate functional autocrine growth factor loop(s) *in vivo* at the time of resection, then cultures of tumour cells established from such lesions may be incapable of liberating themselves from dependence upon either autologus, or heterologously produced, growth factors. Therefore, tumour cells isolated *in vitro* at this particular stage of malignant progression *in vivo* will produce insufficinet endogenous quantities (or none at all), of the growth factor(s) vital for proliferation *in vitro*. If these factors are not supplied by the growth medium, then proliferation will not occur. This notion is supported by reports in the literature that cell lines are more readily established as *in vitro* cultures from metastatic NSCLC lesions than from primary tumour sites {Shaw *et al.*, (1993)}. It is also consistent with the documented cases of malignant progression outlined above, where the transition from paracrine growth

factor dependency in the premalignant state accompanies the autonomous production of these growth factors by malignant cells.

A candidate for such an autocrine loop in squamous cell carcinomas of the lung, is transforming growth factor α. Overexpression of the receptor for this ligand (EGF receptor) has been widely reported in human squamous cell lung carcinomas {Berger *et al.*, (1987); Harris (1990); Veale *et al.*, (1989)} and amplification of the EGF-R gene has frequently been associated with overexpression {Ozanne *et al.*, (1986); Hunts *et al.*, (1985)}. These findings have implicated TGFα and amphiregulin as autocrine growth regulators in this cell type. Furthermore, TGFα has been found to be most frequently mitogenic in serum-free media for malignant cells in culture {Herlyn *et al.*, (1990)}. The proliferative response of two lung squamous cell carcinoma cell lines established in this laboratory DLRP and DLKP to TGFα was not very significant.

1.7 THE PURPOSE OF THIS THESIS

In view of the poor success rates reported for the successful primary culture and establishment of permanent cell lines from NSCLCs, particularly squamous cell carcinomas (SCCs), attempts were made in the work described in this thesis to improve the *in vitro* culture methodology for this type of lung carcinoma.

1.7.1 The use of malignant effusions in primary cell culture

A report in the literature presented evidence for the presence of a growth stimulator(s) in malignant effusions (ME) associated with primary tumours of different histopathological type, which stimulated the efficiency of colony formation by fresh human tumour cells in soft agar {Yen *et al.*, (1986)}. This factor(s) appeared to be particularly active on lung and colon cancers, especially at low plating densities, but it was not source specific. Another study presented evidence for a tumour-specific growth factor present in ascitic fluid from patients with ovarian carcinoma, which stimulated the monolayer growth of primary ovarian tumour cells as well as the growth of an established ovarian carcinoma cell line, HEY. On the basis of the change in cytoplasmic Ca^{2+} levels induced in HEY cells by fluid-derived growth factor(s), the species involved appeared to be distinct from purified prepartions of PDGF, TGFβ, EGF, TNF, IL-1, IL-2, FGF, insulin, thrombin, vasopressin, angiotensin, as well as α and γ-interferons {Mills *et al.*, (1988)}.

In view of the need to improve methods for primary culture of lung tumour tissues, the possibility that malignant effusions might contain growth stimulators for the squamous cell type of lung cancer cells in monolayer cultures, was investigated in this project. Three human lung SCC cell lines DLRP, DLKP, and SK-MES-1 were used as indicator cell lines in preliminary experiments. Two of these lung cell lines, DLRP and DLKP, had recently been established in this laboratory; the former in the course of this project.

The long-established cell line HEp-2 {Moore *et al*,. (1955)} was used as a reference cell line to assess the specificity of stimulatory activity in MEs, and when tested, was found to respond to mitogenic species in all of MEs assayed. HEp-2 displays marker chromosomes for the cell line HeLa (which appears to have contaminated many of the cell lines established during this period) and is considered therefore to be a variant of HeLa. In practice, HEp-2 was found to be a useful indicator cell line for the detection of growth-promoting activity in MEs. This was due to the growth characteristics of this cell line, which include its shorter doubling time and

reduced serum requirement at clonal plating densities, relative to the lung cell lines used. In addition, the minimum number of HEp-2 cells required to give a detectable level of growth in a monolayer assay system was ten-fold lower than that required for the lung cell lines DLRP, DLKP, and SK-MES-1.

1.7.2 The potential of the Hep-2 cell system as a source of growth factors for human lung squamous epithelial cells *in vitro*

Pilot experiments with HEp-2 at low serum concentrations and low plating densities were impressive in terms of proliferative capacity. The growth performance of HEp-2 under these stringent conditions provided an additional incentive for using HEp-2, because it suggested the production of autocrine growth factor activity by this cell line *in vitro*. This was supported by the mitogenic response of HEp-2 to a panel of growth factors, including at least two growth factors (TGFα and IGF-I) which may be important in the proliferation of NSCLCs *in vitro*. In addition, HEp-2 growth was stimulated by media conditioned by a variety of histologically different carcinoma cell lines, including the lung cell line DLKP (data not shown).

It was in this context that the HEp-2 cell system was examined in the hope of isolating a hitherto unidentified growth factor(s) for primary lung epithelial cells. An obvious starting point in the search for a putative NSCLC growth factor(s) would have been in cell lines derived from NSCLC tumours. Unfortunately, preliminary attempts to identify autocrine stimulatory species in the two newly estasblished NSCLC cell lines DLKP and DLRP were not encouraging (Section 3.1.8). The inability to detect autocrine activity in either DLRP or DLKP, together with the relatively slow growth rates associated with the maintenance of lung SCCs, mitigated against further investigation of these systems in the pursuit of growth factor(s) with the potential for improving lung SCC growth *in vitro*.

By comparison, HEp-2 provided a promising alternative to either of the lung cell lines tested. The elucidation of the HEp-2 autocrine mechanisms and the partial characterisation of the responsible growth factors supported the original suppostion that multiple growth stimulatory species might be active in the autocrine stimulation of this cell line. Fractionation of HEp-2 conditioned medium (CM) on the basis of molecular mass was performed by ultrafiltration. This provided initial evidence for the presence of several autocrine stimulatory species. These appeared in the intermediate (10-30 kDa or R10-30 fraction) and large molecular weight (MW) range (i.e. $>$ 30 kDa, i.e. the R30 fraction). The stimulatory activity in a 10x concentrate of

the R30 fraction was masked by an inhibitory activity which could be removed by dilution to manifest the presence of the stimulatory species in the same fraction. A low MW inhibitory species was also identified in the 1-5 kDa MW range, but there was no evidence for bifunctional growth regulation in this fraction. When these findings were verified by gel filtration chromatography, the HEp-2 autocrine stimulatory activity was separable into several sizes, and one band of low MW inhibitory activity was confirmed. Physicochemical stability studies on stimulatory activity in CM revealed that the 10-30 kDa fraction might be more sensitive to protease than the larger MW fraction. A similar difference in sensitivity was noted between the mitogenic activity in late versus early collections of unfractionated (crude) CM taken from the same culture, with the earlier collection being more resistant to proteinase inactivation. The autocrine effect in the 10-30 kDa fraction, and to a lesser extent that in the $>$ 30 kDa fraction, was sensitive to inhibition by soyabean trypsin inhibitor, suggesting a role for a trypsin-like proteinase in HEp-2 autocrine growth control.

A number of chromatographic systems were employed to try to separate and characterise the stimulatory activities in HEp-2 CM. Phenyl-Sepharose chromatography of the 10-30 kDa fraction confirmed the presence of several stimulators; one of which appeared to be hydrophobic, while crude CM was found to contain several autocrine stimulatory species with varying degrees of affinity for immobilised heparin. Apart from the FGF and non-FGF family of heparin-binding growth factors, several other classes of proteins are known to have an affinity for immobilised heparin {Zhou *et al.*, (1992)}. These include adhesive matrix proteins, and serine proteinase inhibitors; both of which are implicated in the control of cellular proliferation. The elaboration of several heparin-binding mitogens by HEp-2 also supported the possibility that more than one such species might be active in the autocrine effect. It was important therefore to further characterise the nature of this activity.

When cation-exchange chromatography was investigated as a potential step in the separation of the active stimulatory species in crude HEp-2 CM, two mitogenic activities and a third species with inhibitory activity bound to the matrix. The most active mitogenic fraction from the ion-exchange step was further separated by heparin affinity chromatography. This protocol had been used by others in the purification of acidic and basic FGFs, and it was attempted here to check for growth factor activity with similar affinity in the HEp-2 system. The results of an exploratory two-step separation protocol demonstrated the presence of heparin-binding mitogens with affinities coincidental with those of FGF-1 and FGF-2 at 1.0 M and 1.5 M NaCl

respectively {Gospodarowicz *et al.*, (1984); Klagsbrun & Shing (1985)} but it was clear that not all of the autocrine mitogenic activity in this semi-pure ion-exchange fraction was heparin-binding.

The heparin-binding HEp-2 mitogens were considered of more interest in terms of further characterisation, given that abundant levels of two members of the FGF receptor family, FGFR-3 and FGFR-4, have been detected in human foetal lung tissue {Partanen *et al.*, (1991)}. More recently, the FGFR-1 gene product was detected by immunolocalisation in the basal epithelial cell layer of the human respiratory tract {Hughes & Hall (1993)}. In the context of the FGF receptor expression pattern in lung tissue, and the known redundancy of FGF receptors due to exon swtiching, the prospect of isolating a novel FGF with mitogenic activity for lung epithelial cells in medium conditioned by HEp-2 was considered worthy of pursuit. Although little is known of the receptor specificities of the oncogenic FGFs, as distinct from the prototypic acidic and basic FGFs, a complex network of ligand/receptor interactions is implied from the data gathered to date. In addition, a role for the normally developmentally regulated FGFs in the carcinogenic process has been emerging in recent publications.

Before any further purification work was contemplated, the contribution of FGF-1 (aFGF) and FGF-2 (bFGF) to the overall autocrine effect in crude HEp-2 CM was examined. The use of anti-FGF neutralising antibodies revealed that a significant amount of the autocrine stimulatory activity in crude HEp-2 CM, and fractions separated by ultrafiltration, could be attributed to a bFGF-like species. However, attempts to investigate the effects of more specific inhibition of aFGF and bFGF gene expression using antisense oligodeoxynucleotides did not support a major role for bFGF in HEp-2 cell proliferation.

2.0 MATERIALS AND METHODS

2.1 PREPARATION OF CELL CULTURE MEDIA

2.1.1 Water quality

Highly purified water is essential for the preparation of cell culture media. Even trace quantities of some elements can result in toxicity if not removed from the water source. Water used in the production of media and all solutions coming in contact with cells in culture was purified to HPLC grade before use. The qualitiy of mains water supply necessitated prefiltration of the supply to remove gross particulates. This was followed by a double distillation step (ELGA) or by passage through a reverse osmosis system (Milli-Q). It was then passaged through two ion-exchange filters, and a carbon filter to remove organic solutes. Finally a 0.22 μm cellulose acetate filter produced 'ultrapure' water which was monitered for purity with an on-line conductivity meter. Water with a resistivity in the range 10-18 megaOhms/cm was sufficiently pure for use in tissue culture media preparation.

2.1.2 Glassware Treatment

Items of glassware used for cell culture (roller bottles, media bottles, spinner flasks, glass universals for conditioned media treatment etc.) were first soaked in a warm 1% (v/v) solution of the non-toxic detergent RBS (R. Borghraef) for 1-2 hours. They were then individually scrubbed with bottle brushes before being rinsed in tap water (at least once). This was followed by three separate rinses in distilled water before a final rinse in ultrapure water. Metal bottle caps were washed and rinsed separately to avoid contamination of bottle rinses with leached metal ions.

2.1.3 Sterilisation

All non-sterile glassware such as media bottles, spinner flasks, roller bottles, etc. as well as non-sterile plastics were autoclaved before use (121°C/15 psi/20 minutes). Instruments for primary cell culture including scalpels, blades, scissors, were sterilised under the above conditions, as were phosphate buffered saline (PBS-A, Ca^{2+} and Mg^{2+} free), HEPES (4-[2-HydroxyEthyl]-PiperazineEthaneSulphonic acid) buffer, $NaHCO_3$, EDTA (EthyleneDiamineTetraacetic Acid), HCl and NaOH solutions as well as water for media preparation. For unstable reagents such as cell culture media, protein solutions etc, filter-sterilisation was employed using a sterile disposable 0.22 μm filter (Millex-GV, Cat.No:SLGVO25BS). Low protein-binding filters were used where appropriate. Larger volumes of liquid for filter-sterilisation, such as culture medium were filtered through a 'bell' filter (Gelman, G.14238) into sterile 500 ml bottles using a peristaltic pump (Braun FE411).

2.1.4 Siliconisation

Spinner flasks washed in the prescribed way must be coated with trimethyldichlorsilane before use to avoid attachment and growth of cells on the vessel walls. To this end a 2% solution of this agent in 1,1,1-trichloroethane (BDH, Cat.No:33164) was rinsed onto all the inner surfaces of the vessel. This procedure must be performed in a fume hood using a glass pipette. Excess reagent is poured off and the vessel allowed to dry at room temperature in the fume hood followed by 2 hours at 37°C. The vessel was then rinsed three times in distilled H_2O followed by a rinse in ultrapure H_2O before autoclaving.

2.1.5 Basal growth media

The preparation of basal media were carried out under sterile conditions in a class II laminar flow cabinet. Basal media are available commercially as 10x sterile liquid concentrates or in powdered form. The liquid concentrates are convenient for the preparation of smaller quantities but sometimes problems arise with solute precipitation during storage. Powdered media require filter-sterilisation but have the advantage of being prepared fresh. In this work a 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle Medium (DMEM) was used for routine maintenance of most cell cultures. Most basal media were prepared from liquid concentrates but DMEM was prepared from both liquid and powdered stocks.

2.1.5.1 Preparation of media from powder

Ham's F12 was made up exclusively from a powdered stock in batches of 5 litres (Gibco, Cat.No: 072-1700). The powder was dissolved together with 5.88g of $NaHCO_3$ in 4750 mls of ultrapure H_2O while stirring. 100 mls of 1M-HEPES (Sigma, Cat.No: H9136) was added and the pH adjusted to 0.2-0.3 pH units below the desired final pH (pH 7.4 - 7.5) using 5M-NaOH. The solution was brought up to 5 litres with ultrapure H_2O and then sterilised by filtration in a laminar flow cabinet. The entire batch was dispensed into 500 ml lots and aliquots of each were removed for sterility checks. The freshly prepared media were labelled with the date and pH at preparation and stored in the dark at 4°C. They were used only after the sterility checks showed no signs of contamination.

2.1.5.2 Preparation of media from liquid concentrates

Table 2.1.5.2 contains a list the constituents for the preparation of 500 ml lots of three basal media used in these studies.

Table 2.1.5.2 Preparation of basal media from 10x liquid stocks

BASAL MEDIUM	MEM	DMEM	RPMI-1640
Supplier	**Gibco**	**Gibco**	**Gibco**
Cat. No:	**042-01430M**	**042-02501M**	**042-2511M**
10x Stock	50 mls	50 mls	50 mls
Ultrapure H_2O	420 mls	420 mls	420 mls
1M-HEPES	10 mls	10 mls	10 mls
7.5% (w/v) $NaHCO_3$	4.5 mls	4.5 mls	4.5 mls
NEAA(100x)[a]	10 mls	-	-
Adjust to pH 7.4-7.5 with 1M-NaOH/1M-HCl			

[a] NEAA:- Non-essential amino acids (Gibco Cat.No: 043-1140H).

Preparation of HEPES

HEPES buffer was made up as a 1M solution in ultrapure H_2O. 23.83g of solid are dissolved in 80 mls of H_2O and then autoclaved. The volume of the solution after sterilisation should be 95 mls (aprox). 5 mls of sterile 5M-NaOH (BDH Cat.No: 301667), also prepared with ultrapure H_2O are added to bring the pH to 7.2.

Preparation of $NaHCO_3$

7.5g of solid $NaHCO_3$ (Riedel-de-Haen Cat.No: 31437) was dissolved in 100 mls of ultrapure H_2O and autoclaved before use.

pH adjustment of media

1M-NaOH (BDH, Cat.No: 30167) and 1M-HCl (BDH Cat.No: 28507) were prepared from analar stock solutions in ultrapure H_2O and sterilised by autoclaving. These solutions were used dropwise to adjust the final pH of the media.

2.1.6 Supplements to basal media

L-Glutamine

A 200 mM L-glutamine solution (Gibco Cat.No: 043-05030H) was added to basal media as a 1/100 dilution for the preparation of **growth media** for cell cultures. This amino acid is a relatively unstable component of culture media and is therefore stored in 10 ml lots at -20°C and thawed before use.

Sodium Pyruvate

Additional carbohydrate sources were added to some basal media. The Krebs cycle precursor pyruvate, in its sodium salt form was purchased from Gibco (Cat.No: 043-01360H) and used to supplement the growth medium for the cell lines SK-MES-1 and SK-LU-1.

Hydrocortisone

Hydrocortisone (Sigma, Cat.No: H-0135) was prepared by dissolving 1mg in 250 μl of absolute ethanol and bringing the volume to 25 mls with serum-free basal growth medium (DMEM:Ham'sF12). This stock solution at 40 μg/ml (100x) was filter-sterilised and stored at -20°C. It was diluted in the growth medium of some squamous cell carcinoma cell lines such as SCC-9 to a final concentration of 0.4 μg/ml.

2.1.7 Serum

Serum is a costly, highly variable but essential supplement to the majority of animal cell culture media. Batch to batch variability necessitated routine batch testing to select a batch which suited the particular cell lines being used, and where possible to achieve this aim with economy. The batches selected for use in this work are listed in Table 2.1.7. These were purchased in bulk (minimum amount 5 litres) and stored at -20°C until required for the preparation of growth media.

Table 2.1.7 Serum batches and sources used in this work

SERUM TYPE	BATCH №	MANUFACTURER / SUPPLIER
Foetal Calf	801017	Seralabs / P.J. Brennan
Foetal Calf	701113	Seralabs / P.J. Brennan
Newborn Calf	10F6182	Gibco / Medlabs
Foetal Calf	10F	Gibco / Medlabs
Foetal Calf	AF1201	Advanced ProteinProducts / Medical Supply Co.

2.1.8 Sterility checks

Prior to the preparation of growth medium (i.e. addition of serum and L-glutamine to basal medium), all bottles of freshly prepared basal medium were tested for sterility. A portion of the aliquot removed for testing was incubated with thioglycollate broth (Oxoid, CM173) to test for anerobic bacteria; another was incubated with Sabourand broth (Oxoid, CM143) to test for fungal contamination; and a the remainder was streaked on blood agar plates (Medlabs) which supports the growth of aerobic bacteria and yeast. Another aliquot of medium alone (in a sterile universal) was incubated to test for bacterial contamination. All samples for sterility checks were incubated at 37°C. Bacterial contamination was normally visible after 3-7 days, but tests for yeast or fungal contamination were left for a minimum of 14 days. In practice media were only used after they had not manifested signs of contaminants in any of the sterility checks.

2.1.9 Preparation of growth media

Growth medium consisted of a suitable basal medium supplemented with 5-10% serum plus 2 mM L-glutamine and any other supplements required by the particular cell type. The supplements were added to the basal media 2-3 days before they were needed for cell culture to allow sufficient time for another sterility check to be performed after supplementation. A sample of the growth medium was removed asceptically to a sterile universal for this purpose and incubated as described in the Section 2.1.8 above.

2.2 ROUTINE MAINTENANCE OF ESTABLISHED CELL LINES

2.2.1 Cell lines used in this work

All cell culture work performed here was carried out in Class II vertical recirculating laminar flow cabinets. Table 2.2.1.1 contains a list of the human cell lines used in this work.

Table 2.2.1.1 Source and description of Human Cell Lines used and basal media required for their *in vitro* maintenance

CELL LINE	Source	Description	BASAL MEDIUM[a]
HEp-2	ATCC[b] (CCL 23)	Epidermoid carcinoma of Larynx	DMEM:Ham's F12
DLRP	This work	Poorly differentiated Lung SCC[c]	DMEM:Ham's F12
DLKP	NCTCC[d]	Poorly differentiated Lung SCC	DMEM:Ham's F12
SK-MES-1	ATCC (HTB 58)	Lung Squamous cell carcinoma	DMEM:Ham's F12[e]
SK-LU-1	ATCC (HTB 57)	Poorly differentiated Lung Adenocarcinoma	DMEM:Ham's F12[e]
MRC-5	ATCC (CCL 171)	Foetal Lung Fibroblast Cell Line	DMEM:Ham's F12
RPMI-2650	ATCC (CCL 30)	SCC of nasal septum	MEM
SCC-9	ATCC (CRL 1629)	SCC of the tongue	DMEM:Ham's F12
A-431	ATCC (CRL 1555)	Vulval Epidermoid carcinoma	DMEM
BAC	This work	Breast Adenocarcinoma	DMEM:Ham's F12

[a] Basal medium used here, all supplemented with 10% FCS. [b] American Tissue Culture Collection, Rockville, MD, USA. [c] Squamous cell carcinoma. [d] Cell Depository at the National Cell & Tissue Culture Centre, Dublin City University. [e] Supplemented with 1 mM Sodium pyruvate.

A list of rodent cell lines used in this work is given in Table 2.2.1.2 below. The rapidly proliferating NRK and 3T3 cell lines grew as adherent cultures. The hybridoma cell lines attached only loosely without spreading out, retaining a 'rounded up' morphology.

Table 2.2.1.2 Source and description of Rodent Cell Lines used and their basal media requirements

CELL LINE	Source	Description	BASAL MEDIUM[a]	% FCS[a]
NRK	Ian Pragnall[b]	Normal Rat Kidney Fibroblasts	DMEM	5
NIH/3T3	ECACC 85111801	Mouse Embryo Fibroblasts	DMEM	10
Ep-16	ATCC (HB 155)	Mouse Hybridoma	RPMI 1650	15
703D4	ATCC (HB 8301)	Mouse Hybridoma	DMEM:Ham'F12	10
704A1	ATCC (HB 8302)	Mouse Hybridoma	DMEM:Ham'F12	20

[a] Basal medium and serum concentration used in this work. [b] At the Beatson Institute for Cancer Research, Glasgow, Scotland.

2.2.2 *In vitro* culture systems

Several types of culture vessels are available commercially. Most are made from polystyrene, electrostatically treated to make it negatively charged and wettable and thereby facilitate cell attachment. The so-called 'open' culture systems are useful in particular applications where gaseous exchange is critical, e.g. CO_2 exchange for the buffering of certain media pH. This can be supplied in special CO_2 incubators which provide a positive CO_2 pressure and humid atmosphere. Covered petri dishes such as the 35 mm size used in soft agar assays (Section 2.12.3) and the flat-bottomed 24-well (Costar, Cat.No: 3524) and 96-well plates (Costar, Cat.No: 3599) are examples of open culture systems used in this work.

Sealed flat-bottomed flasks were used for routine cell maintenance to reduce the risk of contamination associated with open culture systems. 7-10 mls of growth medium was used for start-up cultures in 25 cm^2 flasks (Cell Cult, Cat.No: 32025) and 20-25 mls for cultures in 75 cm^2 flasks (Costar, Cat.No: 307S) which were employed for general stock maintenance.

The use of glass as a substrate for cells in culture is nowadays restricted to non-static culture systems. Examples of these include roller bottles (Bellco) and spinner flasks (Sections 2.7.2 and 2.7.3 below).

2.2.3 Feeding cultures

Cells in culture metabolise media nutrients and produce waste products such as lactic acid. The inclusion of the pH indicator phenol red in basal media provides an indirect means of estimating nutrient depletion and pH acidification. Medium was changed before the pH fell below pH 7.2. Rapidly proliferating cultures such as HEp-2 required more regular feeding than slower growing cell lines such as SCC-9.

2.2.4 Subculturing

When confluency is reached in monolayer cultures, further growth of most cell lines is restricted either by contact inhibition (e.g. in non-transformed cells) and/or nutrient depletion. Some malignantly transformed cell lines derived from carcinomas may continue to grow by forming multilayers after confluency. To ensure that a higher number of viable cells are recovered, cultures are generally subcultured before they reach confluency (i.e. while they are in log phase growth). Suspension cultures (e.g. hybridomas) can be subcultured easily by a simple dilution, but adherent cell lines must be detached from their substrata.

Adherent cells were detached from flasks using a 0.25% (w/v) trypsin/0.02% (w/v) EDTA solution in PBS-A. Briefly, cell monolayers were rinsed in sterile PBS-A to remove traces of serum (which contains trypsin inhibitors) followed by rinsing in a small volume of the trypsin/EDTA solution at 4°C (e.g. 2-3 mls per 25 cm^2 flask). 2-3 mls of trypsin/EDTA were then added and incubated until a single cell suspension was achieved (monitored microscopically). The incubation temperature for subculturing (trypsinisation) was either room temperature or 37°C, depending on the cell line being used. Cold trypsinisation prevents excessive damage to cells by slowing down the rate of proteolysis but pre-warmed trypsin and/or incubation at 37°C was employed for cell lines which were more difficult to detach. Tapping of the flasks helped to dislodge the monolayers and facilitated the generation of a single cell suspension.

The single cell suspensions were removed from the culture flask and transferred asceptically to sterile universals. Cells were then pelleted by centrifugation in a bench top centriguge at 800-1000 rpm for 4-5 minutes. The supernatnant medium was decanted to a waste bottle, taking care not to dislodge the cell pellet which was resuspended in 5 mls of fresh growth medium (pre-warmed at 37°C). If a cell count was required, an aliquot of this suspension was removed using a sterile pasteur pipette. Subcultured cells were reseeded at the desired concentration. (Note: In the maintenance of routinely used cell lines, a specific split ratio was employed for each cell line based on experience of their individual growth characterisitics).

Stock Solutions

Crude trypsin (2.5% w/v) was purchased from Gibco (Cat.No: 043-05090) and stored frozen at -20°C. PBS-A was purchased (Oxoid, Cat.No: BR12a) in tablet form and dissolved in ultrapure H_2O (1 tablet per 100 ml) before autoclaving. 1% (w/v) EDTA (Sigma, Cat.No: ED2SS) was prepared in ultrapure H_2O, autoclaved and stored at room temperature.

Preparation of working stocks of trypsin/EDTA

Periodically as required, 500 ml lots of 0.25% trypsin/0.02 (w/v) EDTA in PBS-A were prepared from stock solutions. For practical purposes these working stocks were dispensed into 20 ml sterile universals {from Sterilin (Cat.No: 128A) or Greiner (Cat.No: 201175)} and stored at 4°C for short-term use (1-4 weeks) or at -20°C for longer storage periods.

2.2.5 Counting cell suspensions

A homogeneous single cell suspension was needed for accurate determination of cell numbers. Therefore, before cell numbers were measured, every effort was made to ensure that the cell suspension was adequately mixed. A small aliquot (aproximately 50 μl) was withdrawn for counting using a sterile pasteur or micropipette tip and removed to a Waber haemocytometer (improved Neubauer). The suspension was allowed to move into the space between the haemocytometer grid and the coverslide by capillary action. Using 40x magnification on the inverted microscope the number of cells on each of the four corner squares (subdivided into 16 smaller squares) was counted. The volume of medium covering each of these squares is 10^{-4} ml. Cells on either two of the sides of each square (1 $\times$ 1 mm) were scored and the average value for the four counts was calculated and multiplied by 10^4 to give the cell number per ml.

2.2.5.1 Determination of viable cell numbers

After the preparation of a single cell suspension, a percentage of the total cell number will always be non-viable. Viability of cells cannot be determined absolutely by microscopic appearance but the determination of plasma membrane intergity gives a better indication of non-viable cells. Exclusion of the dye Trypan Blue (Gibco, Cat. No: 043-05250H) from viable cells distinguishes them visibly from cells with damaged or 'leaky' membranes which take up the dye and stain a dark blue/brown colour.

Counting the total cell number of cells and the number of 'dead' or stained cells using the haemocytometer as described, above permits the number of viable cells/ml to be calculated. The percentage viability after cell manipulations was also be determined in this way. The dye solution was added to an aliquot of uniform cell suspension at a ratio of 1:6 (v/v) and left for 5 minutes before counting. The percentage of viable cells in the suspension was calculated as follows;

$$\%\ \text{VIABLE CELL NUMBER} = \frac{\text{Total Cell Number/ml - Dead Cells/ml}}{\text{Total Cell Number/ml}} \times \text{DILUTION FACTOR} \times 100$$

2.3 LONG TERM STORAGE OF CELL LINES

Animal cells may be stored long-term at -196°C in a liquid N_2 refrigerator. Master stocks were generated for all cell lines used in this work. Thawed vials were replaced as soon as possible after their successful recovery from liquid N_2. Only mycoplasma-free cultures in good condition (i.e. harvested in log growth phase) were used for freezing. Vials were labelled indelibly with the cell line name, passage number, date of storage, and the operator's initials and all vials removed from the liquid N_2 refirgerator were meticulously recorded together with the storage loction of vials deposited. Protective visors covering face and neck as well as gloves were used during the retrieval and deposition of ampoules in liquid N_2. While the percentage recovery from frozen stocks varies between different cell lines (being especially low for hybridoma cell lines) a number of parameters which contribute to successful freezing and recovery can be controlled.

2.3.1 Cell freezing

Cells for freezing were trypsinised and the viability of these single cell suspensions determined as described in Section 2.2.5.1 above. Cell suspensions with a minimum concentration of 4×10^7 viable cells/ml were prepared in growth medium supplemented with 25% FCS. To these cell supsensions an equal volume of a 10% (v/v) DMSO (dimethylsulphoxide, Sigma, Cat.No: D8386) in growth medium with 25% FCS, was added dropwise while swirling. No more than half of the DMSO solution was added in the first minute. After all the DMSO solution was added, the cell suspension was transferred asceptically in 1 ml aliquots to cryovials (Greiner, Cat.No: 122278). These were sealed and labelled before being placed in the vapour phase (at -135°C) of the liquid N_2 refrigerator. After 3 hours they were moved to the liquid phase and catalogued. Liquid N_2 levels in the refrigerator were monitored and replenished regularly.

2.3.2 Cell thawing

The presence of the cryoprotectant DMSO in freezing medium prevents the potentially destructive formation of intracellular ice crystals and osmotic effects in cells during freezing. However, this agent is highly reactive at room temperature and for this reason must be removed as quickly as possible after thawing. The thaw process therefore, should be as rapid as possible. Frozen vials were thawed in water at 37°C taking care not to submerge the seal. Ampoules were swabbed in 70% IMS and brought into the laminar flow cabinet. Their contents were transferred to sterile universals, diluted in 5 mls of pre-warmed growth medium containing 10% FCS and then centrifuged at 1000 rpm for 4 minutes. After decanting the supernatant medium, the cell pellet was resuspended in 5 mls of growth medium and an aliquot removed to determine the percentage viability as described in Section 2.2.5.1. Cultures were set up at high inoculum concentrations (10^6 cells per 25 cm^2 flask) in a minimum volume of medium (5 mls per 25 cm^2 flask). Thawed cultures were incubated overnight and refed the following day with fresh medium, after inspection for signs of contamination. The same precautions were taken with thawing frozen stocks as for the use of live cultures (Section 2.4.3.1).

2.4 CONTAMINATION CONTROL

2.4.1 Microbial

Because of the rich media used, bacterial contamination of animal cell cultures generally becomes rapidly obvious due to sudden pH changes and gross cloudiness in the growth medium. Vigilant examination of cultures using an inverted microspope (especially before feeding) should alert the trained observer to lower levels of contamination. Evidence of cryptic bacterial contamination include 'motile grains' or clusters of dark debris-like material. Other forms of microbial contamination including yeast and fungus are usually slower to appear and may be more difficult to detect due to their slower growth rates. Such contaminants may go undetected for long periods, especially if the culture is being refed or subcultured frequently. Yeast are characterised by their distinctive 'budding' morphology and are often seen in 'chains' of such 'budding' cells. Fungal species are more diverse but become visible macroscopically after sporulation when they produce dendritic-like structures.

Good asceptic technique, thorough sterility checks and working in a class II vertical laminar flow cabinet minimise such contamination incidents. However in this work, when contamination was detected the cultures in question were discarded unopened and autoclaved. All media and solutions that had come in contact with these cultures were re-screened for sterility and discarded if positive. Fresh sterile stocks of all solutions were used if master stocks were thawed from frozen stocks.

2.4.1.1 Use of antibiotics

The use of antibiotics was restricted in this work to bioassays and primary cell culture. Antibiotics used in bioassays were penicillin/streptomycin (Gibco, Cat.No: 043-05140) or gentamicin (Gibco, Cat.No: 043-0570D). General prophylactic use of antibiotics was avoided in cell line maintenance because this practice can mask the presence of cryptic contamination and lead to the evolution of more virulent antibiotic resistant strains. In general the presence of microbial contamination almost certainly indicates co-infection with mycoplasma (Section 2.4.2).

In the case of primary cell cultures, the use of antibiotics and other antimicrobial agents is recommended because absolute sterility cannot be assured. This is due to the nature of the sample collection process. Every effort was made to prevent contamination of primary tissues in this work, but the possibility of endemic levels of bacteria or yeast etc. in some samples could not be excluded. For this reason, all primary tissue samples were collected in a transport medium which contained a cocktail of antibiotics and anti-fungal agents.

2.4.1.2 Anti-fungal agents

The anti-fungal agent amphotericin B was purchased commercilly as fungizone (Gibco Cat.No: 043-05290D). It was included at a concentration of 250 μg/ml in 'transport' medium for the carriage of primary tissue cultures from the hospital theatre to the laboratory. Fungizone is active against fungus and yeast, but it also has a cytostatic effect on mammalian cells and for this reason its use even in primary cultures was avoided where possible.

2.4.2 Mycoplasma

Unlike other forms of microbial contamination, mycoplasma are not visible microscopically even at high-powered magnification under the inverted microscope. Although mycoplasma-infected cultures may continue to grow without any obvious effects, decreased proliferation rates, rapid changes in medium pH after feeding, changes in cell morphology and granulation of the cytoplasm are all indicative of this type of contamination. Despite the often benign effects of this insidious contaminant, the validity of studies using cultures harbouring mycoplasma must be suspect, if not invalid.

Sources of mycoplasma infection include primary tissue material, animal sera, media, trypsin and the operator. They are difficult to remove due to their small size (typically $< 0.1\mu$m long) and plasticity (they lack a cell wall), but double filtration through a 0.1 μm filter is recommended for this purpose. Given the many possible sources of infection, such a procedure is not a practical proposition. Instead, cell lines used in this work (as in other laboratories) were regularly screened for the presence of mycoplasma during *in vitro* maintenance and before the preparation of frozen cell stocks.

2.4.2.1 Detection of mycoplasma

An 'indirect' test based on the method of Chen (1977) was used in this work to detect mycoplasma contamination. This procedure employs a bisbenzimidazole fluorochrome stain (Hoeschst 33258) for DNA. Mycoplasma DNA appears as particulate or fibrillar fluorescent staining in the cytoplasm of infected cells. In heavily infected cultures, this may be localised at the plasma membrane and intracellular spaces. Only nuclear staining is apparent in uninfected cells.

To avoid the problems of interpretation associated with 'direct' staining of suspect cultures (arising from the different growth kinetics, spreading patterns, and fragility of nuclear membranes of some cell lines to the fixation process) a mycoplasma-free rodent cell line, NRK was used as an 'indirect indicator' cell line. Low passage NRK can be infected with mycoplasma derived from the spent medium of contaminated cells and many such tests can be carried out in the same cellular background, thereby facilitating comparative staining. It is important that growth medium from suspect cultures has been in contact with the respective cells for at least 3 days and is free from antimycoplasma agents before testing (to avoid false negative results).

Method:

Preparation of indicator NRK monolayers

22 mm^2 coverslips (Chance Nº1) were pretreated by washing in 2% RBS followed by rinsing in ultrapure H_2O, and then in 70% IMS (Industrial Methylated Spirits, Lennox, Cat.No: 1170). They were dried in lint-free cloth and then autoclaved in a glass petri-dish.

The sterile coverslips were then asceptically transferred to individual 30 mm sterile petri dishes (non-tissue culture grade). 1 ml of low passage mycoplasma-free NRK cells at 5×10^2 cells/ml were seeded in DMEM + 5% FCS on each dish and these were incubated in a 5% CO_2 atmosphere at 37°C overnight.

After removal of the overnight growth medium, 1 ml of antibiotic-free spent growth medium from suspect cultures was addded to duplicate dishes. A control in the form of fresh growth medium (DMEM + 5% FCS) was set up on duplicate wells.It is important that cultures do not become confluent because the cells do not spread sufficiently in this case for adequate staining with Hoechst 33258. Cells were allowed to reach 20-50% confluency before they were fixed and stained. This took 3-4 days approximately.

Fixing, Staining and Mounting

After the removal of growth medium, cells on the coverslips were rinsed twice in PBS, followed by a single rinse in a 1:1 dilution of cold Carnoy's reagent (Glacial Acetic acid:Methanol, 1:3 v/v). Glacial acetic acid (BDH, Cat.No: 27013) and methanol (BDH, Cat.No: 10158) were both purchased from BDH}. The cells were then fixed in 2 mls of Carnoy's reagent for 10 minutes. It is important that the cells are well covered and not allowed to dry out before fixing as this may induce artifacts.

After removal of fixative the coverslips were air-dried and washed twice with ultrapure H_2O and then stained with 2 mls of a 50 ng/ml solution of Hoeschst 33258 (Sigma, Cat.No: B2883) in PBS for 10 minutes. The Hoechst 33258 stain is sensitive to light and heat. For this reason, staining was carried out in the dark (under aluminium foil).[It was also stored wrapped in aluminium foil at 4°C as a 1000x concentrate (50 μg/ml) and diluted on the day as required].

The stain was aspirated and excess liquid removed by rinsing three times in ultrapure H_2O. The coverslips were mounted on a microscope slide in a drop of glycerol mounting medium (50% v/v glycerol (Sigma, Cat.No: G5516) in 0.044 N citrate, 0.111M phosphate at pH 5.5). The cells were first examined under the phase contrast condenser of the fluorescent microscope at 40x to check for cellular integrity before being examined under oil immersion at 100x using the fluorescence filters {a 50 barrier filter LP 440 nm, and a BG12 exciter 330/380 should be used}. The absence of extranuclear fluorescence in the controls was confirmed before any staining patterns in the test samples could be verified.

Positive identification of mycoplasma contamination was followed by disposal of those stocks and the cleaning and fumigation of incubators and laminar flow cabinets used for their manipulation. In the case of unique primary cell lines, mycoplasma contamination was treated with a mycoplasma removal regime purchased in kit form. Contaminated cultures and associated solutions were quarantined from all other routine cell culture work (using separate primary culture facilities).

2.4.2.2 Elimination of mycoplasma

A two-solution antibiotic kit called BM-Cycline was purchased from Boehringer Mannheim (Cat.No: 799050). According to the suppliers this combination of antibiotics is effective against 25 mycoplasma species, bacteria and candida.

250x stocks of BM-Cycline solutions 1 & 2 were made by dissolving the powdered sample in 10 ml steile PBS. To ensure sterility, the PBS was injected through the rubber cap as instructed by the manufacturers. This procedure was performed in a laminar flow cabinet and 100 μl lots of the respective solutions were aliquoted into sterile eppendorfs and stored at -20°C until required for use. These antibiotics are stable to repeated freezing and thawing for at least 12 months.

Treatment Protcol:

Three cycles of exposure to BM-Cycline 1 and 2 solutions in tandem were employed. In each case 40 μl of the stock solution was added to cells in 10 ml of growth medium in a 25 cm^2 flask (= 1/250 dilution). Solution 1 was added on Day 1 and this was incubated without a change of medium for three days. On Day 4 the medium was removed as completely as possible and 10 ml of BM-Cycline 2 containing medium added. After 4 days in the second solution, the cycle was repeated(on Day 8). Cell cultures treated with two such cycles of BM-Cycline containing medium were removed and rinsed with sterile PBS before being returned to antibiotic-free medium. An aliquot of the culture medium from treated cultures was removed for mycoplasma analysis after one medium change in antibiotic-free growth medium. Clean cultures were exposed to a third cycle of BM-Cycline treatment before they were used for experiments. Cultures which remained mycoplasma positive after two cycles were exposed to further cycles of BM-Cycline treatment until they were clear, and an extra cycle was then employed before they were used for experiments.

As with the use of other antibiotics, the continuous use of BM-Cycline was not employed in these studies. Similarly the simultaneous use of BM-Cycline solutions 1 & 2 was avoided on the manufacturer's advise.

Other Anti-mycoplasma agents used

Two other antimycoplasma agents used in this work were tylocine (Anti-PPLO agent, Gibco, Cat.No: 043-05220) purchased as a 100x liquid concentrate (6000 μg/ml) and gentamicin (Gibco, Cat.No: 043-0570D) also purchased as a 100x concentrate (50000 μg/ml).

2.4.3 Cross-contamination by other cell lines

The inadvertant mixing or cross-contamination of different cell lines is a relatively rare but nonetheless likely risk in the culture of animal cells. Judging from the presence of marker chromosomes for HeLa (one of the first human carcinoma cell lines established) in many of the other cell lines derived during this period, this constitutes a real risk unless proper vigilence is maintained in separating different lines. Cross-contamination occurs due to human error. An obvious inference from this is that an increase in the number of cell lines being handled by any one operator and/or laboratory where facilities are shared, increases the potential for this form of error. Cross-contamination between two animal cell lines may not always be immediately obvious. A number of changes in culture properties should alert the experienced cell biologist to the possiblity of this event. Changes in growth rates and morphology in particular lines, with perhaps the associated loss of a particular differentiated function may all result from a cross-contamination event.

Traditionally cell line integrity was assessed using isoenzyme analysis and the cytogenetic analysis of marker chromosomes. More recently the advent of DNA fingerprinting technology has provided a more rapid and unequivocol method for the validation of unique cell lines. This method was employed in the characterisation of new cell lines derived from human primary tissues in this laboratory. Certified cell lines purchased from Cell Depositories such as the American Tissue Culture Collection (ATCC) were expanded immediately after receipt to prepare master stocks of the particular cell line. Enough vials were frozen to ensure a continuous back-up during the anticipated period of use. Master stocks were deposited in liquid N_2 as described in Section 2.3.1 above.

2.4.3.1 Procedures employed to avoid cross-cell line contamination

In order to minimise the possibility of cross-cell line contamination the following guidelines were incorporated into standard operating procedures used in this lab.

1. Cultures were clearly labelled and indexed in a catalogue before freezing and after thawing from liquid N_2 storage. Dubiously labelled or illegibly labelled vials were discarded.

2. Separate **labelled** growth media, trypsin, waste bottles and any other solutions coming into contact with cells were used for each cell line.

3. No more than one cell line was handelled in the laminar flow cabinet at any one time. After removal of **all** materials, the cabinet was swabbed with 70% IMS (Industrial methylated spirits) and left for 15 minutes before being used again.

4. Disposable pipettes which came into contact with cell suspensions or with culture supernatants were discarded immediately after use (avoiding the temptation to re-use the same pipette to feed another flask of the same cell line and risk introducing cells into the stock growth media).

5. Clear labelling of **all** cell-containing universals before removal from laminar flow cabinet for centrifugation and discarding of used and unlabelled universals.

6. Culture flasks were labelled with the name of the cell line and incubated in discrete positions in incubators. Markings were checked before and after manipulations.

7. Preparation of stock media and other solutions in the laminar flow cabinet were performed **only** after a 15 minute quarantine, as for other sterile procedures in the hood.

2.5 PRIMARY CELL CULTURE TECHNIQUES

2.5.1 Collection of tumour tissue samples and transportation medium

Primary tissue was collected in theatre directly after surgical resection at St Vincents Hospital, Elm Park, Stillorgan, Dublin 4. This material was kindly provided by thorasic surgeon Mr. Vincent Lynch only after the requirements of the hospital's histopathology service for such tissue had been met. Tissue samples were placed in a transportation medium immediately and placed on ice or at 4°C until they were taken to DCU for processing on the same day.

Transportation Medium:

This consisted of a 1:1 DMEM/Ham's F12 basal medium supplemented with 20% FCS, 2 mM L-glutamine and containing 500 U/ml penicillin and 0.5 mg/ml streptomycin {diluted from a combination stock of both antibiotics (called P/S) at 100x} and 500 μg/ml fungizone.

2.5.2 Dissection of tumour tissue

Samples were rinsed in PBS (2-3 times) to remove blood cells before dissection. Fatty deposits, necrotic tissue and blood vessels were removed where possible using sterile surgical scalples. Care was taken to avoid tearing of the tissue as this may trigger fibroblast proliferation. These manipulations were performed on sterile petri dishes (100 mm Greiner, Cat.No: 633171) in sterile PBS.

2.5.3 Mechanical disaggregation

The tissue remaining after removal of the unwanted components was sliced into the smallest sized pieces possible (typically in the order of 1 mm^3). These manipulations were performed in 10 mls of growth medium (plus 2% P/S) on a fresh sterile petri dish. Cells which had spilled into the medium during disaggregation were recovered by aspirating the medium and pelleting the suspension in a sterile universal (5-10 minutes at 1000 rpm). This fraction of the tissue was called 'Disection Medium' (DM) and in some cases the cell pellet showed signs of erythrocyte contamination. Such pellets were resuspended in growth medium and separated on a Ficoll gradient (Section 2.5.6) to remove blood cells. Other DM fractions were plated directly after resuspension in growth medium.

Some of the tissue fragments produced by mechanical disaggregation were used to set up explant cultures, but the bulk of the sample was incubated with proteolytic enzymes in an effort to release more cells from the tissue matrix.

2.5.4 Explant technique

Small tissue fragments ($\approx$ 1mm^3) with clean (straight) edges were asceptically transferred to the growth surface of 25 cm^2 flasks which had been pre-wetted with 2.5 mls of growth medium and allowed to stand upright. 15-20 such fragments were added per flask using either the tip of the scalpel blade or a sterile needle. They were allowed to adhere in this position for approximately 30 minutes with the lids loosened slightly. A further 2.5 mls of medium was then added, taking care not to dislodge the explants, and the flasks were incubated at 37°C.

2.5.5 Enzymatic disaggregation

A 200 U/ml solution of type V collagenase (Sigma, Cat.No: C9263) in sterile PBS was used alone or in combination with a 0.2% (w/v) dispase (neutral protease, Boehringer Mannheim, Cat.No: 165-859)) to digest the extracellular matrices of tumour tissue. The tissue was incubated while stirring with a sterile magnetic stirrer at 37°C in flat-bottomed 60 ml universals (Sterilin, Cat.No: 125AP). Periodically (every 30 minutes at most) small aliquots of the supernatant were removed to a 35 mm petri dish and examined under the inverted microscope for release of single cells and/or cell aggregates. When sufficient numbers of cells appeared in the supernatant, the incubation mixture was allowed to settle by gravity. Large pieces of tissue and cell clumps were separated from the smaller single cells and aggregates in solution by decanting the supernatant into a sterile universal, leaving the last 1-2 mls behind. Cells released in this way were recovered by centrifugation at 1000 rpm for 5 minutes, resuspended in fresh growth medium (prewarmed to 37°C) and then plated at high density (at least 10^6 cells per 25 cm^2 flask). The enzyme solution remaining after recovery of cells was returned to the undigested tissue in the flat-bottomed universal. Further quantities of cells were liberated from the matrix up to three hours after the start of enzyme treatment but the viability and yields in these fractions decreased with time.

2.5.6 Ficoll density gradient

Blood cells in tumour cell suspensions were removed by density gradient centrifugation using Ficoll (Pharmacia, Cat.No:). Tumour cells suspended in 5 mls of growth medium were layered carefully, using a sterile pasteur pipette, on top of 10 mls of Ficoll in a sterile universal. Without disturbing the interface which forms between the two solutions, the universal was spun at 3500 rpm for 10 minutes. After centrifugation and careful removal of the universal, the layer of cells which had formed at the interface ('buffy layer') was recovered by aspiration using a sterile pasteur pipetted (blood cells should be visible in a pellet at the bottom of the universal). 5 mls of growth medium was added to the buffy layer and the cells were pelleted by centrifugation as before. A further round of Ficoll treatment was performed if the recovered pellet still showed signs of blood cell contamination after the initial treatment.

2.5.7 Harvesting cells from malignant effusions

Malignant effusions were centrifuged at 2000-3000 rpm in 50 ml sterile universals at 4°C to pellet cells in suspension. In many cases the cell pellets recovered were heavily blood stained. Blood cells were removed as described in the Section 2.5.6 above.

2.5.8 Fibroblast elimination

2.5.8.1 Selective trypsinisation of fibroblasts and epithelial cells

The progress of trypsinisation in primary cultures was monitored continuously under 40x and 100x magnification using an inverted microscope. The procedure was terminated by the addition of growth medium as soon as the majority of either fibroblasts or epithelial cells appeared to have detached. Unnecessary agitation of the flasks was avoided until the detached cells had been removed by aspiration of the medium. The different compositions of trypsin/EDTA and the protcols used here are described in more detail in Section 3.1.5.1.

2.5.8.2 Selective toxicity of fibroblasts to G418 sulphate

Geneticin (G418 sulphate) was purchased from Gibco (Cat.No:066-1811). A 100x stock solution (10mg/ml) was prepared in complete medium. Mixed fibroblast/epithelial cultures were treated with 100 μg/ml G418 in complete medium. After 6 days without medium change, the agent was removed and the cultures returned to normal growth medium.

2.5.9 3T3 feeder layers

NIH3T3 cells were set up in 25 cm^2 flasks at 2.3×10^5 cells/flask. The cells were exposed to Mitomycin C (Sigma, Cat.No: M0503) for 3 hours at a concentration of 4 μg/ml in growth medium (prepared fresh from a 200 μg/ml stock solution in PBS stored at -20°C in the dark). After treatment, the Mitomycin C-containing medium was completely removed and cultures were washed with 10 mls of sterile PBS (x2) and refed in fresh growth medium. On the next day they were examined for signs of growth and growth-arrested cultures were seeded with primary cultures of tumour cells at a minimum concentration of 10^6 cells per flask.

2.5.10 Collagen coating of tissue culture flasks

Collagen type III (from rat tail) was used to coat 25 cm^2 flasks for primary cultures of tumour cells. A 100 μg/ml solution was made up in PBS and filter-sterilised before use. 2 mls was added to each flask for coating and after all the growth surface had been wetted, they were left for 30-40 minutes before the solution was removed. The flasks were allowed to dry, with lids slightly loosened, in the laminar flow cabinet. Dry coated flasks were stored at room temperature with lids sealed until required for use.

2.6 CELL LINE CHARACTERISATION

2.6.1 Immunohistochemical identification

Antibodies directed against differentiation-specific antigens can be used to discriminate between different cell types and lineages and different species. Binding of specific 'primary' antibodies raised in one species (e.g. rabbit) can be visualised indirectly by the use of a second antibody raised against antibody types of the primary antibody species. Labelling of the secondary antibody avoids the need to label the primary antibodies and provides even more specific staining patterns by minimising non-specific binding. Commonly used labels which are conjugated to the secondary antibody include enzymes with chromogenic substrates (e.g. alkaline phosphatase) and fluorescent dyes. A secondary antibody labelled with the fluorescent dye, fluorescein thiocyanate (FITC) was employed in this work.

2.6.1.1 Preparation of slides

Eight-well H.T.C. blue autoclavable 6 mm slides were used for mounting adherent cells. These were sterilised by autoclaving in glass petri-dishes. Individual slides were transferred asceptically to sterile plastic petri-dishes before seeding with cells. These slides were re-usable if soaked in 5% (v/v) RBS for 1-2 hours followed by rinsing in distilled water for 10 minutes. Such slides were stored in methanol at 4°C until required and then sterilised by passage through a bunsen flame or by autoclaving.

20 μl of cells at a concentration of 10^5 cells/ml in complete medium were seeded per well using a micropipette with sterile tips and the slides were incubated at 37°C in a covered perti dish for 24 hours in humidified, 5% CO_2 atmosphere. Non-adherent cells were mounted on non-sterile Gold star microslides at a concentration of 10^5 cells/ml in PBS and air-dried on the day of analysis.

Slides were washed ($\times$3) in PBS and excess solution removed using filter paper. Care was taken not to remove the cell monolayer. The cellular location of the target antigen dictated the fixation method used.

2.6.1.2 Surface epithelium specific antibody

The antigen recognised by the Ep-16 IgMκ monoclonal antibody is present on the plasma membrane of cells which express it. The procdure employed to fix cells for analysis with an antibody directed against as surface antigen was as follows. A 5 minute soak in 3% (v/v) formalin (BDH, 101113) at room temperature followed by 5 minutes in acetone (BDH, Cat.No: 27023) at -20°C. The slides were then air dried and 20 μl of EP16 antibody (in the form of Ep-16 CM) was added to all but one of the eight wells. The eighth well was left to act as a control for non-specific binding with the labelled secondary antibody. The slides were incubated with the primary antibody in a humid environment for 1 hour at 37°C.

2.6.1.3 Anti-cytokeratin antibody

Anti-cytokeratin №18 (Bohreingher Mannheim, Cat.No: 814-385) recognises an intracellular protein expressed in epithelial but not fibroblast cell types. The fixation procedure employed

for analysis of intracellular antigens was as follows. Slides were placed in methonal (BDH, Cat.No: 10158) at -20°C for 7 minutes followed by 2 minutes in acetone at -20°C. To all but one of the eight wells, 20 μl of a 1μg/ml solution of anti-cytokeration antibody was added and incubated for 1 hour in a humid environment at 37°C. The remaining well served as a control for non-specific binding with the second antibody as above.

2.6.1.4 Non-small cell lung carcinoma specific antibody

Two murine IgG2Aκ monoclonal antibodies 703D4 and 704A1, raised against a human large cell lung cancer line (NCI-H157) are secreted by hybridomas produced by Mulshine *et al.*, (1983). 703D4 and 704A1 were obtained from the ATCC. The antibodies were reactive against non-small cell lung carcinomas but not small cell carcinomas. Their staining pattern suggested that the epitopes they recognised were associated with cytoskeletal elements. The cells for this analysis therefore were fixed as for the anti-cytokeratin antigen above. CM was used as a source of the antibodies and 20 μl of the relevant CM was added to all but one of the eight wells on each slide and they were incubated for 1 hour at 37°C as described above.

2.1.6.5 Visualisation of primary antibody binding pattern with an FITC-labelled second antibody

After the 1 hour incubation unbound primary antibody was removed by three 10 minute washings in PBS. The binding pattern was visualised by the addition of 20 μl to all wells of an FITC-labelled goat anti-mouse antibody IgM (Sigma F9259) used at a dilution of 1/30, The second antibody was incubated in the dark for 1 hour in a humid environment followed by three 10 minute washes in PBS under darkness. Slides were then mounted in a 9:1 glycerol:PBS mixture and the coverslips sealed with clear nail varnish. The staining patterns were viewed and photographed in the dark using a Nikon fluorescent microscope. An oil immersion lens was used for higher resolution.

2.6.2 Preparation for shipment of cell lines and tumour biopsy samples for DNA fingerprint analysis

Cell lines for DNA fingerprint analysis were shipped to Cellmark Diagnostics (ICI, Abingdon, U.K.) as live cultures. One confluent 75 cm^2 flask provided sufficient DNA for analysis. Cultures were left in the incubator until just before shippment. Flasks were completely filled with growth medium plus antibiotics and lids sealed with parafilm before being packed in sturdy boxes with adequate shock absorbant insulating material. An overnight express delivery service was used. Frozen tissue was sent on dry ice in cryovials (Greiner, Cat.No: 122278).

2.7 COLLECTION OF CONDITIONED MEDIUM (CM)

Serum-free basal medium used for the preparation of conditioned medium (CM) was checked for sterility before use as described in the preparation of growth medium. Antibiotics were not used in order to avoid cryptic contamination. The basal medium was supplemented with 2 mM L-glutamine just before the collection commenced.

2.7.1 75 cm^2 flasks

For the preparation of small volumes (< 100 mls) of CM, cultures were grown to 70-80% confluency in 75 cm^2 flasks. Growth medium was removed and cell monolayers washed twice with 20 ml lots of sterile PBS followed by a 30 minute incubation in 20 mls of PBS. 20 mls of serum-free basal medium was used for conditioning, and this was replaced every 24 hours with fresh medium. CM was centrifuged in sterile universals at 5000 RPM for 10 minutes at 4°C to remove cell debris. The clarified CM was stored at 4°C (or at -20°C) until required.

2.7.2 Roller bottle culture

Bellco glass roller bottles with an internal surface area of 670 cm^2 were routinely used for the collection of larger volumes of CM (> 100 mls). Bottles were washed as described for media bottles and autoclaved with the lids slightly ajar. Cells were seeded in prewarmed bottles to prevent cell clumping. 100 ml aliquots of growth medium were used per roller bottle. Inoculum concentrations of single cells depended on the growth properties of the cells being used. HEp-2 cells could be set up at 1×10^7 cells (approximately 1 confluent 75 cm^2 flask, but in practice 2 subconfluent 75 cm^2 flasks were used). In general, higher inocula were needed for cell lines with longer doubling times (typically a minimum equivalent of 2-3 confluent flasks were used for such cell lines). Bottles were rotated for 24 hours initially at a speed of 0.25 rpm (revolutions per minute) on a roller bottle apparatus. After this time (or after the cells had attached) the rotation speed was increased to 0.75 rpm.

Cultures were grown to 70-80% subconfluency (as judged by microscopic observation) with regular feeding. Before CM collection was initiated, the growth medium was entirely removed and the cell monolayer was washed with 2×100 mls of sterile PBS followed by a final 30 minute incubation, also in 100 mls of sterile PBS. 100 mls of serum-free basal medium supplemented with 2 mM L-glutamine was used for each collection of CM. The first 24 hour collection was discarded to avoid carry over of serum-derived proteins, but subsequent lots of CM were collected at regular intervals thereafter (24-48 hours) until the cultures showed signs of deterioration (i.e. detachment). CM lots were centrifuged as described in Section 2.7.1 and stored at 4°C (or at -20°C) before processing for assay.

2.7.3 Suspension cultures

HEp-2 were grown in suspension culture for CM collection. The 250 ml and 500 ml spinner flasks used for this purpose were supplied by Techne (Cambridge, U.K.). Pre-warmed growth medium was added to the flasks while the single cell suspension was being prepared. Inocula of 50 mls and 100 mls were started in the 250 and 500 ml vessels respectively at a density of 1×10^5 cells/ml. The spinner flasks were incubated at 37°C with continuous stirring at 20 rpm initially for a 100 ml culture.

Suspension cultures were monitered for cell growth by periodically removing an aliquot of the cells in suspension. When these cultures had reached a density of 4×10^5 cells/ml (day 3) the agitation was increased to 30 rpm. When the culture density reached 8.5×10^5 cells/ml, cells were removed from suspension by centrifugation at 1000 rpm for 10 minutes in sterile 50 ml universals (Elkay, Cat.No:2094-STR). The cell pellets were washed twice by resuspension and centrifugation in sterile PBS and were finally set up in serum-free Ham's F12 plus 2 mM L-glutamine and stirred at 40 rpm during the conditioning process. CM was collected by centrifugation of the suspension cultures.

2.7.4 Cell factory system for very large scale CM production

This system has some advantages over the roller bottle and spinner systems for collecting large volumes of CM from adherent cells. There is less physical stress to cells in this static system, a factor which may influence the growth factor productivity of such cultures. Manipulation of large cell numbers is greatly facilitated by the increased growth surface area in a single vessel and unlike roller and suspension cultures, no specilised mechanical equipment is required with this system.

A ten-tier Nunc Cell Factory system was used for 'litre volume scale' production of CM. Each pre-sterilised unit consists of ten 335 × 205 mm polystyrene trays (total surface area 600 cm^2 per tray). The compartments formed by the stacked trays are interconnected via two openings into vertical channels at opposite corners of one end of the trays. The design is such that, with the liquid connection in one position, an equal volume of medium or cell suspension will fill each compartment. The vertical channels terminate in two adaptor caps which allowed for the insertion of a bacterial airfilter (Gelman Cat.No:4210) and a Nunc tube connector (Cat.No: 171838) respectively. Media and cell suspensions were added through the latter from an aspirator bottle mounted with sterile tubing, tube connector and clamp. {Note: All the accessories such as filters, adaptor, caps, tube-connector, silicone tube, and clamp can be purchased as a 'Start-up kit' from Nunc (Cat.No: 170769)}.

Filling and emptying the unit through the inlet is achieved by raising the aspirator bottle to approximately 1m above the Cell Factory or vice versa. Care must be taken to ensure adequate distribution of cells during the inocculation procedure by mixing the cell suspension well beforehand. All these procedures were performed in a laminar flow cabinet. The inlet can be sealed with a cap or attached to a CO_2 supply if gassing is required during incubation and the entire unit can be housed in a regular 37°C warm room.

HEp-2 cells were loaded at a density of 5×10^4/ml in 1.5 litres of growth medium. After 7 days the medium was removed and the subconfluent monolayers were washed with 1 litre of sterile PBS (prewarmed to 37°C) followed by a 30 minute incubation in the same. 1.5 litres of serum-free Ham's F12 (supplemented with 2 mM L-glutamine) was added. After two days the CM was removed asceptically and replaced with fresh serum-free medium. CM was centrifuged and stored as described in Section 2.7.1 above.

2.8 PROCESSING OF CM

CM was stored in sterile glass bottles (or plastic universals for small quantities) at 4°C. In general, lots of CM were stored for up to 6 months under these conditions without appreciable loss of growth-promoting activity (i.e. HEp-2 autocrine activity). CM fractionated by ultrafiltration or column chromatography was only stored at 4°C for short periods (1-4 weeks) depending on the degree of separation (purification). In general such samples were stored at -20°C.

2.8.1 Ultrafiltration

Ultrafiltration was used to concentrate and fractionate the CM collected. A stirred cell apparatus (Amicon, Cat.No: 8499) was employed for this purpose. This apparatus consisted of a chamber with a 400 ml volume capacity which contained a magnetic stirring bar. CM was filtered under a N_2 pressure head, through one of a series of membranes with a variety of molecular weight (MW) cut-off points (Amicon or Millipore). To avoid the build up of a hydrated gel layer at the surface of the membrane, CM in the chamber was stirred during filtration by placing the ultrafiltration unit on a magnetic stirring apparatus. Molecules in the CM with a molecular weight above the nominal cut-off point for the membrane used were concentrated by being retained in solution while the overall solution volume was reduced by filtration. Such a concentrate was called a retentate. Ultrafiltration was always performed at 4°C.

2.8.1.1 Fractionation of CM by ultrafiltration

By taking advantage of membranes with different MW cut-off points it was possible to generate CM retentates that contained molecules within defined (approximately) molecular weight ranges. This was achieved by taking the filtrate produced by passage through one membrane and subsequently filtering it through a second membrane with a lower MW cut-off point. The following is a list of the different membranes used in this work together with their nominal MW cut-off points and the designation of the retentates and filtrates produced using these membranes.

Amicon Membrane	*Nominal MW cut-off*	*Retentate & Filtrate Designations*
YM 30	30,000 daltons	R30 & F30
YM 10	10,000 daltons	R10 & F10
YM 5	5,000 daltons	R5 & F5
YM 1	1,000 daltons	R1 & F1

Using the same lot of CM, filtration of the F30 through the YM 10 membrane produced a retentate with molecular species > 10 kDa but < 30 kDa. This retentate was designated R10-30 and the filtrate from this process was designated F10(F30). Similarly, CM was separated into fractions with MWs between 1 and 5 kDa, 5 and 10 kDa and 10 and 30 kDa were designated R1-5, R5-10 and R10-30 respectively. All concentrated and/or fractionated CM samples were filter-sterilised immediately using a low protein binding 0.22 μm filter (Millex-GV).

2.8.2 Dialysis

Concentrated CM and fractions from column chromatography were dialysed against 50 volumes of PBS or double distilled H_2O followed by 25 volumes of serum-free medium (Ham's F12) using either (benzoylated) 1,200 MW cut-off (Sigma, Cat.No: D7884) or 10,000 MW (visking) dialysis tubing. Dialysis was performed at 4°C with two changes of buffer daily.

Dialysis tubing needed to be pretreated before dialysis to remove toxic residues (resulting from the manufacturing process and/or present in the storage solution). For this reason the tubing was soaked in ultrapure H_2O and then boiled in 10 mM EDTA for 5-10 minutes. It was then washed several times in distilled H_2O and allowed to soak overnight in ultrapure H_2O. It was rinsed again several times in ultrapure H_2O before use.

2.8.3 Lyophilisation

Samples for lyophilisation in open vessels were covered in parafilm which was pierced many times using a fine a needle. They were then place in a Consol 4.5 freeze-drier (Virtis Comp Inc, Gardiner, HY, USA) and frozen to - 40°C overnight. The following day the condenser was switched on and the refrigeration unit was switched off. When the condensor had reached -50°C (10-20 mins) the vacuum was switched on and the air evacuated from the chamber. The pressure in the chanmber reached 50 miliTorr within 1 hour if the samples were sufficiently frozen. After all the liquid had evaporated, the vacuum was released slowly and the samples retrieved and sealed immediately to avoid absorption of moisture from the atmosphere.

2.9 PHYSICOCHEMICAL TREATMENT OF CM

A series of physicochemical treatments were performed on crude (unprocessed) and fractionated CM (from HEp-2) in an attempt to characterise the active growth stimulator(s) therein. A combination of the treatments described below was performed for some experiments.

2.9.1 Temperature treatment

CM and control medium for heat treatments were transferred in aliquots of 10 mls to clean sterile glass universals (30 mls). A water bath set to 65°C was used for heat treatment at this temperature and the universals were sealed during the incubation periods which lasted 10, 20, 30, and 60 minutes. Treatment by boiling was performed by placing the opened universals into a beaker of boiling water over a bunsen flame. A thermometer placed in a control universal was used to monitor the rise in temperature to 100°C, and the treatment time began at that point. Boiled samples were centrifuged at 3,000 rpm for 15 minutes to remove insoluble precipitate and the samples were filter-sterilised after cooling.

2.9.2 Adjustment of CM pH

The pH of CM and control medium was adjusted and exposed to acid and alkaline pH using HCl and NaOH respectively. Initially 1M solutions of acid and alkali were used but this resulted in significant volume changes in the media. A freeze-drying step was then required in order to reconstitute samples at their original concentration.

To avoid the need for a freeze drying step, 5 M acid and alkali were used thereafter. Significant changes in osmolarity resulted from the use of solutions with higher molarity so these values were monitored as described in the next Section. Exposure to a range of pHs was performed at 4°C and the pH of samples then re-adjusted to pH 7.4 after the appropriate exposure time.

2.9.3 Measurement of Osmolarity

Osmolarity determinations were performed using an Gonotec Osmomat 0300 osmometer (Clandon). The instrument was calibrated immediately before use using a standard salt solution (0.300 Osmols/Kg H_2O). The basis for the measurement of osmolarity is the depression of the freezing point of water by the presence of dissolved solutes.

2.9.4 Exposure to proteinase

Samples of CM and control medium were mixed with a crude trypsin preparation (Gibco, Cat.No: 043-05090H) to give a final concentration of 10 μg/ml trypsin and this was incubated at 37°C for 2-3 hours (in one case 250 μg/ml trypsin was used). In some experiments, purified bovine trypsin (Sigma grade III, from bovine pancreas, Cat.No: T5266) was used at a final concentration of 10 μg/ml in CM. The action of trypsin in all cases was stopped by the addition of soyabean trypsin inhibitor (Sigma, Cat.No: T6522) added to give a final concentration of 20 μg/ml.

2.9.5 Addition of heparin

Heparin from bovine intestinal mucosa (Sigma, Cat.No: H5271) was prepared as a stock solution in Ham's F12 basal medium at 1 mg/ml. It was added to CM and control media to a final concentration of 10 μg/ml before other treatments were performed to assess its ability to inhibit the negative effects of such treatments on the autocrine species.

2.10 PREPARATION OF CELL EXTRACTS

Cells for the preparation of extracts were grown in 75 cm^2 flasks until confluency was reached. For HEp-2 extracts, three flasks were used per preparation.

2.10.1 Extraction buffers

The two extraction buffers used were modifications of those reported by Tiesman *et al.*, (1988). Both contained 0.1% w/v CHAPS {(3-[(3-Cholaminodopropyl)dimethylammonio]-1-propane sulfonate; Sigma, Cat.No: 1300C-2} and the following cocktail of protease inhibitors; Leupeptin (Sigma, Cat.No: L2023) at 1 μg/ml; Pepstatin A (Sigma, P4265) at 2.75 μg/ml; and 1 mM PMSF (Phenylmethylsulphonylfluoride, Sigma, Cat.No: P7626). The protease inhibitors were diluted from concentrated stocks stored at -20°C (Pepstatin at 1 mg/ml; Leupeptin at 1 mg/ml and PMSF at 0.1 M). The high and low salt buffers were as follows.

High Salt Buffer: 1.5M NaCl adjusted to pH 7.54.

Low Salt Buffer: Ham's F12 (1x) adjusted to pH 7.54.

2.10.2 Cell harvesting and extraction

Flasks were trypsinised and the combined cell suspensions pelleted in growth medium in a bench top centrifuge (at 800 rpm). Cell pellets were resuspended in PBS and pelleted by centrifugation. This step was repeated. After the final wash the supernatant PBS was decanted and the cell pellet was resuspended in the extraction buffer and placed on ice. Using a Labsonic U sonicator (Braun), cell suspensions were treated to three cycles of 20 pulses each, with 20 second intervals between cycles (Power setting = 045). Lysates were then pelleted in polypropylene tubes at 25,000g for 30 minutes in a Sorval using the SM-24 rotor (see Appendix A for calculation of settings in rev/min for this rotor).

2.11 PURIFICATION PROTOCOLS

2.11.1 Gel filtration chromatography

Bio-Gel P30, with a 100-200 mesh gel bed was purchased from Bio-Rad (Cat.No: 150 1340) and used to prepare sufficient matrix for a 58 × 1.9 cm column. The gel was pre-equilibrated in phosphate buffer (100 mM, pH 7.4) before it was poured by a downward flow technique ensuring even packing of the gel.

Two separations were performed using this column, both of which were performed at 4°C. The gel was equilibrated in the running buffer before samples were loaded. In the first separation a 100 mM phosphate buffer (pH 7.4) containing 100 mM NaCl was used. A 20x concentrate of HEp-2 conditioned medium (R1 fraction, see Section 2.8.1.1) was separated in this system. 110 × 1.5 ml fractions were collected at a flow rate of 8 ml/hour using a fraction collector (LKB 2070 Ultrorack II) and protein levels in the column eluate were measured on line by a uv monitor set to read at 280 nm. Fractions 1-3 and every three consecutive fractions thereafter were pooled before being dialysed overnight in low MW pore-size tubing (cut-off 1200 daltons) against distilled H_2O. They were then lyophilised as described in Section 2.8.3 before being reconstituted in 2.5 ml of serum-free Ham's F12.

In the second separation a more concentrated sample of HEp-2 conditioned medium (a 60x R1 fraction prepared by ultrafiltration as described in Section 2.8.1.1 above) was applied to the column (which had been treated by passing 10 column volumes of running buffer through the column). The separation was carried out in 25 mM phosphate buffer (pH 7.4) to avoid the need for dialysis. 60 × 2 ml fractions were collected as before. The protein concentrations of the respective fractions were determined using the BioRad protein assay (Section 2.11.6). All fractions were lyophilised as described in Section 2.8.3 above and then reconstituted into 4 ml of serum-free Ham's F12.

Samples from both separations were filter-sterilised before assay (see Section 2.1.3).

2.11.1.1 Desalting of Conditioned Medium / equilibration in running buffer

A 500 ml Sephadex G-15 (Pharmacia, Cat.Mo:17-0020-02) gel filtration column was used to equilibrate 300 mls of unconcentrated HEp-2 CM in 100 mM phosphate (pH 7.4), the running buffer for heparin-Sepharose chromatography. The CM was applied to the column in 3 × 100 ml lots. The peaks of protein excluded from the gel on each separation were pooled (giving 350 mls total volume). The column was run using a 'BIOPILOT' FPLC system (Pharmacia). Operating parameters for column separations such as sample application, flow rates (15 ml/min), buffer mixing for gradient formation, can be pre-determined using the system's programmable contol unit which operates a system of valves and pumps. On-line uv and conductivity monitors connected to a chart recorder measure protein and salt concentrations in the eluate.

2.11.2 Heparin-Sepharose chromatography

Heparin-Sepharose was purchased in powdered form as Heparin-Sepharose CL-6B from Pharmacia (Cat. No: 17-0467-01). The gel was hydrated before use (1g powder yields 4 mls gel aproximately) and washed extensively in buffer A (100 mM potassium phosphate, pH 7.4) to remove particulate material and preservatives (250 mls of buffer per 1g of powder was recommended by the manufacturers). The swollen gel was stored at 4°C before pouring the column. All separations using this couumn were performed at 4°C.

2.11.2.1 Batch elution

A 5 ml heparin-Sepharose column was prepared and equilibrated in running buffer {buffer A, (100 mM postassium phosphate buffer, pH 7.4)}. A 2ml concentrate (60x, R1 as described in Section 2.8.1) of Hep-2 CM was loaded onto the column and the column was washed with 4 column volumes of running buffer. The 'wash' contained unbound material and was pooled for analysis. Heparin-bound material was eluted using 10 ml batches of buffer A containing incremental increases in NaCl concentrations from 0.1 M through 0.5 M, 1.0 M, 1.5 M, 2.0 M, and 5.0 M respectively. 10 ml fractions were collected after every batch application and these were dialysed as described in Section 2.8.2 above. All fractions were filter-sterilised after dialysis (Section 2.1.3). The volume change in all fractions (due to loss during handling and osmosis effects) was accounted for in determining the dilution factor required to give a 1x equivalent in the assay.

2.11.2.2 Gradient elution

A 10 ml column was poured and equilibrated in running buffer (100 mM potassium phosphate buffer, pH 7.4). The system was controlled using the 'BIOPILOT' FPLC system described in Section 2.11.1.1 above. Pre-equilibrated HEp-2 CM (Section 2.11.1.1) above was applied to the column at a rate of 1 ml/minute (i.e. 300 mls of the total volume of 350 ml). After sample loading the column was washed with 50 ml of buffer A and a 10 column volume continuous gradient was applied using buffer B (2.5M NaCl in 100 mM phosphate buffer, pH 7.4) at a flow rate of 1 ml/minute. The first 132 mls of eluate was collected and pooled. 5 ml fractions were collected thereafter. All fractions for analysis were dialysed as described in Section 2.8.2 above and filter-sterilised before assay (Section 2.1.3).

In the case of cell extracts, the same column was used after it had been washed through with 10 column volumes of 0.5 M-NaCl in 0.1 M-Tris/HCl, pH 8.5 followed by 10 column volumes of 0.5 m-NaCl in 0.1 M sodium acetate, pH 5.5. It was then finally re-equilibrated in 10 column volumes of buffer A {which included 0.1% CHAPS (w/v) for these separations}. The high and low salt extracts were loaded as 8 and 10 ml samples respectively at a flow rate of 1 ml/minute in separate subsequent runs on the same column. After sample application, the column was washed with 75 mls of buffer A. A 0-3M NaCl continuous gradient was developed in this system with 3M NaCl in 100 mM phosphate buffer (buffer B). 3 ml fractions were collected from the start of the wash through to the end of the gradient. Both separations of extracts were run using the same programme. Where appropriate, samples were dialysed as described in Section 2.8.2, or filter-sterilised directly.

2.11.3 Cation-exchange chromatography

CM Sepharose fastflow matrix was purchased from Pharmacia (Cat.No: 17-0719-01) in a pre-swollen form. A 10 ml column of this cation-exchange resin was poured and equilibrated with 50 mM potassium phosphate buffer pH 6.0. To improve the stability of FGF-like or other unstable proteins 0.1% (w/v) CHAPS was included in the sample and running buffer. The conductivity of 200 mls of unconcentrated HEp-2 conditioned medium was reduced from 10 mS to 6 mS by diluting it with 180 mls ultrapure of H_2O and 20 mls of 200 mM potassium phosphate (pH 6.0). The pH of a ½ dilution of CM was reduced to pH 6.0 before it was applied to the column at rate of 5 mls/minute.

After sample application the column was washed with 60 mls of equilibration buffer (50 mM phosphate, pH 6.0 containing 0.1% w/v CHAPS) and then eluted by irrigating with 4 column volumes of buffer containing 0.25M, 0.5M, 0.75M and 1M-KCl respectively. This separation was run at 4°C under controlled conditions using the 'BIOPILOT' FPLC system described above (Section 2.11.1.1). Fractions of 20 mls were collected from the start of the KCl gradient. 5 ml samples of these were dialysed in pretreated low MW pore-size dialysis tubing (Section 2.8.2.) against distilled water, followed by dialysis against Ham's F12 (serum free medium).

2.11.4 Hydrophobic interaction chromatography

Phenyl Sepharose CL4B was purchased from Pharmacia (Cat.No: 17-0467-01). A 1 ml column of this material was poured and equilibrated with Ham's F12 medium (serum-free). A 2 ml sample of HEp-2 CM concentrate (R10-30, 10x) was applied to the column. Fractions of 2 mls were collected as the column was washed through first with 2 mls of Ham's F12 followed by successive 6 ml volumes of 1M and 0.5M $(NH_4)_2SO_4$ (Sigma, grade III, Cat.No: A6387). This was followed by 6 mls of distilled H_2O and by successive 6 ml volumes of 10%, 20% and 30% glycerol (Sigma, Cat.No: G5516). All fractions were dialysed in dialysis tubing with a MW cut-off of 1200 daltons (pretreated as described in Section 2.8.2 above) against three changes of distilled H_2O followed by overnight dialysis against serum-free Ham's F12 medium (buffered to pH 7.45 with 20 mM HEPES). All samples were filter-sterilised (Section 2.1.3) before being tested for mitogenic activity in the HEp-2 assay.

2.11.5 Ammonium sulphate fractionation of a biological fluid

A 100 ml sample of an effusion derived from a patient with ovarian cancer was subjected to several salt cuts using ammonium sulphate (Sigma, grade III, Cat.No: A6387) as follows.

The percentage $(NH_4)_2SO_4$ in the effusion sample was brought to 20% (w/v) by adding 25 mls of a saturated $(NH_4)_2SO_4$ soultion in a dropwise manner, while stirring the fluid on ice. The sample was then centrifuged at 15,000 rpm in a Sorval for 30 minutes and a 25 ml aliquot of the supernatant removed. The remaining supernatant was returned to the beaker on ice and the protein pellet resuspended in 10 mls ultrapure H_2O (thereby concentrating this fraction $\times$ 10). The % salt in the fluid sample was increased to 30% as described above by the addition of 14.3 mls of the saturated $(NH_4)_2SO_4$ solution and then centrifuged as before. 14.4 mls of the supernatant was removed before bringing the % salt to 50% by the addition of another 40 mls of the saturated salt solution. 10 mls of the 50% saturated fluid supernatant was removed after centrifugation. Precipitate collected at the respective salt concentrations were centrifuged for 30 minutes at 15,000 rpm and then resuspended in 10 mls ultrapure H_2O. All samples were then dialysed against distilled H_2O and lyophilised before being resuspended in PBS (to ensure a uniform and physiological salt concentrations in all samples).

2.11.6 BioRad protein assay

The BioRad protein microassay was used in this work to determine the protein concentration of ultrafiltration concentrates, fractions eluted from the separation of HEp-2 CM on the Biogel P30 column, as well as biological fluids. A standard curve for protein was generated using bovine serum albumin (Sigma, Cat.No: A7031) as a reference. Stock BSA was serially diluted in the range 1-25 μg/ml. 0.2 ml of dye reagent (BioRad, Cat.No: 500-0006) was added to 0.8 ml of standards and samples in clean dry test tubes. Test tubes covered in parafilm were mixed by gentle inversion and left at room temperature for 5 minutes before the absorbance at 595 nm was determined. All measurements were taken within 1 hour from the time of adding the dye and the absorbance versus protein concentration then plotted. The concentration of unknowns were read from this standard curve.

2.12 BIOASSAYS

2.12.1 Pretreatment of cells

All stocks of cells used for bioassay were mycoplasma-free and in log phase growth at the time of harvesting. To minimise inter-assay variability and to ensure that cultures were actively dividing when harvested, cells for use in bioassays were pretreated by subculturing two days before required for assay and refed on the morning of the preceeding day. The plating densities used in each bioassay depended on the particular cell line used. Growth medium used for cell pretreatment was supplemented with 10% serum. It is important to maintain this level even with cell lines such as HEp-2 which otherwise will grow at much lower concentrations. For example, HEp-2 pretreated in 5% FCS will not grow at the low plating density used in the autocrine assay. Table 2.12.1 shows the plating densities used for the different cell lines used here.

Table 2.12.1 Plating densities of cell lines used for bioassays in this work

CELL LINE	ASSAY TYPE	Cell №/75 cm^2 flask
HEp-2	Monolayer growth	2.0×10^6
DLRP	Monolayer & Soft agar growth	2.0×10^6
DLKP	Monolayer & Soft agar growth	1.0×10^6
SK-MES-1	Monolayer growth	1.5×10^6
NRK	Soft agar growth	0.9×10^5

2.12.2 Preparation of cell suspensions

In general it was easier to prepare single cell suspensions from subconfluent monolayers. Longer exposure times to trypsin also caused difficulties in generating single cell suspensions because individual cells tended to become 'sticky' and formed clumps. After pelleting cells following trypsinisation (Section 2.2.4) cells for bioassay were washed by being resuspended and pelleted in serum-free medium (to remove traces of serum and debris). They were then resuspended in plating medium. A small volume of serum-free medium (1ml) was used initially to make the stock cell suspension with a micropipette tip and the final volume was brought to no more than 2-5 mls depending on the expected yield. The viable cell number in this 'stock' suspension of cells (often diluted to facilitate counting) was determined (Section 2.2.5.1) before the appropriate diltions were made to give the 'working' cell suspensions in the relevant assay plating medium.

2.12.3 Soft agar assays

The ability of cell lines to form three-dimensional colonies in semi-solid media has been correlated with the tumourigenicity of such cells in syngeneic hosts. Consequently this property has been used as an index of malignant transformation. The double agar method developed by Hamburger *et al.*, (1978) was used here to quantify this property in the new lung carcinoma cell lines established in this lab.

2.12.3.1 Colony counting in soft agar

Colonies formed in soft agar were viewed at 40x using a CK Olympus inverted microscope, (Tokyo, Japan). The eye-piece graticule had a stage micrometer which was used to score colonies of a particular size. A transparent grid was placed over the light aperture on the microscope stage. The grids formed 4 mm^2 squares which were visible through the agar and facilitated the counting of colonies on the plate. 35 such squares were viewed by moving the focus through the agar and the number of colonies per square were recorded. The total number of colonies per plate was extrapolated from the number of colonies on this protion of the plate (= 14.55% of the total plate area). An even distribution of cells at plating was assumed.

The percentage colony forming efficiency (% C.F.E.) was then calculated from this figure as follows:

$$\% \text{ C.F.E.} = \frac{\text{Number of colonies per plate}}{\text{Number of cells plated}} \times 100$$

For every sample set up (in triplicate) the mean % C.F.E. was determined and standard error of the mean for these determinations were calculated using the following formula.

$$S.E.M. = \frac{\sqrt{\frac{\sum x^2 - (\sum x)^2/n}{(n-1)}}}{\sqrt{n}}$$

Where n = number of replicates per sample.
x = C.F.E. of each replicate.
Σ = sum of the samples from 1 to n.

2.12.3.2 Transforming growth factor α-like assay

Only cultures of NRK cells between passages 19 and 21 were used in this assay.

Assay Protocol:

On the day of assay, 1.548g of bacto-agar (Difco, Detroit USA) was dissolved in 100 mls of ultrapure H_2O, autoclaved and allowed to cool to 44°C in a water bath at this temperature.

A sufficient volume of an 'agar growth medium' (AgM) was prepared in the following ratio;

Preparation of Agar Growth Medium:		*Preparation of 2x DMEM:*	
DMEM 2x	50 mls	DMEM 10x	20.0 mls
HEPES (1 mM)	2 mls	Ultrapure H_2O	73.0 mls
$NaHCO_3$	1 ml	HEPES (1 mM)	4.0 mls
P/S (100x)	1 ml	$NaHCO_3$	2.2 mls
Growth mediun (-FCS)†	14 mls	Adjust to pH 7.4	(1M-NaOH)

† This was DMEM supplemented with 2mM L-glutamine and 2% P/S.

Freshly prepared AgM was equilibrated in the water bath at 44°C.

Samples were assayed in triplicate on appropriately labelled 35 mm petri dishes (Greiner, Cat.No: 627160). The empty labelled dishes were arranged on clean trays which had been swabbed with 70% IMS in the laminar flow cabinet.

The thermo-labile components of the AgM, L-glutamine (1 ml) and FCS (10 mls) were added just before equal volumes of the agar and AgM were mixed.

The AgM/agar solution was mixed well and 1.5 mls/plate of this mixture dispensed quickly before it was returned to the water bath. The temperature in the water bath was reduced to 41°C and allowed to equilibrate while the agar on the plates was allowed to set. The agar in this layer was 0.6% (w/v).

The NRK cells were trypsinised and a single cell suspension prepared and counted as described in Sections 2.2.4 & 2.2.5 above. A concentration of 4×10^4 NRK cells/ml was prepared in growth medium without serum such that the final plating number per 35 mm plate would be 6×10^3 cells per plate after the following dilutions; 0.5 ml of this suspension was added to each test sample (2mls) in a sterile universal and this was mixed with 2.5 mls of agar growth medium (plus serum). After adequate mixing (achieved by pipetting up and down), 1.5 mls/plate of this diluted cell suspension was dispensed onto the relevant plates (x3). Cells were thus plated in 0.3% (w/v) agar at 41°C. This effectively diluted the original test sample concentration by a factor of 1/5.

The AgM solution was re-equilibrated at 41°C after every five samples to avoid solidification.

Trays were covered in aluminium foil before being incubated for 10 days at 37°C in a humid atmosphere with 5% CO_2. The number of colonies which were 50 μm (microns) or greater in diameter were counted as described in Section 2.12.3.3 below.

2.12.3.3 Clonogenic assay

The protocol for the clonogenic assays was the same as that described for the TGFα-like assay above except that a range of cell concentrations were plated in the top agar layers. Cell suspensions were made up at 6.66x the desired final number/ml to allow for dilution in the agar growth medium. Serial dilutions of the highest cell concentration were made in growth medium.

Colonies with diameters of 50 μm and greater were counted (i.e. scored as a colony) in the clonogenic assay and the percentage colony forming efficiency was calculated as described in Section 2.12.3.1.

2.12.4 Monolayer assays

Monolayer staining with crystal violet

Growth medium was aspirated from plates, taking care not to remove cells attached in monolayer. Wells were rinsed with PBS (×1) and fixed with 3% formalin (BDH, Cat.No: 101113). They were then stained for 10 minutes with a 0.25% (w/v) aqueous solution of crystal violet (Gurr, BDH Cat.No:30424). The crystal violet solution was pre-filtered through Wattman №1 filter paper before use. The plates were washed in tap water to remove excess dye and then dried, inverted on tissue paper to avoid water stains.

2.12.4.1 Measurement of colony area and number by image analysis

The area (mm^2) covered by crystal violet-stained colonies was measured using an AMS 40-10 image analyser. The instrument was callibrated to read in a defined area (in this case a circular area delineated by the circumferance of a well on the 24-well culture plate) These settings were programmed into the microprocessor and recalled for subsequent readings. The image scored by the instrument is calculated by the relative difference in the degree of shading between the amount of dark staining on the plate (i.e. the area covered by cells) and the background unstained surface. Detection sensitivity was set immediately before reading the assay. The choice of setting for sensitivity was a compromise between the detection of very low staining levels (e.g. in control wells of the autocrine assay) and that of higher levels of staining (e.g. in growth stimulated wells). In practice this was adjusted for optimum detection. Sensitivity was also subject to variations in voltage. In order to minimise these fluctuations, a voltage stabiliser was employed and the instrument was switched on overnight before use.

2.12.4.2 HEp-2 autocrine assay in 24-well plates

A single cell suspension of HEp-2 cells was prepared (Section 2.2.5.1) at a concentrations of 8×10^2 cells/ml in DMEM containing supplements at 2x their final concentration such that there was 1% FCS, 500 U/ml penicillin/500 μg/ml streptomycin or 250 μg/ml gentamicin and 2 mM L-glutamine in the assay medium after addition of the test samples. 0.5 ml of cell suspension was plated in each well with mixing of the cell suspension after every 6 wells plated.

Test samples of CM or growth factors were added to the plated cells in a total volume of 0.5 ml (in quadruplicate or triplicate per determination), bringing the final volume per well to 1 ml. All dilutions of test samples for assay were diluted in serum- and supplement-free Ham's F12. Controls consisting of assay medium only (DMEM/Ham's F12 plus supplements) were included on each plate. When all the wells had been plated with cells and test samples the individual plates were mixed carefully to ensure even spreading of cells. These were then incubated at 37°C in a humidified 5% CO_2 atmosphere. After 7 days the plates were removed and checked for signs of contamination before they were stained with crystal violet (Section 2.12.4) and the colony areas determined by image analysis (Section 2.12.4.1 above).

2.12.4.3 Monolayer assays for human lung squamous cell carcinomas cell lines

The protocol for all the monolayer growth assays was essentially the same. Indicator cells were plated in DMEM with 2x supplements such that the final concentrations in the assay were 1% FCS, 500 U/ml penicillin/500 μg/ml streptomycin or 250 μg/ml gentamicin and 2 mM L-glutamine. This was to allow for dilution by the addition of test samples in a total volume of 0.5 ml/determination, bringing the final volume per well to 1 ml.

Samples for testing in the growth assays were added either as undiluted solutions or diluted in serum- and supplement-free Ham's F12. Growth factors diluted from stock solutions (Section 2.13.2 below) containing 1 mg/ml bovine serum albumin (Sigma, fraction V, Cat.No: A9418) were diluted to 2x their desired concentration in the assay to allow for the dilution factor upon addition to the cell suspension in the assay.

The plating densities used for the three human lung squamous cell carcinoma cell lines in the various applications of the 24-well monolayer growth assays are given in Table 2.12.4.3.

Table 2.12.4.3 Plating densities of DLRP, DLKP and SK-MES-1 in monolayer growth assays used for differnent applications

CELL LINE	ASSAY TYPE	Cell N°/well
DLRP	Growth factor assay	3.0×10^3
DLRP	Autocrine assay and ME†	5.0×10^3
DLKP	Autocrine assay and ME	1.0×10^3
SK-MES-1	Growth assay with ME	5.0×10^3

† Growth response to malignant effusions.

2.12.5 Acid phosphatase measurement

The acid phosphatase activity was determined by measuring the dephosphorylation of the substrate PNP (p-nitrophenyl phosphate) resulting in the appearance of the product p-nitrophenol with a λ_{max} for absorbance at 405 nm. The absorbance measured at this wavelength is proportional to the activity of acid phosphatase which in turn is proportional to cell number.

Method:

Preparation of Substrate

A 10 mM p-nitrophenyl phosphate (Sigma, Cat.No: C104) was prepared **just before use** in a lysis buffer containing 0.1% Triton X-100 (BDH, Cat. No: 30632), 0.1M Sodium Acetate (Sigma, Cat.No: S2889) at pH 5.5. The lysis buffer was stored at 4°C before use and the phosphatase substrate was stored in the dark at -20°C.

Measurement of Acid Phosphatase Activity

Growth medium was carefully removed from wells to avoid disruption of cells on the monolayer (This is particularly important for loosely adherent cell lines). Wells were rinsed with 100 μl of PBS and this was carefully removed as per growth medium.

100 μl of 10 mM PNP substrate in lysis buffer was added to each well and the plates incubated at 37°C for 2 hours. After this time bubbles were removed using an 18 gauge syringe needle (to avoid light scattering effects) and the absorbance was measured. If after 2 hours the intensity of absorbance due to the p-nitrophenol chromophore was insufficient for adequate measurement, then the reaction was either incubated for a longer time (e.g. a further 1-2 hours) or 'stopped' by the addition of 50 μl of 1M-NaOH per well which has the effect of causing an electrophillic shift in the chromophore and therefore increases the absorbance in the yellow wavelength range (405 nm).

Absorbance at 405nm was measured on a dual wavelength ELISA plate reader (Titertek Multiskan) using the 620nm filter as a reference wavelength. Absorbance at 620 nm was due to the plastic plate and automatically is subtracted from the total absorbance before readout of the results.

2.12.5.1 HEp-2 autocrine assay in 96-well plates

HEp-2 cells for use in the 96-well autocrine assay system were pretreated as described for the 24-well assay. The working cell suspension was prepared at 3×10^3 cells/ml in DMEM plus 2x supplements. 100 μl of this cell suspension was plated per well on the 96-well plate using an eight-well multichannel micropipette. Test samples were added in 100 μl of Ham's F12 and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2.

2.12.6 ^{125}I-EGF radioreceptor assay

This assay is based on the competition for binding between ligands with an affinity for the Epidermal growth factor receptor. TGFα competes with EGF for the EGF receptor on a mole per mole basis {Todaro *et al.*, (1980)}. Displacement of a fixed concentration of radioactively labelled EGF by increasing concentrations of unlabelled EGF permits a standard curve to be drawn from which the concentration of an unknown EGF competitor can be estimated.

Method:

A431 cells were plated in 24-well plates (Costar 3524) at 10^5 cells per well in 1 ml of DMEM + 10% FCS. Cultures were incubated overnight at 37°C in an atmosphere of 5% CO_2. The next day the medium was removed carefully (to avoid removing the attached cells) and the cell monolayer was washed in cold binding buffer {DMEM + 1mg % BSA, (Sigma A7031)}.

^{125}I-EGF (Amersham 1M 124) was prepared in binding buffer to give a final activity of 0.01 μCi per 100 μl of binding buffer. EGF standards were prepared from a 10 μg/ml stock of 'unlabelled' EGF (Sigma, Cat.No: E1257) freshly thawed from storage at -20°C (prepared in lots of 450 μl). The following dilutions were prepared for the standard curve; 10000, 500, 100, 50, 10, 5 and 1 ng/ml. All standards and samples were kept on ice until required.

100 μl of each standard was added to triplicate wells together with 100 μl of ^{125}I-EGF solution. Samples were mixed by swirling and incubated at 20°C for 1 hour. 100 μl of CM concentrates for analysis were mixed with 100 μl of ^{125}I-EGF solution and treated in the same way.

Non-specific binding of ^{125}I-EGF was determined in the presence of excess unlabelled EGF. For this purpose 100 μl of 10 μg/ml EGF was incubated instead of the standard EGF concentration.

Supernatants were removed without disturbing the cell monolayers and these were rinsed three times with 1 ml of cold binding buffer. Finally the cells in each well were dissolved in 0.5 ml of 0.5M NaOH and transferred to counting vials (Rohren tubes 55.44 Starsted). Each well was rinsed twice with 0.5 M NaOH and combined with the original extract for the respective well (in the counting tube).

Samples were counted for 1 minute on a mini gamma counter (LKB 125). The results were calculated as the percentage inhibition of each sample over total ^{125}I-EGF bound in the absence of a competitor.

$$\% \text{ INHIBITION} = 100 - \frac{\text{CPM bound per sample - Non-specific CPM}}{\text{TOTAL CPM - Non-specific CPM}} \times 100$$

2.13 PURE GROWTH FACTORS

2.13.1 Source

Purified growth factors with the exception of TGFα (which was kindly donated by Majorie Winkler, Genentech) were purchased from several commercial suppliers. A list of suppliers and the source of the purified growth factors are given in Table 2.13.1.

Table 2.13.1 Source of pure growth factors used in the course of this project

GROWTH FACTOR	Source/Supplier	Catalogue №
Acidic FGF (aFGF)†	Bovine brain/British Biotechnology Ltd. (BBL)	BDP-12
Basic FGF (bFGF)†	Bovine brain/British Biotechnology Ltd.	BDP-13
Interleukin 1α (IL-1α)†	Human recombinant protein from *E.coli*/BBL.	BDP-15
Interleukin 1β (IL-1β)†	Human recombinant protein from *E.coli*/BBL.	BDP-17
Insulin	Bovine pancreas/Sigma.	I-1882
Insulin-like growth factor I (IGF-I)	Human recombinant, *E.coli*/Boehringer Mannheim.	BCL1048-066
Insulin-like growth factor II (IGF-II)	BRL-3A cell conditioned medium/Sigma.	M-9145
Epidermal growth factor (EGF)	Mouse submaxillary gland/Sigma.	E-4127
Transforming growth factor α (TGFα)	Human recombinant/Genentech.	PD-1
Transforming growth factor β (TGFβ)	Human platelets/Peninsula Labs, Europe Ltd.	IP-1085
Platelet-derived growth factor (PDGF)	Human platelets Peninsula Labs, Europe Ltd.	IP-1080
Bombesin/Gastrin releasing peptide	Sigma.	B-5508

† Manufactured by R&D systems, inc, Minneapolis, MN, USA.

2.13.2 Preparation

Stock solutions of growth factors were prepared in the appropriate buffers, all of which contained bovine serum albumin (fraction V, Sigma, Cat.No: A9418) at a concentration of 1 mg/ml (except in the case of Bombesin). Bovine serum albumin (BSA) was included to prevent loss of growth factors due to non-specific binding of these proteins to the walls of storage vials at low concentrations.

In the case of particularly 'sticky' and labile proteins such as the FGFs, the zwitterionic detergent CHAPS was included in the buffer for the preparation of stock solutions at 0.1% w/v. Matuo *et al.*, (1988) reported that this agent stabilised FGFs during frozen storage. All growth factor stocks were stored at -20°C and thawed as required. Repeated freeze/thaw cycles were avoided by dispensing stocks in smaller volumes after initial preparation.

A list of the buffers and concentrations used in the preparation of growth factor stock solutions is given in Table 2.13.2.

Table 2.13.2 Preparation of stock solutions of pure growth factors

GROWTH FACTOR	Reconstitution buffer	Stock concentration
Acidic FGF (aFGF)	PBS-A + 0.1% (w/v) CHAPS	10 μg/ml
Basic FGF (bFGF)	PBS-A + 0.1% (w/v) CHAPS	10 μg/ml
Interleukin 1α (IL-1α)	PBS-A	1 μg/ml
Interleukin 1β (IL-1β)	PBS-A	1 μg/ml
Insulin	0.1 M Acetic Acid	200 mg/ml
Insulin-like growth factor I (IGF-I)	10 mM HCl	100 μg/ml
Insulin-like growth factor II (IGF-II)	10 mM HCl	20 μg/ml
Epidermal growth factor (EGF)	DMEM/Ham's F12 (1:1)	1 μg/ml
Transforming growth factor α (TGFα)	Phosphate buffered saline (PBS)	100 μg/ml
Transforming growth factor β (TGFβ)	0.1 M Acetic Acid	10 μg/ml
Platelet-derived growth factor (PDGF)	0.15 M-NaCl	25 U/ml
Bombesin/Gastrin releasing peptide	DMEM + 1 mg% BSA	1 mg/ml

2.14 NEUTRALISING ANTIBODY EXPERIEMTS

The ability of antibodies to neutralise the mitogenic activity of the growth factors against which they are directed depends on the target cell line and the incubation conditions used. The two different lots of anti-FGF neutralising antibodies used in this work were purchased from British Biotechnology Ltd (Abingdon, Oxon OX14 3YS, England).

2.14.1 Source and specifications of anti-FGF antibodies

Anti-aFGF (Cat.No: BDA 6) and anti-bFGF (Cat.No: BDA 4) IgG antibodies raised in rabbits against highly purified bovine bFGF (homogeneous on silver stained SDS-PAGE gels) were isolated by Protein A chromatography and filter-sterilised by passage through a 0.2 μm filter (R&D systems inc, Minneapolis, MN, USA). They were supplied lyophilised from a solution containing PBS, pH 7.5, in 1 mg quantities (British Biotechnology Ltd, and stored at -20°C (Stable for 6-12 months lyophilised).

Lot 1 Efficacy

The efficacy of the first lot of anti-FGF antibodies purchased had been determined by the manufacturers using the NR6-3T3 fibroblast cell line. The amount of antibody required to neutralise 80% of acidic FGF mitogenic activity (when aFGF was present at a concentration sufficient to give maximal stimulation) was quoted as 20-30 μg/ml for anti-acidic FGF antibody. Using the same criteria, anti-basic FGF at 30 μg/ml inhibited 90% of the mitogenic response to bFGF. According to the manufacturers specifications both anti-FGF antibodies were non-cross reactive with a number of different polypeptide growth factors from various growth factor classes (as determined by Western blotting or ELISA). Similarily neither anti-FGF antibody cross reacted with the other. Furthermore neither antibody demonstrated mitogenic activity in their normal working concentration range (0-100 μg/ml) for the indicator cell line used, NR6-3T3.

Lot 2 Efficacy

The efficacy of the second lot of antibodies purchased (Lot.No:P083, anti-aFGF; Lot.No:Q177, Anti-bFGF) was expressed in terms of their ***neutralising dose***$_{50}$ (ND_{50}) values. These values had also been determined using the NR6-3T3 fibroblast cell line. The ND_{50} value was defined as,

> **" that concentration of antibody required to yield one-half maximal inhibition of the cytokine, when that cytokine is present at 5 times its normal ED_{50} concentration"**

In the NR6-3T3 fibroblast system, 5 times the ED_{50} concentration of cytokine (growth factor) gave 100% activity (stimulation). Under these conditions the ND_{50} values determined were quoted as 2 μg/ml for both neutralising antibodies respectively.

2.14.2 Preparation of antibodies

The antibodies were reconstituted in 1 ml of sterile ultrapure H_20 to make stock solutions of 1 mg/ml. The recommended storage time for these solutions at 4°C was 1 month or 3-6 months at frozen at -20°C.

2.14.3 Preincubation of antibodies with CM and pure growth factors

Anti-FGF neutralising antibodies stocks were thawed from previously unused (since initial preparation) frozen stocks on the day of the experiment and were diluted in Ham's F12 (minus BSA) to working stocks of 1 μg/ml and 0.1 μg/ml. Similarly fresh stocks of acidic and basic FGF growth factors were thawed on the day and working stocks made by dilution only after the antibody dilutions were complete. Growth factor dilutions were made in Ham's F12 with and without BSA to give working stock solutions at 1 μg/ml and 0.1 μg/ml respectively. These working stocks were kept at 4°C and diluted to the final concentrations just before mixing with the antibodies. Growth factor controls were mixed with the appropriate amount of antibodies in a total volume of 40 μl incubated for 90 minutes at 37°C before being brought to 1.5 mls with serum-free Ham's F12. The CM samples were also incubated with antibodies for 90 minutes in a total volume of 1.5 mls of the respective sample. After incubation with antibody, all samples were added directly to pre-plated HEp-2 cells set up for the autocrine assay (Section 2.12.4.2).

2.15 ANTISENSE OLIGODEOXYNUCLEOTIDES

2.15.1 Source

Customised 15 base pair oligonucleotides were produced by British Biotechnology Ltd in an unmodified form on an Applied Biosystems 380B DNA synthesiser. Approximately 400 μg of each HPLC-purified oligodeoxynucleotides were recovered from a 1.0 μmole scale synthesis and supplied in a lyophilised form.

2.15.2 Preparation

Oligonucleotides were reconstituted in 100 μl of sterile ultrapure H_2O. The DNA concentration was determined spectrophotometrically as described below. 1 μl was removed for testing the acidity of this solution (using indicator paper).

2.15.3 Spectrophotometric quantification of oligodeoxynucleotides

Oligonucleotides were quantified by measuring the absorbance of a dilute aqueous solution (in ultrapure H_2O). Concentrations were calculated on the basis that one OD_{260} unit corresponds to 30 μg/ml of single-stranded DNA (for short sequences). Absorbance measurements were performed in quartz cuvettes using a dual beam spectrophotometer (UV-160A, Shimadzu, Japan). 3 μl aliquots of the stock solutions were diluted in 3 ml of ultrapure H_2O ($\times$ 1000 fold) and mixed well by inversion before absorbance was measured.

2.15.4 Calibration of multichannel micropipettes

The percentage errors on plating for an electronic multiwell pipette (Rainin EDP plus), used with the appropriate tips (Rainin RT 96) was compared to that of a manual multichannel micropipette (SLT 40-200 μl, Labinstrunebts, Austria). The coefficient of variation with each pipette was determined by dispensing 100 μl/well of a 0.2% w/v solution (in 1% acetic acid) of the dye Sulforhodamine B (Sigma, Cat.No: S9012). The difference in absorbance was at 570 and 620 nm was measured using a Titertek Multiskan ELISA plate reader which calculated the coefficient of variation for each channel on the micropipette. The errors with both instruments was generally very low but the performance of the electronic pipette was better in this respect and this instrument was used to dispense the small volumes employed in the antisense experiments.

2.15.5 Protocol for antisense experiments with HEp-2

A 2 $\times$ 10^4/ml cell suspension in DMEM/Ham's F12 medium plus 1% heat-inactivated FCS, 2 mM L-glutamine and 500 U/ml penicillin/500 μg/ml streptomycin was plated at 50 μl/well in 96-well plates 24 hours prior to the addition of the oligonucleotides. No cells were plated on the outside wells but instead 50 μl of growth medium was plated here. Although the optimum time for addition of the oligonucleotides was not known, the oligonucleotides were addded after the cells had been plated and allowed to attach overnight. This was in order to avoid any potentially inhibitory effect on cell attachment. On the next day, all the growth medium was removed before the addition of oligonucleotides.

Oligonucleotides were diluted from stock solutions in growth medium (DMEM:Ham's F12 + 1% heat-inactivated FCS, 2 mM L-glutamine, 250 μg/ml gentamycin). In parallel, a control with no added oligonucleotide was refed with fresh medium. Separate controls were included on each plate. Acidic FGF sense and antisense oligonucleotides were assayed on the same plate and the bFGF oligonucleotidess were tested on a second plate to avoid plating errors resulting from variations due to cell density changes.

3.0 RESULTS

3.1 PRIMARY CULTURE OF HUMAN THORASIC TUMOUR TISSUE

Over a three year period from 1986-1989, 55 solid human tumour tissue samples were received from thorasic surgery. The samples were taken directly at the time of resection and collected in cell culture growth medium containing antibiotics and an antifungal agent (Section 2.4.1). While the majority of samples were of lung origin, non-lung thorasic samples were also obtained. These included oesophageal and thymic tissue samples. Attempts were made to develop a regime for the *in vitro* culture of the tissue obtained using a number of established techniques. In addition, 8 malignant effusions were collected during this period from patients with different carcinomas and an effort was made to culture cells obtained from these fluids.

3.1.1 Source and composition of tissue samples

The nature of the samples obtained varied considerably in terms of size, texture, accessory adipose tissue, degree of necrosis and vascularisation. In addition, the histological status of the tissue received was not always known at the time of processing for *in vitro* culture (i.e. immediately after carriage to the laboratory). This was generally the case with investigative surgical procedures where patients first presented with disease symptoms. These include bronchoscopy, involving the removal of tissue with a bronchoscope lowered down the trachea into the bronchus, and mediastinoscopy, which requires an incision in the upper chest to examine and/or resect mediastinal nodes for histology. Where appropriate, both procedures were often performed by the surgeon in tandem. The amount of tissue removed for these biopsies was small and that available for tissue culture (after sufficient material was removed for hospital histology) was limited (usually less than 1 cm^3). More tissue was generally removed during mediastinoscopies than bronchoscopies, but as a rule, neither procedure provided as much material as the resection of entire tumours.

In the case of lung cancers, removal of tumour masses were performed by surgical procedures which excised the entire lung (pneumonectomy), or one or more of the constituent lobes (lobectomy). The composition of such tumour material was no less heterogeneous than biopsy sized samples. However, resection for the removal of entire lung tumours was normally only considered where non-small cell lung carcinoma had been diagnosed following bronchoscopy or mediastinoscopy. For this reason large samples formed a histologically selected subset of lung tumours.

A small number of the tumours resected from the lung were benign or were metastases from primary tumours in other organs. For presentation purposes samples were classified as follows;

(1) Pneumonectomy / Lobectomy

(2) Mediastinoscopy / Bronchoscopy

(3) Thoracotomy

(4) Oesophageal

(5) Malignant effusions

3.1.2 TISSUE PROCESSING TECHNIQUES

Samples were carried to the cell culture laboratory in transportation medium on ice. All manipulations of the tissue were carried out under sterile conditions as described in Section 2.5. The amount of tissue received dictated the type of processing employed.

After the removal of vascular, adipose, and necrotic tissue, the objective was to release the epithelial cell component from the tissue matrix with minimum cellular damage. Two methods have been used traditionally to achieve this end, namely **mechanical** and **enzymatic** disaggregation. In this work, the former method was the method of choice where the amount of tissue was small and/or loosely bound. Where the texture was tougher, mechanically sliced fragments were also subjected to enzymatic treatment in an effort to digest the stromal extracellular matrix and release the epithelial cell component.

A common problem with primary epithelial cell cultures not grown in selective media is overgrowth by cells from the stromal cell component of the tissue, namely fibroblasts. These cells appear to have less stringent growth requirements than their epithelial counterparts. It is also possible that the isolation conditions and/or the culture media components used, mitogenically activate fibroblasts in preference to epithelial cells. Because the majority of human tumours arise from epithelial cells, these are the cells which are of interest in growing. However, excessive proliferation of fibroblasts causes problems with nutrient depletion of the medium and limited growth surface area (which can lead to a physical restriction on growth of epithelial cells). The percentage of stromal cells in different tissues varies. The same is true for malignant tissue. The following investigation considered this problem with respect to the methodology used, but also looked at ways to remove unwanted fibroblasts. Other sources of growth stimulators for primary epithelial cells were investigated, with a view to identifying factors that might promote *in vitro* growth of lung squamous cell carcinomas.

3.1.2.1 Mechanical disaggregation of tissue samples

This procedure was performed in cell culture growth medium or in sterile PBS in the case of heavily bloodstained samples as described in Section 2.5.3. Experience during this work has shown that mechanical disaggregation did not yield a higher number of fibroblasts compared to the enzymatic treatment of the same tissue samples.

In cases where the total sample mass was less than 1 cm^3 mechanical disaggregation was used to produce tissue fragments of approximately 1 mm^2 which were suitable for setting up as **explants** (Section 2.5.4). The explant technique was found to be time consuming and unreliable in terms of successful attachment, and the degree of subsequent outgrowth of epithelial cells. It was therefore only used for very small samples with a hard texture. A more successful approach derived from the observation that mechanical dissection of many samples resulted in the spillage of tiny tissue fragments, smaller clumps of cells, and single cells into the medium. This fraction of the dissociated tissue was called the **dissection medium**. It was found that tissue disrupted in this way resulted more frequently in successful attachment and subsequent proliferation of epithelial cells.

3.1.2.2 Enzymatic disaggregation of tissue samples

Even with soft tissue, the ability to completely disaggregate a sample mechanically is limited. Further digestion of tissue pieces was attempted using a mixture of proteolytic enzymes. Type IV collagenase and dispase were used for this reason. This cocktail of enzymes was particularly useful for samples with a hard texture that yielded few cells after mechanical disaggregation. Enzymatic disaggregation was attempted after mechanical disruption with all samples of sufficient size (1 cm^3 or greater).

Incubations in enzyme were carried out for 1-3 hours depending on tissue texture. Cells released from the tissue matrix in this way were harvested periodically as described in Section 2.5.5. It was observed that later collections from the same tissue samples contained greater numbers of fibroblasts. For this reason, but also to avoid cell damage, incubation times were kept to a minimum. In spite of this, treatment with enzymes resulted in more rapid establishment of fibroblast overgrowth than purely mechanical procedures.

An example of this can be seen in photographs in figures 3.1.1 & 3.1.2, which are taken after three days in culture. In the dissection medium fraction, outgrowth can be seen at the edge of the attached cell clumps with a noticeable absence of fibroblasts at this stage. However, in the 1 hour incubation with collagenase/dispase, the single cell suspension achieved using this procedure resulted in widespread proliferation of fibroblasts with few epithelial cells interspersed.

Enzymatic treatment of samples did not always result in such a good dispersion of the tissue into single cells. In other samples disruption under the same conditions (1 hour with collagenase/dispase) often resulted in a mixture of single cells and smaller clumps of cells and/or tissue fragments. This pattern of disruption may reflect the intrinsic composition of the tissue sample, but it also appeared to favour the establishment of localised epithelial colonies. An example of this in figure 3.1.3 shows a squamous cell lung carcinoma after four days in culture. In this case epithelial cells are found associated with a small tissue fragment. A considerable number of fibroblasts are also visible but many of these were successfully removed from the sample in question on the tenth day in culture (figures 3.1.4 & 3.1.5 Section 3.1.5.1).

3.1.2.3. Malignant effusions as a source of epithelial cells for primary culture

Pleural effusions and peritoneal (ascites) fluids produced symptomatically in association with malignancy are often a source of metastatic cancer cells. Samples taken from such patients for palliative purposes were used initially as a source of cells for primary culture. Cells were separated from the fluids by centrifugation. Fibroblast contamination was not a problem with these samples (in the limited number of cases studied). In later work the remaining fluid was also assayed for growth factor activity (Sections 3.1.9 and 3.1.10 below).

3.1.3 REMOVAL OF BLOOD CELLS

Some tissue samples were so bloodstained, or vascularised to such an extent, that dissection resulted in spillage of large numbers of red blood cells (RBC) into suspension. Initial experience suggested that epithelial cell attachment was not successful in the presence of blood cells. Erythrocytes therefore were separated in such cases by differential centrifugation on a ficoll gradient (Section 2.5.6). This technique was also usefully employed in the removal of erythrocytes from cell pellets from pleural effusions and ascites fluid samples.

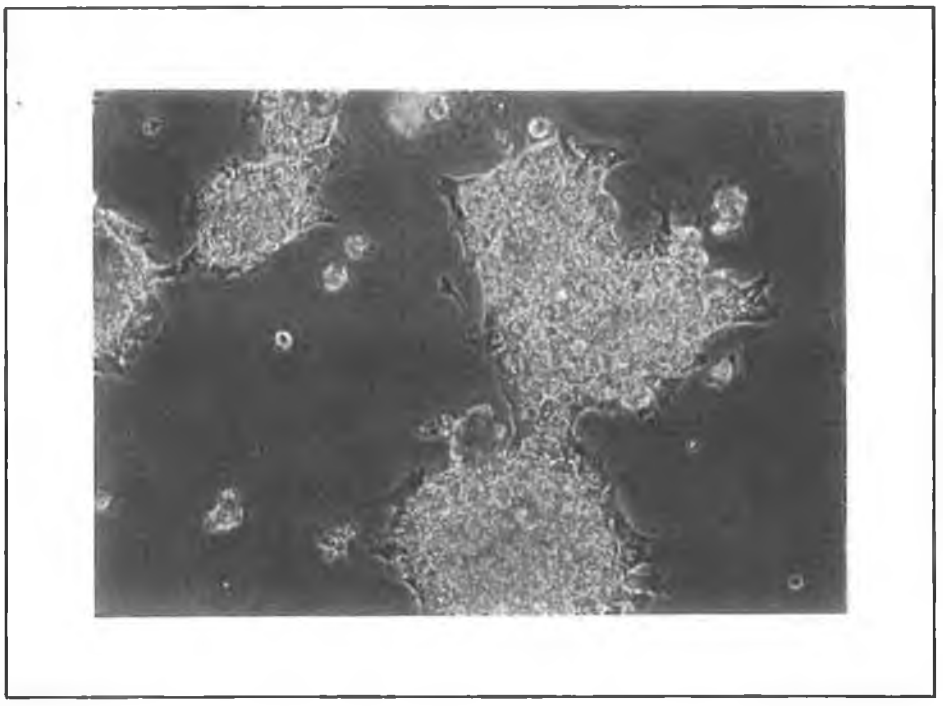


Figure 3.1.1 Growth of mechanically disaggregated squamous cell lung carcinoma after 3 days in culture, showing absence of fibroblasts. (150X magnification).

Figure 3.1.2 Growth of the same lung tumour sample as in figure 3.1, three days after digestion with collagenase/dispase. Arrows indicate the few epithelial-like cells, the majority are fibroblasts (150X magnification).

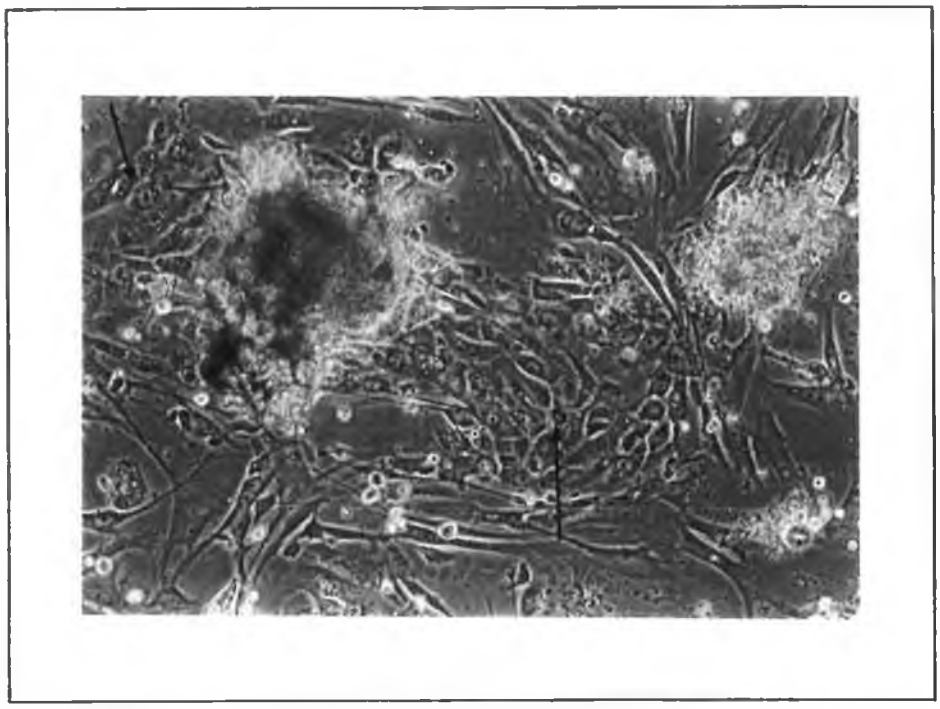


Figure 3.1.3 Outgrowth of primary lung epithelial and fibroblast cells from a tissue fragment three days in culture after incubation of tumour with collagenase/ dispase (150X magnification). Arrows indicate areas of epithelial cell monolayers.

3.1.4 *IN VITRO* GROWTH OF TUMOUR SAMPLES

The growth performance of thoracic tissue samples is outlined in Table 3.1.4.1. Samples have been catergorised according to the site/operative procedure used, as outlined in Section 3.1.1 above. In this scheme performance was graded from no growth {NG} through to the various degrees of attachment and proliferation thereafter. In general, no growth assumes no attachment, but in one case (a small cell lung carcinoma, cultured from a bronchoscopy sample) cells grew as clumps in suspension. This is the typical growth pattern for small cell carcinoma *in vitro*. This type of lung carcinoma was only expected in bronchoscopy and mediastinoscopy samples for the reasons outlined above.

Cells that attach and demonstrate signs of metabolism (acidification of the growth medium) are called 'primary cultures'. These are taken to be at 'passage zero'. After these primary cultures reach confluency they are subcultured and replated (usually at a lower density), and from this point onwards they are considered a 'cell line'. However, a distinction is made between 'continuous' or 'established' cell lines, and those with a limited capacity to proliferate *in vitro* before terminal differentiation occurs. In this project, where cell attachment was observed, it was categorised as follows.

(1) Cultures where only fibroblasts were obtained {Fibs}, and epithelial cells were absent. In all cases where fibroblasts attached, they continued to proliferate and could be subcultured for several passages before eventually differentiating and/or senescing.

(2) In some cultures primary epithelial colonies were obtained {1°Ep} which displayed a limited capacity for subsequent proliferation. These too were eventually lost before they could be subcultured (passaged). In all cases however, fibroblasts were also present. Loss of such epithelial cultures was associated with overgrowth by fibroblasts but this factor alone did not contribute to the demise of epithelial cell proliferation. In most cases signs of differentiation were visible and this may be the cause of senecence in epithelial monolayers.

(3) Other cultures with primary epithelial cells proliferated sufficiently to permit one or more subcultures {Ep > 1°}. This was achieved with a relatively small number of samples {5/55 solid tumour samples and 1/8 malignant effusion-derived cultures, excluding one continuous cell line established in each category.

(4) A continuous cell line {CL} is assumed to be immortal in terms of its capacity to continue replication while maintained as an *in vitro* culture. Two permanent cell lines were established in the course of this work, one from a lymph node taken from a patient with prolific metastases of a poorly differentiated squamous cell lung carcinoma, and the second from a pleural effusion from a patient with breast adenoacarcinoma. The establishment of these cell lines and their subsequent characterisation is described in Sections 3.1.6, 3.1.11 and 3.1.12 respectively).

Table 3.1.4.1 Growth performance of solid tissue samples and malignant effusions *in vitro*

GROWTH	C.L.	Ep > 1°	1° Ep	FIBS	N.G.	TOTAL
PNEUMONECTOMY/ LOBECTOMY	0	3 (13.0)[a]	12 (52.2)	3 (13.0)	5 (21.8)	23
BRONCHOSCOPY/ MEDIASTINOSCOPY	0	0 (0.0)	7 (30.4)	5 (21.7)	11 (47.8)	23
OESOPHAGEAL	0	2 (33.3)	2 (33.3)	1 (16.7)	1 (16.7)	6
THORACOTOMY	0	0	1	0	1	2
MALIGNANT EFFUSIONS	1	1	4	1	1	8
OTHER[b]	1	0	0	0	0	1

Abbreviations: C.L, Cell line established. Ep > 1°, Epithelial cells passaged after primary colonies. 1° Ep, Primary epithelial colonies only. Fibs, Fibroblasts only. N.G, No growth. [a] Figures in brackets are the numbers expressed as a percentage of the total in that category. [b] A secondary lung tumour (poorly differentiated squamous cell carcinoma) metastatic to the cervical lymph node.

A number of general empirical observations were made with respect to successful establishment of primary epithelial cultures. These apply particularly in the case of unpromising techniques attempted where the observation was made three times or less.

(1) Epithelial colonies became visible within the first seven days after inititiation of the primary culture from the tissue sample.

(2) When differentiation occured in primary epithelial colonies it became apparent 2-3 weeks after the initiation of the culture.

(3) Substitution of the foetal calf serum (FCS) component of the growth medium with autologus serum did not appear to improve epithelial growth substantially with respect to the growth of the same culture grown in FCS-containing medium. However, the level of fibroblast growth did appear to be reduced with autologus serum substitution.

(4) The use of growth-arrested mouse 3T3 fibroblasts as feeder layers did not inhibit overgrowth of the epithelial component by tumour-derived stromal cells (Section 2.5.9).

(5) Collagen coating of culture flasks (Section 2.5.10) was similarly ineffective in retarding fibroblast proliferation but epithelial cells were trypsinised from these flasks in preference to fibroblasts using only 0.25% (w/v) trypsin at 4°C.

(6) Only large samples gave rise to cultures of epithelial cells which could be passaged beyond the initial primary culture. Details of the histology of this set and the ultimate passage number achieved are shown in Table 3.1.4.2.

Table 3.1.4.2 Histology of 6 samples passaged after initiation of primary epithelial culture

SAMPLE ORIGIN	OPERATIVE PROCEDURES	SAMPLE HISTOLOGY	FINAL PASSAGE Nº
Oesophagus	Oesophageal brushings	Moderately Differentiated Squamous Cell Carcinoma	P1
Oesophagus	Oesophageal tumour removal	Moderately Differentiated Squamous Cell Carcinoma	P1
Lung	Lobectomy	Squamous Cell Carcinoma	P3
Lung	Lobectomy	Recurrent Malignant thymoma	P1
Lung	Lobectomy	Benign tumour	P2
Lung	Pleural effusion	Moderately Differentiated Squamous Cell Carcinoma	P2

Although this represents only a small number of samples, and may not predict growth performances in a larger survey, preliminary indications would suggest that oesophageal tumours tend to establish with a higher frequency compared to other thorasic samples. Clinical histology of the pleural effusion did not detect any malignant cells but this patient was subsequently found to have a tumour in the right main bronchus. The growth of a benign tumour sample from the lung emphasises the need for *in vitro* characterisation of primary cultures (see Section 3.1.12).

3.1.5 CONTROL OF FIBROBLAST PROLIFERATION

As mentioned earlier, stromal fibroblasts proliferate with greater facility *in vitro* than their epithelial counterparts from the same tissue. This behaviour causes problems where the objective is to selectively cultivate the epithelial element of a tissue sample.

Studies with established cell lines suggest that the number of cells (malignant or normal) in a culture at the time of plating (plating density) is critical to their successful cycle of proliferation thereafter. A lower limit to the number of epithelial cells which can effect this sustained growth cycle is characteristic of every tissue type *in vitro*. This phenomenon is thought to reflect the production of self-stimulating (autocrine) growth factors elaborated by a stem cell population in normal regenerative tissue such as the skin, and, by analogy, malignantly transformed cells in tumour tissue.

The approach taken here was to endeavour to restrict fibroblast growth and/or to stimulate epithelial proliferation. It was reasoned that if a tumour contains a subpopulation of 'immortal' cells, then it should be possible (in theory) to expand this fraction with suitable *in vitro* culture conditions, so that they might eventually outgrow all other cell types present in the initial primary culture. The first step in this approach therefore, was the selective removal of fibroblasts from cultures displaying epithelial cell growth. In this way it was hoped to maintain epithelial cell density at the expense of fibroblasts.

3.1.5.1 Differential trypsinisation of epithelial and fibroblast cell types

Epithelial cells tend to grow in tightly associated monolayers with outgrowth occuring from the edges of the colony, whereas fibroblasts produced migrating "whorls" as well as very motile individual cells which proliferated rapidly, encircling epithelial colonies and thereby physically restricting further epithelial outgrowth. This often resulted in piling up and/or tight packing of the epithelial cells, causing senescence or necrosis at the centre of colonies.

A number of methods using different combinations and concentrations of crude trypsin and/or EDTA were employed in the selective detachment of epithelial and fibroblasts respectively. A description of these trypsin/EDTA formulations and methodology used is given in Table 3.1.5.1 below. All the following protocols were monitered microscopically as described in Section 2.5.8.1.

Table 3.1.5.1 Methods used to selectively remove fibroblasts from epithelial cells in primary cultures

Method №	PROTOCOL DESCRIPTION
1	0.04% EDTA for 5 minutes at room temperature, followed by incubation in 0.01% trypsin/0.04% EDTA at R.T.
2	0.01%trypsin/0.04%EDTA at 4°C
3	0.02% trypsin/0.04% EDTA at 4°C.
4	0.001% trypsin/0.04% EDTA at 4°C.
5	0.25% trypsin only at 4°C.

The relative success in the physical separation of fibroblast and epithelial cell populations is summarised as follows:

Method 1
Under these conditions fibroblasts and epithelial cells deteched simultaneously upon the addition of the trypsin/EDTA mixture. No preferential detachment was noted.

Method 2
In one case > 90% of the fibroblasts were removed using this technique but typically fewer than this could be detached before epithelial cells were also removed. The incubation time for this method varied between samples but 4 minutes was the average exposure time.

Method 3
Similar to (2) above but fibroblasts left after treatment appeared to be resistant to this procedure in subsequent attempts to remove them.

Method 4
Longer incubation periods were required for this protocol (typically 30 minutes). This methodology was only partially selective in the removal of fibroblasts. Fibroblasts removed in this way were seen to contain epithelial cells three days after replating in growth medium.

Method 5

Where the number of epithelial cells exceeded that of fibroblasts (as judged empirically by eye) this method was successful in selectively detaching epithelial cells in preference to fibroblasts.

It was also generally observed that selective trypsinisation was less successful where epithelial cells were scattered in small colonies or as single cells interspersed by fibroblasts. Differential separation of both cell types was easier to achieve when epithelial cells formed large compact and/or discreet colonies. In these cases the epithelial-fibroblast contact was limited to the edges of such colonies. A successful procedure following protocol Nº2 above is illustrated in figures 3.1.4 & 3.1.5.

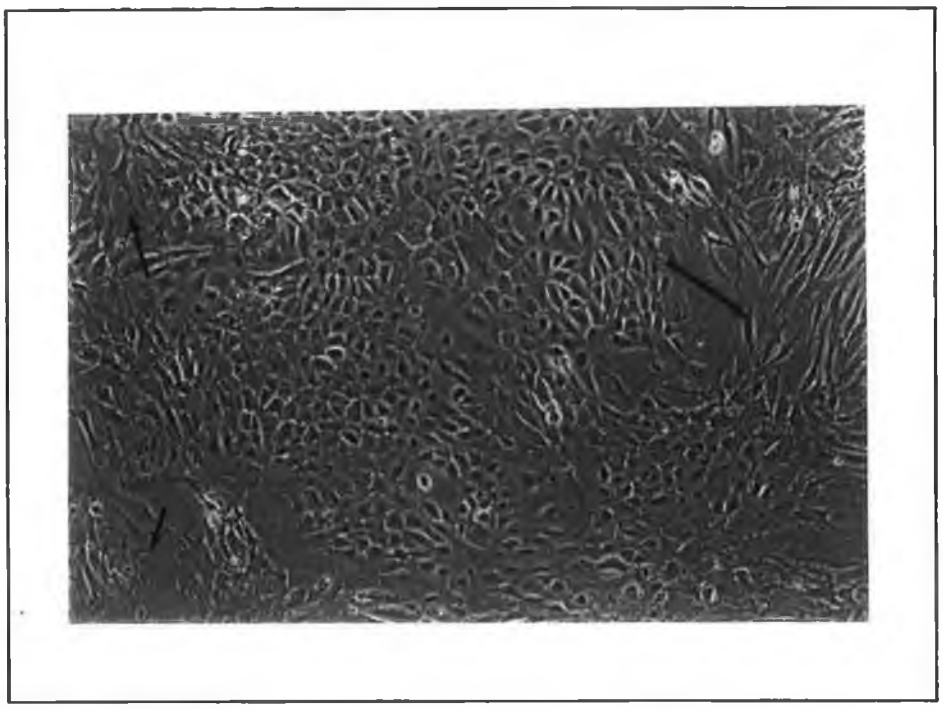


Figure 3.1.4 A colony of human lung squamous cell epitlelial surrounded by fibroblasts Arrows indicate the presence of fibroblasts. (60X magnification)

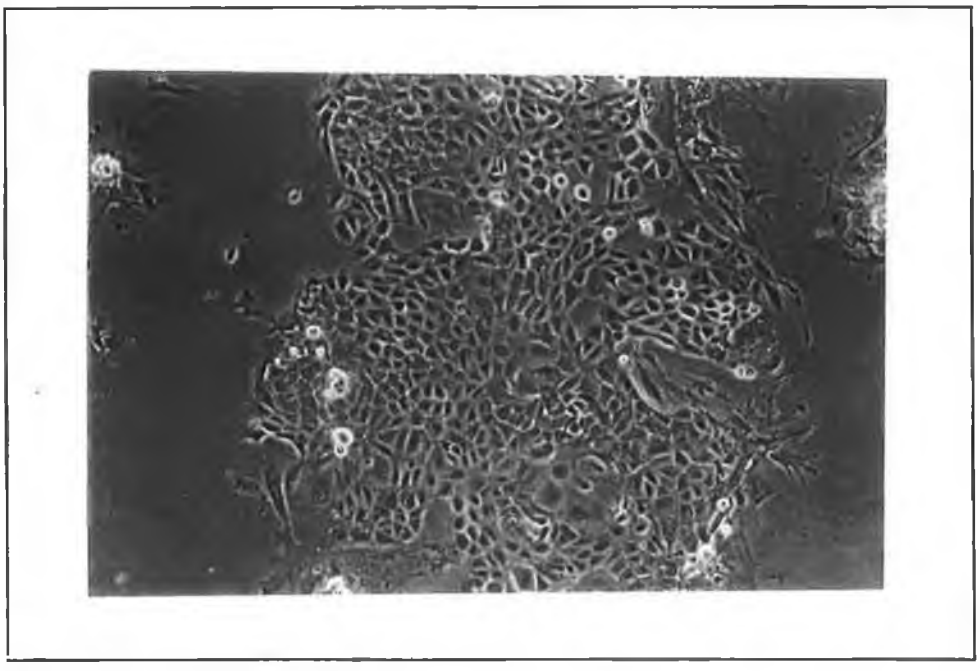


Figure 3.1.5 The same colony of squamous epithelial cells as shown in figure 3.1.4, after removal of fibroblasts using 0.01% trypsin/0.04% EDTA at 4°C. (60X)

3.1.5.2 Differential toxicity of geneticin to fibroblasts and epithelial cells

An alternative approach to the elimination of fibroblasts from epithelial cultures was based on work carried out in this laboratory with primary fibroblasts and established epithelial cell lines {McDonnell (1987)}. McDonnell found a differential in the toxicity of fibroblasts and the cell lines to geneticin (G418); 100 μg/ml G418 for two days was preferentially toxic to fibroblasts. In this work preliminary experiments with the same concentration of G418 displayed an encouraging reduction in the number of fibroblasts after a 6 day incubation in G418. However, complete recovery of fibroblast numbers were seen 24 hours after removal of the selective agent.

Further work by Martin (1992) using this treatment with lung fibroblast cultures indicated that higher concentrations of this agent might be required (200-250 μg/ml) to kill 80-100% of fibroblasts, but a variation in the efficacy of G418 toxicity on different fibroblast cultures was seen.

This method was not preferred to the use of selective trypsinisation in this work for a number of reasons; (1) the variation in efficacy, (2) the rapid tendancy to recovery of fibroblasts after treatment, (3) the lack of data on the selective effects of G418 toxicity in primary epithelial cells, and (4) the potential hazzards associated with its use.

3.1.6 ESTABLISHMENT OF A CONTINUOUS HUMAN LUNG SQUAMOUS CELL CARCINOMA CELL LINE - DLRP

3.1.6.1 Clinical History of DLRP

A continuous cell line was established from a cervical lymph node biopsy taken from a sixty-one-year-old male with a history of smoking. Bronchoscopy revealed the presence of tumour at the orifice of the bronchus to the left upper lobe. A biopsy performed on the left cervical node showed that the lymph node architecture was completely replaced by metastatic poorly differentiated squamous cell lung carcinoma. The patient died one month after bronchoscopy.

The following is a description of the primary culture history of this sample which was subsequently passaged forty times and is considered to be an established cell line.

Day 1. The sample of tissue received was devoid of vascular tissue and seemed to have an outer layer encapsulating its inner mass. There were no macroscopic signs of necrosis visible. Approximately half of the entire lymph node was made available for cell culture (2-3 cm^3 of tissue). The outer layer was dissected separately after careful removal, but both sections were processed identically thereafter.

Dissection was performed in complete medium (Section 2.5.2) and explants were set-up from tissue fragments of suitable size (1 mm^2). Larger fragments were allowed to settle under gravity in complete medium and the supernatant (dissection medium) was plated directly into tissue culture flasks. The remaining tissue pieces were digested in collagenase (Sigma type IV, at 2000 U/ml) for 3 hours with stirring. The resulting cell suspension was pelleted by centrifugation and the cells were resuspended in complete medium. Enzyme digests of the outer layer yielded fewer cells (as judged empirically by eye).

Day 2. Epithelial colonies were visible in the dissection medium (DM) platings and these appeared to be growing outward from cell clumps. The medium in these cultures contained many unattached cells and was therefore replated into fresh flasks and the original flasks were refed.

Day 5. A small number of fibroblasts were visible in DM platings but epithelial monolayers continued to expand. Limited epithelial growth was seen in enzyme digest of the inner node mass and in the explant cultures. All cultures were refed.

Day 6. Epithelial colonies in cultures from the DM fraction began to reach confluency. Evidence of colonies arising from smaller groups of cells, with individual cells dividing away from the main colonies was also noted in these flasks. Explants and enzyme fractions continued to form epithelial monolayers but many fibroblasts were also visible.

Day 8. Attempted to remove fibroblasts present in enzyme digest culture using method Nº1 in Section 3.1.5.1 above. This resulted in the simultaneous removal of fibroblasts, therefore all cells were replated. These cells at passage 1 (P1) were plated onto collagen coated tissue culture flasks (Section 2.5.10).

Day 12. Second attempt to remove fibroblasts from an enzyme digest using method Nº 2 in Section 3.1.5.1 above. This was partially more successful than method Nº 1.

Day 13. Removed an aliquot of spent growth medium from passage 1 cells for mycoplasma analysis.

Day 19. Subcultured primary cultures from DM fraction using method Nº 5 {0.25% trypsin only at 4°C}, Section 3.1.5.1. The majority of fibroblasts were left behind by this procedure.

Day 22. Passage 1 cells on collagen-coated flasks (day 8) were subcultured (P1→P2) using 0.25% trypsin only at 4°C. The trypsinisation process took 30 minutes but fibroblasts remained firmly attached.

Day 26. Removed culture medium from P2 cells for mycoplasma analysis. Passaged these to P3 using 0.25% trypsin only at 4°C.

Day 34. Passage 3 cells are free of fibroblasts but appear very 'grainy' in their cytoplasm and their medium was becomming quickly very acidic. This suggested mycoplasma contamination. All flasks of this cell line were refed with fresh medium containing 60 μg/ml of the antibiotic tylocine (anti-PPLO agent) {Section 2.4.2.2}.

Day 40. Medium from passages 1 & 3 from this cell line were positive for mycoplasma.

Day 42. Improvement in growth rate and less granulation in cultures treated with tylocine. Gentamicin included in growth medium of all stocks (with tylocine).

Day 46. Made frozen stocks from cultures at passages 3, 4 & 5.

Day 47. Frozen stocks made from cultures at passage 6 made.

Day 54. All stocks remaining in culture with antibiotics showed continued improvement in growth rate with less granulation in their cytoplasmic space.

Day 56. Discontinued the use of gentamicin and tylocine.

Day 58. Made frozen stocks at passages 6 & 8. Yields from 25 cm^2 flasks (approximately 70-80% confluent) were in the order 7.5 x 10^5 cells per flask.

Day 62. All stocks in antibiotic-free medium show no signs of deterioration with respect to growth rates and granulation of the cytoplasm. Frozen stocks prepared from cultures at passages 6, 8 & 9 respectively.

Day 63. Morphology change noted at passage 10. Some of the cell population has become elongted with dendritic-like protrusions ('spindly'). The original squamous (cuboidal) phenotype can still be distinguished in the population.

Day 68. Returned all stocks to tylocine-containing growth medium after increased granulation noted in the cytoplasm. Passaged all P10 stocks into new flasks.

Day 75. Passage 11 cells in new flasks have improved growth rates and the original squamous cell morphological phenotype was the only one discernable.

Day 79. Frozen stocks were prepared from cultures at passage 13.

Day 84. Stocks prepared from cultures at passage 14 for frozen storage.

Day 89. All remaining stocks were discarded due to a rapid deterioration in their growth rates and gross moprhology changes similar to those seen at passage 10. Rapid acidification of culture medium immediately after plating suggested mycoplasma contamination.

3.1.6.2 Elimination of mycoplasma contamination in the cell line DLRP

Passage 9 DLRP cells (frozen on day 62 after six days in antibiotic-free growth medium) were thawed. Recovery from frozen stocks was extremely poor (< 40% attached). The culture was refed with fresh medium after 24 hours and anti-mycoplasma agent BM cycline was added three days after thawing. The procedure took three weeks and is described in Section 2.4.2.2. Cultures were passaged during this treatment process and the previously noted change in morphology (i.e at passage 10 on day 63) was seen again in this stock culture at P10. Cells proceeded through this 'crisis' and by passage 12 had recovered their original phenotype.

After three cycles of BM Cycline treatment cultures were mycoplasma negative as determined by the indirect detection method (Section 2.4.2.1). A separate stock (passage 6, from day 62) was thawed and treated in this way. The morphology change observed in previous transtitions to passage 10 was recorded yet again. All subsequent frozen stocks were derived from the latter cultures.

This cell line consistently grew better when plated regularly into new culture flasks. This was contrary to the generally observed phenomenon (in animal cell culture folklore) that replating the cells into the same flasks permits better attachment and faster growth thereafter. The squamous cell-like morphology which is characteristic of DLRP can be seen in figures 3.1.6 & 3.1.7.

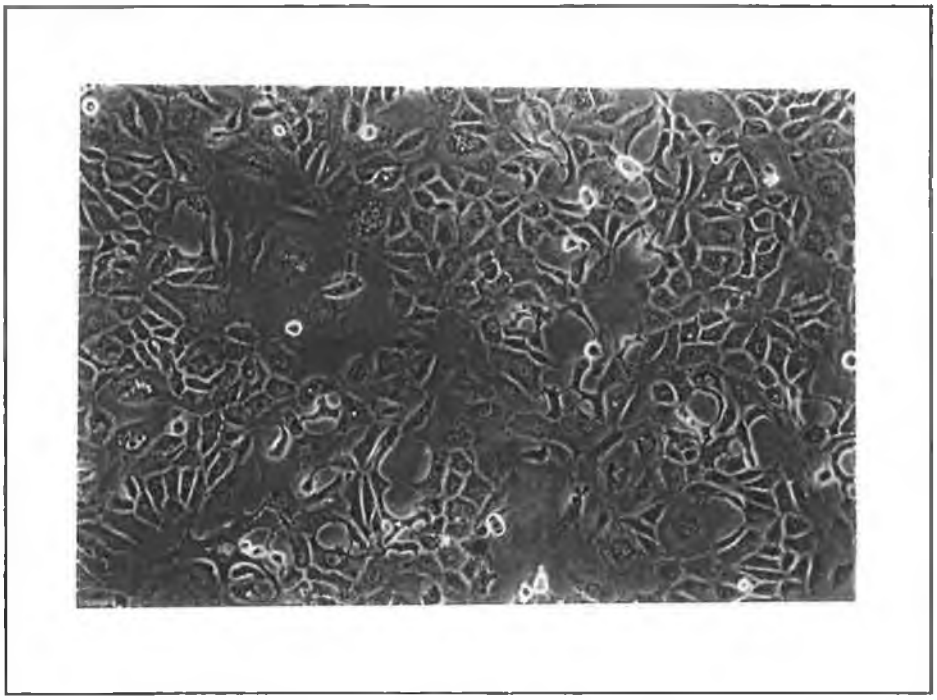


Figure 3.1.6 Monolayer growth of DLRP at passage 7, grown in antibiotic-free medium (60X magnification).

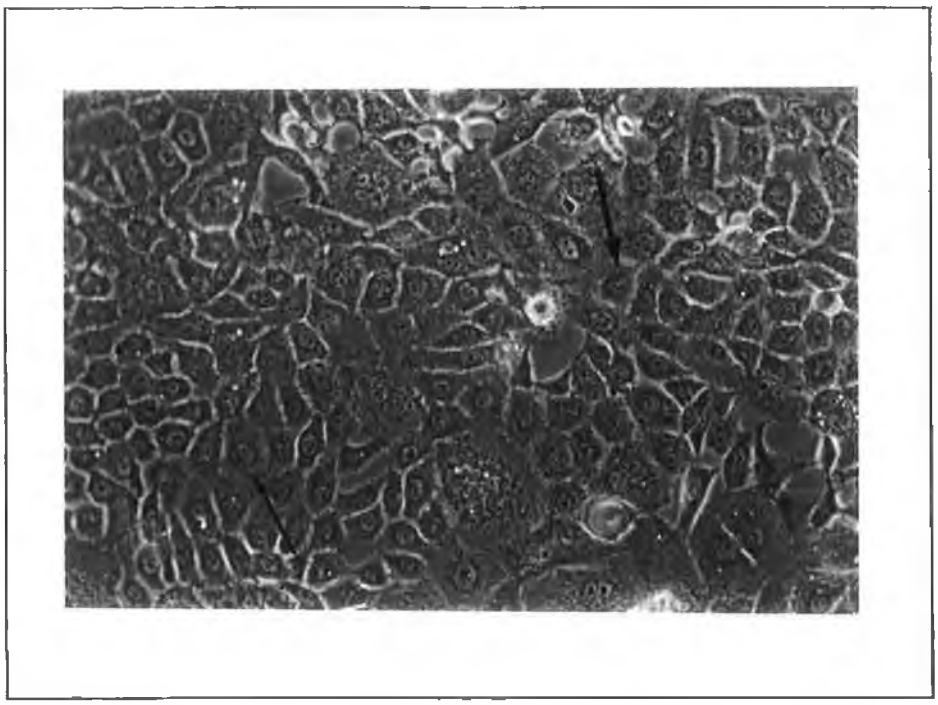


Figure 3.1.7 The same culture of DLRP as figure 6 above with cells at passage 9 Cells with multiple nucleoli indicated by arrows (150X magnification).

3.1.6.3 Measurement of epidermal growth factor-like activity in medium conditioned by the cell line DLRP

The high levels of expression reported in human squamous cell lung carcinomas for epidermal growth factor receptor (EGF-R) {Berger *et al.*, (1987)} have implicated the ligands for this receptor as potential autocrine growth factors as mentioned earlier. Among these are the mitogens epidermal growth factor, transforming growth factor α. Many studies have demonstrated the production of TGFα by carcinoma cells but until recently EGF production had not been detected in tumour tissues.

In the context of successful *in vitro* growth of DLRP, it was of considerable interest to establish whether this cell line produced an EGF-like mitogenic activity. To this end, medium conditioned by the cell line at passage 21 was concentrated 100x by ultrafiltration (Section 2.8.1). A radioreceptor assay for the detection of EGF-like activity based on competitive binding to the EGF-R-rich membrane of the cell line A431 was used for this purpose (Section 2.12.6). Conditioned medium (CM) from the rapidly growing human epidermoid carcinoma cell line HEp-2 (concentrated 50x by ultrafiltration) was also assayed in this experiment. The results are presented in Table 3.1.6.3 and a standard curve for 'cold' EGF is presented in figure 3.1.8.

Table 3.1.6.3 Detection of EGF-like activity in CM from DLRP and HEp-2

CONCENTRATION COLD EGF (ng/ml)	C.P.M. ± S.E.M.[a]
10000	61 ± 4
500	282 ± 11
100	924 ± 35
50	1064 ± 72
10	1251 ± 19
5	1343 ± 90
1	1523 ± 55
Control Medium[b]	1615 ± 67
DLRP CM	
100x	1478 ± 27
75x	1505 ± 33
50x	1490 ± 23
25x	1632 ± 37
10x	1592 ± 95
HEp-2 CM	
50x	1507 ± 72
25x	1585 ± 74

[a] Counts per minute ± standard error of the mean. [b] Ham's F1 (n=3).

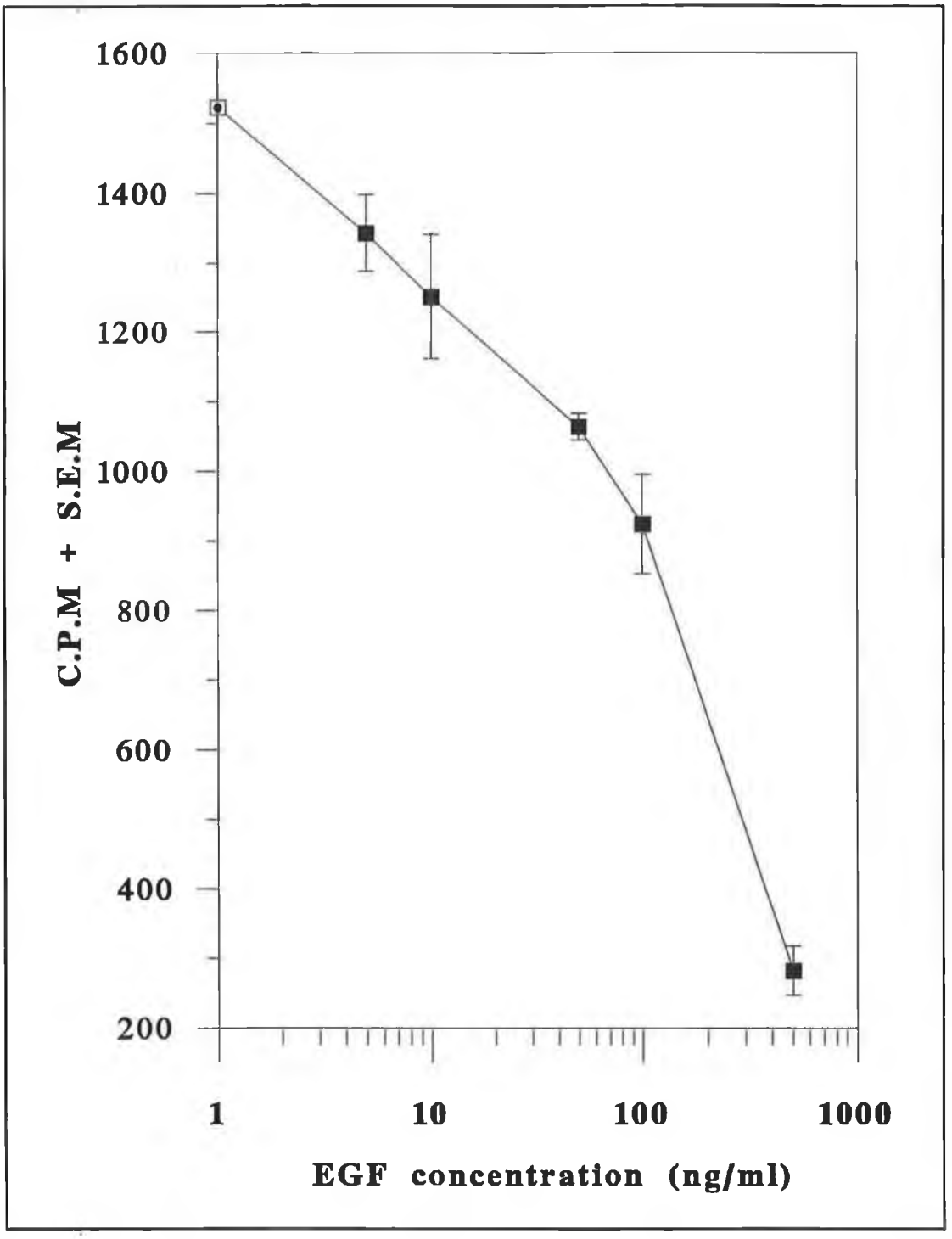


Figure 3.1.8 Standard curve of EGF concentration (ng/ml)

The presence of EGF-like activity was indicated in the 100x concentrate of DLRP conditioned medium (CM). The reading indicated that between 1-5 ng/ml of EGF-like activity was present in this sample, but this measurement was at the lower end of the detection range on the standard curve (figure 3.1.8). Accurate quantification cannot be assured at this level of detection. No acitvity was detected in the 50x HEp-2 CM concentrate.

3.1.6.4 Comparison of the percentage colony forming efficiencies of DLRP and another poorly differentiated human lung squamous cell carcinoma line DLKP in soft agar

A characteristic of the growth of malignant cell lines *in vitro* is their abililty to form colonies in semi-solid agar growth medium. This property has been used as an indicator of malignancy by workers in the field. DLRP and another human lung squamous cell carcinoma line, DLKP, (also recently established in this laboratory) were plated in a 0.3% agar overlay of 0.6% agar on 35 mm petri dishes and incubated for 10 days in a CO_2 incubator (Section 2.12.3.3). Both cell lines were plated at several densities. Colonies greater than 50 μm were counted (i.e. scored as a colony) and the percentage colony forming efficiency (%C.F.E.) was determined (see Section 2.12.3.1 for details of calculations). The morphology of DLRP colonies in this soft agar growth system can be seen in figure 3.1.9 and the results of replicate experiments are shown in Table 3.1.6.4.

Table 3.1.6.4 The % colony forming efficiency of DLRP and DLKP in soft agar

Plating Density	Experiment 1	
	DLRP (P33)[a]	DLKP (P11)[a]
	% C.F.E. ± S.E.M.	% C.F.E. ± S.E.M.
5.0×10^5	0.42 ± 0.04	N.R.[b]
2.5×10^5	0.73 ± 0.01	N.R.
1.0×10^5	0.79 ± 0.05	N.R.
5.0×10^4	0.00 ± 0.00	5.34 ± 0.49
1.0×10^4	0.00 ± 0.00	0.00 ± 0.00
5.0×10^3	0.00 ± 0.00	0.00 ± 0.00
	Experiment 2	
5.0×10^5	1.34 ± 0.05	N.D.[c]
2.5×10^5	2.66 ± 0.30	N.D.
1.0×10^5	3.28 ± 0.42	N.D.
5.0×10^4	2.12 ± 0.50	2.32 ± 1.31
1.0×10^4	0.00 ± 0.00	0.00 ± 0.00
5.0×10^3	0.00 ± 0.00	0.00 ± 0.00

n=3 for all determinations. [a] Passage number in the assay. [b] Not readable. [c] Not determined.

Although every effort was made to ensure identical assay conditions between replicate assays significant inter assay variation in growth was noted. This phenomenon is a feature of bioassays but is particularly apparent with soft agar assays. The percentage colony forming efficiency (% CFE) of DLRP was higher in the repeat assay, but the trends were similar between assays. % CFE was maximal at plating densities between 1 x 10^5 and 2.5 x 10^5 cells/plate. Higher plating densities resulted in reduced % CFE for this line. A lower limit to the plating density was reached in both assays below which no colonies formed. This was between 5 x 10^4 and 1 x 10^5 cells/plate for DLRP.

The limiting plating density for DLKP in this system was 5 x 10^4 cells/plate. However, a plating density of 1 $\times$ 10^5 cells/plate gave so many colonies in the first experiment that accurate counting was impossible. For this reason, densities in this range were not plated for DLKP in the repeat assay.

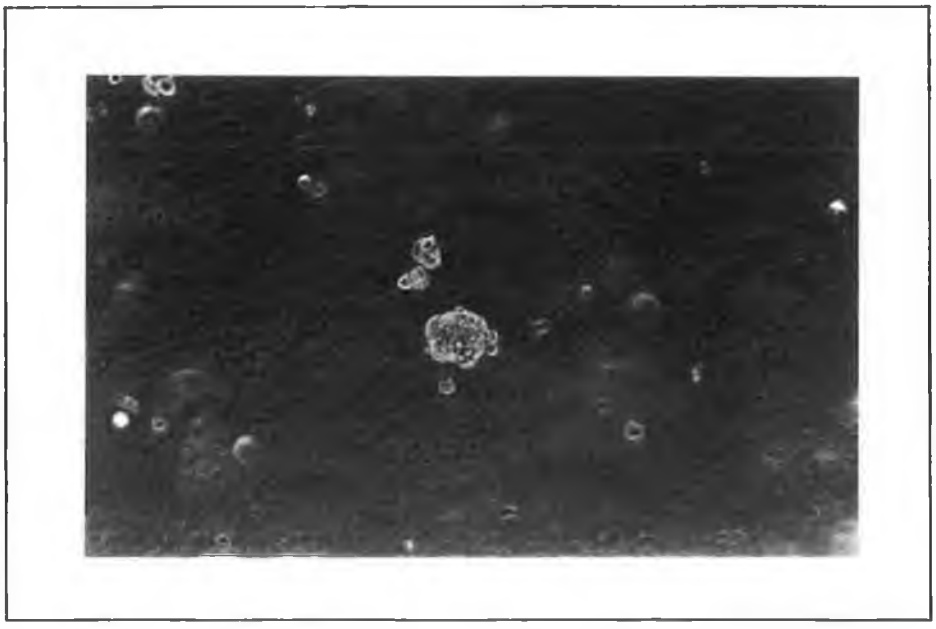


Figure 3.1.9 The growth of a DLRP cell colony after 14 days in agar (150X magnification).

3.1.6.5 Determination of serum batch preference for the cell lines DLRP, DLKP & HEp-2

Animal serum is an essential supplement to the chemically defined component of cell culture growth medium for the majority of animal cell lines cultured *in vitro*. As mentioned earlier, currently there are no commercially available serum-free media formulations for the culture of human lung squamous cell carcinomas. Therefore serum supplementation of basal media for these cell types continues to be necessary.

By virtue of their biological source and subsequent processing, animal sera display considerable variation in their ability to sustain in vitro growth of animal cell lines. Experience in this and other laboratories has shown that batch testing is required to ensure a supply of serum with good growth-promoting properties. In this respect it is important to consider the nature of the work to be undertaken. A batch of serum considered for use in general cell maintenance should be tested for its growth-promoting potential on all the cell lines in use. This is because the growth performance in different serum batches is also dependent on the target cell line. Therefore, a good batch for one cell line may prove to be a poor batch for another in terms of its ability to stimulate cell proliferation.

In this work, batches of serum were selected for use on the basis of their ability to support the growth of monolayer cultures of particular cell lines. In some cases batches were reserved exclusively for use with one cell line, and where appropriate, others were used in general cell line maintenance. Growth was quantified by measuring the area of colonies stained with the dye crystal violet (Section 2.12.4). In order to get a better idea of batch performance it was necessary to measure growth at a number of serum concentrations. To this end batches were tested at low serum concentrations between 1-3% v/v, at routinely used levels, i.e 10% v/v, and one concentration in between, at 5% v/v.

A preliminary experiment was designed to determine the serum batch preference of the new lung cell lines DLRP and DLKP. Cells were plated at a final concentration of 5×10^3 cells per well in a volume of 1 ml on 24-well plates. Cells were plated as describe in Section 2.12.4.3. Sera for testing were prepared at 2x the final concentrationsin Ham's F12. The cell plating densities chosen were designed to give sufficient readable growth at the lowest serum concentrations tested and to avoid excessive growth at the highest concentration (which would also prohibit accurate measurement of colony area by image analysis).

Four batches of bovine serum, two newborn calf (NCS) and two foetal calf (FCS), were tested. The response of a non-lung-derived carcinoma line HEp-2 was measured to check for any tissue specificity in batch performance. The latter cell line is well-established *in vitro* and displays a relatively short doubling time even at low serum concentrations. A much lower plating density, 4×10^2 cells/well was used for batch testing with this line based on the results in Section 3.2.1 above. The results of these these batch tests are given in Table 3.1.6.5.

Table 3.1.6.5 Determination of serum batch preference in the cell lines DLRP, DLKP and HEp-2

Serum Batch		FCS:801017	FCS:701113	NCS:F6182	NCS:30F7079
Cell Line	% Serum	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
DLRP	2.5	4.01 ± 0.80	5.79 ± 0.81	1.50 ± 0.24	1.21 ± 0.10
	5.0	7.05 ± 1.22	9.54 ± 0.45	4.90 ± 0.32	2.21 ± 0.20
	10.0	7.95 ± 0.70	12.76 ± 0.74	6.78 ± 0.72	3.32 ± 0.32
DLKP	1.0	19.77 ± 1.78	20.36 ± 2.26	38.35 ± 2.41	36.62 ± 3.07
	5.0	83.40 ± 4.67	80.66 ± 4.00	84.33 ± 3.00	74.95 ± 5.79
	10.0	86.59 ± 3.68	73.51 ± 5.56	76.17 ± 4.67	67.77 ± 1.56
HEp-2	1.0	0.05 ± 0.06	2.31 ± 0.20	1.22 ± 0.68	2.28 ± 0.39
	5.0	2.33 ± 1.09	4.09 ± 0.76	3.25 ± 0.45	10.83 ± 1.50
	10.0	12.97 ± 2.33	5.26 ± 0.46	19.15 ± 0.94	17.22 ± 3.28

n=3 for all determinations.

The growth of DLRP was clearly better in foetal calf serum than in either of the newborn batches tested. At this plating density, more growth was evident at all concentrations of FCS than at the same concentrations of NCS.

The plating density used for DLKP resulted in very high growth even at low serum concentrations. The only significant differences in growth levels therefore were seen at 1% serum. At this plating density better growth was evident in the NCS batches than in either of the FCS batches. At higher serum concentrations no significant differences between batches are apparent. This suggested that a lower plating density should be used for DLKP in this system (24-well plate). This would facilitate the detection of growth promoters (in serum or as purified growth factors) by making the conditions of growth more stringent.

Batch preference for HEp-2 was not confined to either type (newborn or foetal calf serum), but the plating density used for this cell line discriminated between all batches with the same ranking order at each concentration tested.

Batches 701113-FCS and 10F6182-NCS were purchased in bulk and used for these cell lines.

3.1.7 THE EFFECT OF PURIFIED GROWTH FACTORS ON THE PROLIFERATION OF DLRP AND DLKP

The evidence for an EGF-like activity in the medium conditioned by DLRP (Section 3.1.6.2) suggested that such a growth factor(s) might be important for the proliferation of this and other squamous cell carcinomas of the lung. It was reasoned that the effect of this and other growth factors produced in an autocrine manner might be more easily detected at low serum and low cell densities (minimising any potential self-feeding effect) where their contribution to the overall population growth would be more important. Preliminary experiments designed to define more stringent growth conditions in the 24-well plate system (lowest plating densities giving sufficient 'readable' growth in 1% serum) were performed for DLRP and DLKP using the same batch of FCS (Batch N°AF1201). The results of these experiments are presented in Table 3.1.7.

Table 3.1.7 Growth of DLRP and DLKP at different plating densities using 1% FCS in the 24-well plate system

CELL LINE	DLRP	DLKP
Plating Density Cells/well	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
8×10^3	40.97 ± 2.22	92.39 ± 2.63
6×10^3	19.86 ± 2.53	60.81 ± 14.25
5×10^3	12.77 ± 2.77	78.77 ± 10.36
4×10^3	10.67 ± 1.74	91.38 ± 0.72
3×10^3	3.70 ± 0.77	69.55 ± 5.93
2×10^3	1.67 ± 0.57	42.28 ± 1.66
1×10^3	0.14 ± 0.07	22.32 ± 2.39

n=3 for all determinations.

These experiments illustrate the very different growth rates of these two squamous cell lung carcinoma lines. At plating densities greater than 4×10^3 cells/well, overgrowth of DLKP was responsible for the lack of linearity between colony area measured and the number of cells plated. This may be explained by the fact that DLKP tends to detach very easily when confluency is reached in monolayer cultures. In subsequent growth factor experiments this cell line was plated at 1×10^3 cells/well. (Section 3.1.7.2). The plating density chosen for DLRP was 3×10^3 cells/well (Section 3.1.7.1).

3.1.7.1 The effect of a selection of growth factors on DLRP proliferation

The mitogenic response of DLRP to purified growth factors from three different growth factor classes was determined. Cells were seeded in 24-well plates for this experiment (Section 2.12.4) The growth factors assayed were transforming growth factor α, interleukin 1α (IL-1α) and interleukin 1β (IL-1β), and acidic and basic fibroblast growth factors (aFGF & bFGF).

One growth factor was assayed per 24-well plate. Seven concentrations of each growth factor were assayed together with a 'medium only' control (plus 1 mg/ml BSA, fraction V). Controls on each plate were included to allow for interplate variations in growth (n=3 determinations per plate). An experiment control mean was calculated from the control readings (in colony area) from all 5 plates in the experiment (n=15).

Growth factors were scored as mitogenic if the numerical value of the sample mean ***minus*** the standard error on that determination was in excess of the control mean value ***plus*** the standard error on that value. The results of this experiment are presented in Table 3.1.7.1.

Table 3.1.7.1 The effect of TGFα, IL-1α & IL-1β, and aFGF & bFGF on DLRP growth

Growth Factor Conc[a]	Colony ± S.E.M. Area mm^2 mm^2	Growth Factor Conc[a]	Colony ± S.E.M. Area mm^2 mm^2	Growth Factor Conc[a]	Colony ± S.E.M. Area mm^2 mm^2
ng/ml	Acidic FGF	pg/ml	Basic FGF	pg/ml	TGFα
25.00	4.65 ± 0.39	500	1.94 ± 0.72	500	2.21 ± 0.33
5.00	6.11 ± 0.89	250	2.65 ± 0.17	250	3.07 ± 0.33
2.50	6.35 ± 1.14	100	3.41 ± 0.24	100	2.62 ± 0.19
1.50	4.25 ± 0.72	50	2.83 ± 0.74	50	3.19 ± 0.94
0.50	5.21 ± 0.76	10	3.08 ± 0.65	10	3.44 ± 1.01
0.25	5.19 ± 0.72	5	4.49 ± 1.68	5	3.02 ± 0.20
0.10	4.77 ± 0.74	1	3.79 ± 0.22	1	3.42 ± 0.43
Control + BSA	3.39 ± 0.64	Control + BSA	2.34 ± 0.22	Control + BSA	2.41 ± 0.06
pg/ml	Interleukin 1α	pg/ml	Interleukin 1β	EXPERIMENT CONTRLS	
500	3.63 ± 0.92	500	3.27 ± 0.86	**Control Medium + 1 mg/ml BSA** (n=15) 2.23 ± 0.89	
250	4.43 ± 0.34	250	3.75 ± 0.39		
100	4.85 ± 1.19	100	3.44 ± 1.00		
50	2.56 ± 0.66	50	2.02 ± 0.15		
10	3.00 ± 0.65	10	3.20 ± 0.50	**Control Medium minus BSA** (n=16) 1.66 ±0.72	
5	3.44 ± 1.03	5	3.49 ± 0.71		
1	3.11 ± 0.15	1	2.38 ± 0.37		
Control + BSA	1.81 ± 0.87	Control + BSA	1.21 ± 0.34		

n=3 unless otherwise stated.

A considerable inter-plate variation in control growth was seen in this experiment. Increased growth was scored using the experiment control and the plate control for each growth factor assayed. Positive stimulation was scored only if the level of growth in growth factor wells was greater than both controls as outlined. On this basis, aFGF was mitogenic for DLRP in monolayer. The stimulation appeared to be biphasic because significant growth over the controls was detected between 0.25 and 0.5 ng/ml and in a higher concentration range between 2.5 and 5.0 ng/ml. At 25 ng/ml aFGF stimulation was still detected but at a reduced level. Maximal stimulation with growth factor under these conditions was detected at 2.5 ng/ml and this induced a 2.85 fold increase in growth with respect to the experiment control mean.

Basic FGF was also mitogenic for DLRP. At 1 pg/ml the fold increase in growth was 1.7 (w.r.t experiment control mean). The differential in the concentrations of acidic and basic FGF required to induce mitogenic activity in DLRP was consistent with the pattern of stimulation seen in other cell systems for these homologous growth factors.

Interleukin-1α was mitogenically active between 100 and 250 pg/ml on DLRP inducing 1.98 and 2.17 fold increases in growth at these concentrations respectively. Activity with IL-1β was also detected at 250 pg/ml where a 1.68 fold increase in growth over control (experiment control mean) was measured.

Transforming growth factor α was not mitogenic for DLRP under these conditions.

3.1.7.2 The proliferative response of DLKP to growth factors from three different growth factors classes

The same stocks of growth factors used in the DLRP assay above were tested on the cell line DLKP under the same assay conditions as in Section 3.1.7.1. One growth factor was assayed per plate and medium controls were included on each plate. The results of this experiment are shown in Table 3.1.7.2.

Table 3.1.7.2 The effect of TGFα, IL-1α & IL-1β and aFGF & bFGF on DLKP growth

Growth Factor Conc^n	Colony ± S.E.M. Area mm^2 mm^2	Growth Factor Conc^n	Colony ± S.E.M. Area mm^2 mm^2	Growth Factor Conc^n	Colony ± S.E.M. Area mm^2 mm^2
ng/ml	Acidic FGF	pg/ml	Basic FGF	pg/ml	TGFα
25.00	0.52 ± 0.01	500	0.66 ± 0.03	500	2.44 ± 0.50
5.00	0.90 ± 0.28	250	1.31 ± 0.28	250	2.94 ± 0.47
2.50	1.77 ± 0.32	100	1.77 ± 0.40	100	3.22 ± 0.26
1.50	1.30 ± 0.24	50	2.28 ± 0.65	50	3.19 ± 0.46
0.50	2.75 ± 0.26	10	2.80 ± 0.30	10	3.22 ± 0.43
0.25	3.82 ± 0.52	5	5.22 ± 0.62	5	4.93 ± 1.61
0.10	3.58 ± 0.41	1	4.34 ± 0.67	1	3.24 ± 0.65
Control + BSA	2.69 ± 0.44	Control + BSA	3.07 ± 0.70	Control + BSA	2.69 ± 0.65
pg/ml	Interleukin 1α	pg/ml	Interleukin 1β	EXPERIMENT CONTRLS	
500	2.20 ± 0.73	500	2.69 ± 0.23		
250	2.78 ± 0.21	250	4.06 ± 1.05	Control Medium + 1 mg/ml BSA	
100	3.60 ± 1.42	100	3.47 ± 0.85		
50	2.81 ± 0.39	50	3.16 ± 0.59	(n=15) 2.79 ± 0.69	
10	1.79 ± 0.44	10	3.71 ± 0.17		
5	3.54 ± 0.86	5	5.36 ± 0.20	Control Medium minus BSA	
1	2.91 ± 0.77	1	4.23 ± 0.71		
Control + BSA	2.22 ± 0.34	Control + BSA	3.25 ± 0.73	(n=16) 1.76 ± 0.44	

n=3 unless otherwise stated.

Inter-plate variation in control DLKP growth was not as great as with DLRP in the previous experiment. However, this cell line displayed a different pattern of growth factor sensitivity profile than DLRP. High concentrations of aFGF (> 0.5 ng/ml) were inhibitory for DLKP growth in monolayer. Weak growth stimulation was indicated with 0.25ng/ml aFGF with respect to the plate control (× 1.4 fold), but this was not significant with respect to the experiment control.

A similar sensitivity pattern was seen with bFGF. Concentrations between 1 and 5 pg/ml bFGF were mitogenic for DLKP giving fold stimulations of 1.56 and 1.87 respectively with respect to the experiment control. However, concentrations greater than 10 pg/ml were inhibitory to DLKP with growth reduction directly proportional to the increase in bFGF concentration.

No significant activity was detected with IL-1α, but IL-1β was stimulatory at 5 pg/ml inducing a 1.92 fold increase in growth (w.r.t the experiment control).

TGFα was not active on DLKP over the range of concentrations assayed.

3.1.8 INVESTIGATION OF POSSIBLE AUTOCRINE GROWTH FACTOR PRODUCTION BY DLRP AND DLKP

The possibility that the NSCLC cell lines established in this laboratory produced autocrine growth factors was investigated. If such factors exist, then it they might prove beneficial in supporting the growth of primary lung epithelial cells *in vitro*. Preliminary experiments designed to test this hypothesis using Ham's F12 conditioned by DLRP and DLKP (Section 2.7.2) are described below.

3.1.8.1 Preliminary autocrine assay development for DLRP

DLRP cells were plated as described in Section 2.12.4.3. A number of attempts to measure the effects of DLRP conditioned medium at low serum concentrations were unsuccessful. No growth was obtained in assays using 0.5% FCS but the results of an experiment using 2% FCS (Batch N°701113) are shown in Table 3.1.8.1. In this experiment, collections of Ham's F12 medium conditioned in a roller bottle culture of DLRP were assayed for growth-promoting activity on DLRP in monolayer.

Table 3.1.8.1 The effect of DLRP CM on the growth of DLRP in monolayer (collected from DLRP over 31 days)

CM COLLECTION NUMBER	DLRP Conditioned Medium	
	Undiluted	Diluted ½
	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
CM 1	3.04 ± 1.35	0.75 ± 0.39
CM 2	3.78 ± 1.91	3.75 ± 2.69
CM 3	1.75 ± 0.67	1.37 ± 1.62
CM 4	1.27 ± 0.37	2.87 ± 2.81
CM 5	1.92 ± 0.88	3.56 ± 1.19
CM 6	3.36 ± 2.41	1.29 ± 0.91
CM 7	2.85 ± 1.73	2.35 ± 2.06
CM 8	2.06 ± 1.41	3.06 ± 0.87
CM 9	2.21 ± 1.79	3.66 ± 2.06
CM 10	2.97 ± 1.19	2.37 ± 1.66
CM 11	3.40 ± 3.57	5.37 ± 2.03
CM 12	4.37 ± 0.98	N.D.
Control Ham's F12		3.09 ± 1.73

n=4 for all determinations.

No evidence for autocrine stimulatory activity was detected in any of the 12 collections of DLRP CM.

3.1.8.2 Preliminary autocrine assay development for DLKP

DLKP were plated at three plating densities initially, at 1 × 10^3, 1.5 × 10^3 and 2 × 10^3 cells per well in 24-well plates, as described in Section 2.12.4. Two concentrations of serum were used at each cell density, 1 and 2% FCS (batch Nº 701113). Unconcentrated Ham's F12 conditioned by DLKP was incubated with DLKP in this system for 7 days and growth (colony area, mm^2) in the assay was then measured by image analysis. The results of this experiment are presented in Table 3.1.8.2.

Table 3.1.8.2 The effect of DLKP conditioned medium on the growth of DLKP in monolayer

Plating Density	1 × 10^3 Cells/well	1.5 ×10^3 Cells/well	2.0 × 10^3 Cells/well
DLKP CM	Colony ± S.E.M Area mm^2 mm^2	Colony ± S.E.M Area mm^2 mm^2	Colony ± S.E.M Area mm^2 mm^2
1% Foetal Calf Serum			
Undiluted	1.81 ± 0.30	4.19 ± 0.63	13.56 ± 2.06
Diluted ½	N.D.	8.67 ± 1.00	N.D.
Control	3.38 ± 0.58	9.07 ± 2.40	26.77 ± 2.40
2% Foetal Calf Serum			
Undiluted	11.96 ± 0.73	18.71 ± 4.54	4.73 ± 0.66
Diluted ½	N.D.	22.90 ± 3.14	N.D.
Control	14.75 ± 1.81	23.52 ± 1.87	22.71 ± 1.76

n=3 for all determinations.

No evidence for autocrine activity was detected in the medium conditioned by DLKP. In low serum, addition of undiluted DLKP CM caused growth inhibition. Dilution of this CM restored DLKP cell growth to the control level at a cell plating density of 1.5 × 10^3 cells/well. The degree of inhibition was in the same order at each plating density (aproximately 50%), but it was was less obvious at the higher serum concentration.

3.1.9 EVIDENCE FOR A SUBSTANCE IN HUMAN MALIGNANT EFFUSIONS WITH GROWTH-PROMOTING ACTIVITY FOR HUMAN LUNG SQUAMOUS CELL CARCINOMA CELL LINES *IN VITRO*

The potential benefit of biological fluids in the primary culture of squamous cell lung carcinomas in monolayer culture was addressed in this work. The effect of malignant effusions on human squamous cell lung carcinomas was examined because of the poor success to date of these tumour types *in vitro*. Monolayer assay systems were preferred for this analysis because of the technically cumbersome protocols required for agar assays and the added time factor involved with these systems.

DLRP and DLKP were chosen as model cell lines for this investigation together with another squamous cell carcinoma line of human origin, SK-MES-1. The latter cell line was not established in this laboratory. The cell line HEp-2 was included as a non-lung-derived control to determine the specificity of any effects on the lung cell lines. In a preliminary series of experiments, the effect on monolayer growth of an ascites fluid from a patient with ovarian carcinoma was determined.

3.1.9.1 The effect of ascites fluid on the growth of DLRP in monolayer

An ascites fluid from a patient with ovarian carcinoma (Ovarian 1) was processed as described in Section 2.5.7) before being filter-sterilised (0.22 μm low-protein-binding filter, Millex) and assayed with and without dilution, using the same the monolayer growth assay described in Section 2.12.4.3. A medium only control containing 1% (v/v) FCS was included and the results are presented in Table 3.1.9.1.

Table 3.1.9.1 Stimulation of DLRP monolayer growth by a malignant effusion from a patient with ovarian carcinoma

OVARIAN M.F. (Dilutions)	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]
Undiluted	0.00 ± 0.00	-
Diluted 3/5	0.00 ± 0.00	-
Diluted 1/2	0.00 ± 0.00	-
Diluted 2/5	0.00 ± 0.00	-
Diluted 1/5	0.03 ± 0.02	-
Diluted 1/10	9.99 ± 3.59	9.00
Diluted 1/20	14.52 ± 1.54	13.08
Control[b]	1.11 ± 0.10	-

n=3 for all determinations. [a] Fold stimulation with respect to the growth in the control. [b] Ham's F12.

DLRP was inhibited completely with neat fluid and at all dilutions of this sample down to 1/5. However, a nine-fold increase in growth over control was apparent at a dilution of 1/10 (≡ 5% v/v) fluid. Further dilution to 1/20 increased the apparent fold stimulation to 13.

3.1.9.2 The effect of ascites fluid on DLKP growth in monolayer

The same fluid (Ovarian 1) used in the previous experiment was assayed with DLKP in the 24-well plate system as described in Section 2.12.4.3. Medium controls were included as before and the results of this experiment are shown in Table 3.1.9.2.

Table 3.1.9.2 Stimulation of DLKP growth in monolayer by a malignant effusion from a patient with ovarian carcinoma

OVARIAN M.F. (Dilutions)	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]
Undiluted	0.00 ± 0.00	-
Diliuted 1/2	0.00 ± 0.00	-
Diluted 1/5	0.04 ± 0.03	-
Diluted 1/10	2.75 ± 1.34	2.25
Diluited 1/15	2.06 ± 0.38	1.69
Diliuted 1/20	2.97 ± 0.36	2.43
Diluted 1/40	3.31 ± 0.52	2.71
Control[b]	1.22 ± 0.65	-

n=3 for all determinations. [a] Fold stimulation with respect to the growth in the control. [b] Ham's F12.

A similar response to this fluid was observed with DLKP. Dilutions down to 1/5 were completely inhibitory for growth but at 1/10 a two-fold increase in growth over the control was apparent. However, further dilution of the fluid did not result in significantly greater increases in stimulation.

3.1.9.3 The effect of ascites fluid on the growth in monolayer of SK-MES-1

Under the same conditions as those used in Section 3.1.9.1 above the response of SK-MES-1 to the fluid 'Ovarian 1' was investigated as described in Section 2.12.4.3. The same background serum batch was used here as in Sections 3.1.9.1 & 2 respectively. The results are presented in Table 3.1.9.3

Table 3.1.9.3 Stimulation of SK-MES-1 growth by a malignant effusion from a patient with ovarian carcinoma

OVARIAN M.F. (Dilutions)	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]
Undiluted	0.00 ± 0.00	-
Diluted 1/2	0.00 ± 0.00	-
DIluted 1/5	0.04 ± 0.03	-
Diluted 1/10	16.80 ± 13.64	6.24
Diliuted 1/15	30.24 ± 7.49	11.24
Diluted 1/20	26.29 ± 11.81	9.77
Diluted 1/40	3.51 ± 1.36	1.30
Control[b]	2.69 ± 0.55	-

n=3 for all determinations. [a] Fold stimulation with respect to the level of growth in the control. [b] Ham's F12.

The response of SK-MES-1 was consistent with that observed for DLRP in Section 3.1.9.1 above. Inhibition was seen down to a dilution of 1/5. Fold stimulation over the control was in the same order at equivalent dilutions. In this case the level of stimulation was reduced to the control level at a dilution of 1/40 (≡ 1.25% v/v).

3.1.9.4 The effect of ascites fluid on the growth of HEp-2

The growth response of HEp-2 to the ascites effusion designated 'Ovarian 1' was investigated to determine the specificity of the growth-promoting activity detected with the lung cell lines.

HEp-2 was plated at 2×10^2 cells per well in the 24-well plate system but all other assay parameters were the same as described for the same experiments with the lung carcinoma cell lines (Sections 3.1.9.1, 2 & 3 above). The results of this experiment are shown in Table 3.1.9.4.

Table 3.1.9.4 Stimulation of HEp-2 growth by a malignant effusion from a patient with with ovarian carcinoma

OVARIAN M.F. (Dilutions)	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]
Undiluted	0.00 ± 0.00	-
Diluted 1/2	0.00 ± 0.00	-
Diluted 1/5	12.51 ± 1.45	1.89
DIluted 1/10	26.32 ± 4.83	3.97
Diluted 1/15	24.64 ± 1.32	3.72
Diluted 1/20	21.50 ± 2.41	3.24
Diluted 1/40	16.26 ± 2.02	2.45
Control[b]	6.63 ± 0.14	-

n=3 for all determinations. [a] Fold stimulation with respect to the level of growth in the control. [b] Ham's F12.

The stimulatory effect of this malignant effusion was not specific to lung carcinoma lines. As in the previous experiments with this fluid, a similar pattern of inhibition and stimulation of HEp-2 growth was observed at low and high dilutions of the fluid respectively. In this case however, inhibition was replaced by stimulation below a ½ dilution. Maximal fold stimulation over control was seen at a 1/10 dilution at approximately 4-fold.

3.1.10 HEp-2 AS AN INDICATOR CELL LINE FOR DETECTING GROWTH-PROMOTING ACTIVITY IN EFFUSIONS DERIVED FROM HUMAN CARCINOMAS OF DIFFERENT HISTOLOGICAL TYPE

The preliminary results with the NSCLC lines DLRP, DLKP, and SK-MES-1 (Section 3.1.9 above) indicated that a growth factor(s) activity present in a malignant effusion from a patient with ovarian carcinoma could stimulate the proliferation of these squamous cell lung carcinomas. The laryngeal carcinoma cell line HEp-2 was also shown to be sensitive to growth stimulatory activity in this effusion.

The potential usefulness of HEp-2 as an indicator cell line for the detection of growth-promoting activity in malignant effusions was considered for the following reasons. HEp-2 has a much faster rate of proliferation than any of the lung cell lines used in this work. This cell line could proliferate rapidly even in low serum concentrations and at low plating densities. Such growth properties facilitated the maintenance of *in vitro* stocks on a continuous basis in preparation for the receipt of fluid samples. Samples arrived with only a couple of hours warning, and on an infrequent basis. The HEp-2 assay system also required ten-fold fewer cells than the lung cell lines DLRP, DLKP, and SK-MES-1. HEp-2 therefore had several advantages over the lung cell lines as candidates for use as an indicator cell line.

In addition, at this time the growth performance of HEp-2 under stringent conditions (low serum concentration and low plating density) suggested the possibility that this cell line might be producing a growth factor or a repertoire of autocrine growth factors which diminished its serum requirement and reduced its minimum plating density at low serum levels *in vitro*.

Many of the autocrine growth factors characterised to date have also been shown to be active in cell types whose origins are histologically distinct to the originally identified producer cell type. Preliminary attempts to identify autocrine growth factor activity in the cell lines DLKP and DLRP were not encouraging (Section 3.1.8). For this reason, but also for the technical difficulties already mentioned, and the time factor involved, further development of these systems was not considered.

However, HEp-2 provided a facile cell line with which to work in these respects and preliminary experiments designed to detect autocrine growth factor activity in medium conditioned by this cell line were successful (Section 3.2.1.2). Further investigation of this autocrine system was pursued to characterise and purify the responsible autocrine species involved. The illucidation of such a mechanism, and the prospect of isolating in the process a novel growth factor(s) with growth promoting activity on primary epithelial cells, provided the incentive for this pursuit.

In the following series of experiments HEp-2 cells were plated under autocrine assay conditions (Section 2.12.4.2) and used to screen for growth-promoting activity in malignant effusions from patients with ovarian, breast, lung and kidney cancer, as well as a non-Hodgkins lymphoma. A sample of ascites from a patient with a non-malignant proliferative dissorder in the peritoneal cavity was also assayed. Fluids were filter-sterilised before assay and diluted in serum-free Ham's F12. Because there was a considerable variation in control growth between experiments the results of these experiments were also presented as the fold stimulation with respect to (w.r.t.) the experiment control ($\equiv$ sample mean $\div$ control mean). The results of the first experiment presented in Table 3.1.10(i) include as a reference the original sample of malignant effusion (Ovarian 1) assayed in Sections 3.1.9.1, 2, 3 & 4 above. The protein concentrations of these samples were determined using the BioRad protein assay (Section 2.11.6).

Table 3.1.10(i) Detection of growth-promoting activity for HEp-2 in ascitic fluids associated with malignant and non-malignant disease

Origin of Associated Carcinoma	[Protein] g/L	Sample Dilution	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t Control[a]
Ovarian (1)	N.D.	None	0.00 ± 0.00	-
		1/10	0.12 ± 0.06	-
		1/30	7.89 ± 2.27	4.15
Ovarian (2)	55	None	7.98 ± 1.94	4.20
		1/10	12.11 ± 2.14	6.37
		1/30	9.65 ± 0.32	5.08
Breast (1)	43	None	50.24 ± 6.62	26.44
		1/10	16.21 ± 0.26	8.53
		1/30	6.89 ± 0.10	3.63
Breast (2)	33	None	14.75 ± 3.55	7.76
		1/10	8.81 ± 2.03	4.64
		1/30	4.78 ± 0.74	2.52
Non-Malignant Proliferative Disorder of the Peritoneal Cavity	6.5	None	37.78 ± 5.14	19.88
		1/10	9.39 ± 2.79	4.94
		1/30	4.48 ± 1.05	2.36
Experiment Control Growth[b] (n=28)			**1.90 ± 0.69**	-

n=3 unless otherwise stated. [a] Fold stimulation with respect to the level of growth in the control. [b] Ham's F12.

This experiment confirmed previous observations of growth inhibition with the sample 'Ovarian 1'. In this case the inhibition was detected at an even lower dilution (1/10). This suggested either a loss of stimulatory activity in this sample or an increase in the inhibitory species, or both, over time. Stimulation was also detected in a second ovarian sample but inhibition was not detected in the undiluted sample. However, further dilution of Ovarian 2 (to 1/10) augmented the apparent stimulation detected suggesting that an inhibitory species was being diluted out.

Relatively high levels of stimulatory activity were not confined to the malignant effusions, as shown by the 20-fold stimulation observed with undiluted ascites fluid taken from a patient with a non-malignant proliferative dissorder. Stimulatory activity in this sample was also dilutable.

Stimulatory activity was detected in both sample of fluids from breast carcinoma patients and although this appeared to be greater in breast sample 1, the effect was dilutable in both cases. In the following experiment both of the fluids from breast carcinomas were assayed again together with the first fluid sample tested (Ovarian 1), as well as a non-Hodgkins lymphoma associated peritoneal fluid. The results are presented in Table 3.1.10.(ii).

Table 3.1.10.(ii) Comparison of growth effects on HEp-2 of effusions associaed with non-Hodgkins lymphoma, ovarian and breast carcinomas

Origin of Associated Carcinoma	Sample Dilution	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t Control[a]
Ovarian (1)	None	0.00 ± 0.00	-
	1/10	0.00 ± 0.00	-
	1/30	5.28 ± 1.32	1.55
Breast (1)	None	109.30 ± 1.77	32.15
	1/10	56.63 ± 4.30	16.66
	1/30	27.13 ± 3.12	7.98
Breast (2)	None	72.91 ± 1.14	21.44
	1/10	24.71 ± 1.14	7.27
	1/30	15.75 ± 2.13	4.63
Non-Hodgkins Lymphoma	None	0.00 ± 0.00	-
	1/10	0.00 ± 1.32	-
	1/30	5.30 ± 1.32	1.56
Experiment Control growth [b] (n=57)		3.40 ± 1.08	-

n=3 unless otherwise stated. [a] Fold stimulation with respect to the level of growth in the control. [b] Ham's F12.

This experiment confirmed the loss of stimulatory activity and the concomitant rise of inhibitory activity in the original ovarian sample with time (sample were stored at 4°C). A similar pattern of inhibition was detected in the non-Hodgkins lymphoma sample. The two breast samples were again shown to be stimulatory and this effect was dilutable.

In the last experiment in this series, pleural effusions from two patients with lung cancer were assayed together with pleural effusions from patients with kidney and breast cancers. Also included was an ascitic fluid from an ovarian carcinoma patient. The results are shown in Table 3.1.10(iii).

Table 3.1.10(iii) HEp-2 growth-promoting activity in effusions associated with lung & kidney carcinomas: comparison of activities in ovarian and breast samples

Origin of Associated Carcinoma	[Protein] g/L	Sample Dilution	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t Control[a]
Ovarian (3)	N.D.	None	0.89 ± 0.15	-
		1/10	8.84 ± 2.03	1.40
		1/30	12.43 ± 1.28	1.97
Breast (3)	31	None	55.25 ± 5.08	8.74
		1/10	30.85 ± 3.25	4.88
		1/30	14.01 ± 1.83	2.22
Kidney (1)	35	None	76.48 ± 4.18	12.10
		1/10	40.90 ± 7.38	6.47
		1/30	22.78 ± 3.94	3.60
Lung (1)	N.D.	None	9.60 ± 0.88	1.52
		1/10	7.65 ± 1.61	1.21
		1/30	6.69 ± 1.20	1.06
Lung (2)	N.D.	None	13.08 ± 8.04	2.07
		1/10	10.77 ± 3.45	1.70
		1/30	6.47 ± 1.90	1.02
Experiment Control Growth[b] (n=45)			6.32 ± 2.12	-

n=3 unless otherwise stated. [a] Fold stimulation with respect to the level of growth in the control. [b] Ham's F12.

The highest fold stimulation was detected in the kidney carcinoma sample. Stimulatory activity in this fluid was dilutable. Relatively high activity was detected in a third sample of pleural effusion taken from a patient with a breast adenoacarcinoma (Breast 3). A continuous cell line was established from cells harvested from this effusion (Section 3.1.11). The inhibition seen with the ovarian ascites was consistent with the pattern for other ovarian samples. Dilution of the latter sample to 1/10 abolished the apparent inhibition and at 1/30 this sample gave an apparent 2-fold stimulation in growth over the control. Activity in the lung samples was relatively low in both samples assayed.

3.1.10.1 Ammonium sulphate fractionation of an ovarian malignant effusion

Salt fractionation is a useful first step in the purification of protein from complex mixtures in solutions. The potential with this technique to differentially precipitate proteins from solution by incrementally increasing salt concentrations can be usefully employed to separate interesting proteins if these precipitate at salt concentrations other than those where the bulk of proteins precipitate (i.e. at low or high salt concentrations). Given that the majority of animal cell growth factors identified to date are proteinacious species, it was anticipated that some or all of the active species in human effusions might be protein. For this reason ammonium sulphate fractionation was applied to the task of characterising, by means of separation, the growth-promoting species for human lung squamous cell carcinomas detected in fluids associated with various histological types of cancer. This approach has also been employed by others in the purification and characterisation of growth factors and inhibitors from biological fluids {Mills *et al.*, (1988); Podolsky *et al.*, (1988)}.

A 100 ml sample of 'Ovarian 1' was subjected to several salt cuts as described in Section 2.11.5. Protein pellets from the respective salt cuts resuspended in PBS were filter-sterilised before assay and diluted in Ham's F12 (serum-free). A PBS 'dilution control' was included to allow for dilution of nutrients in the assay and the results are presented in Table 3.1.10.2a.

Table 3.1.10.1 Growth factor activity in a salt fractionated malignant effusion from an ovarian carcinoma patient

SAMPLES	DILUTIONS		
% $(NH_4)_2SO_4$	1/10	1/30	1/60
	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
20% Pellet	4.57 ± 1.07	6.40 ± 0.97	4.87 ± 0.84
20% Supernatant	8.39 ± 1.61	6.00 ± 1.08	6.84 ± 0.64
20-30% Pellet	11.52 ± 1.61	9.59 ± 1.35	6.95 ± 0.70
20-30% Supernatant	7.37 ± 0.54	8.53 ± 0.32	6.33 ± 0.44
30-50% Pellet	9.17 ± 1.12	7.07 ± 1.03	7.30 ± 0.48
30-50% Supernatant	3.25 ± 0.36	5.47 ± 0.76	6.90 ± 0.61
PBS Dilution Control[a]	1.74 ± 0.28	3.05 ± 1.14	4.12 ± 0.68
CONTROLS			
Untreated Effusion	20.64 ± 1.28	14.00 ± 1.83	N.D.[b]
Experiment Control Growth[c] (n=15)			4.56 ± 0.78

n=3 unless otherwise stated. [a] Ham's F12 diluted with PBS to allow for the dilution effect of fluid reconstituted in phosphate buffered saline. [b] Not determined. [c] Ham's F12.

The fold stimulation in all of the above samples assayed in table 3.1.10.1a were calculated with respect to the appropriate control for the sake of comparison.

Table 3.1.10.1b Fold stimulation in the ammonium sulphate fractionated malignant effusion

SAMPLES	DILUTIONS		
% $(NH_4)_2SO_4$	1/10	1/30	1/60
	Fold Stimulation[a]	Fold Stimulation	Fold Stimulation
20% Pellet	2.62	2.10	1.18
20% Supernatant	4.82	1.97	1.66
20-30% Pellet	6.62	3.14	1.69
20-30% Supernatant	4.24	2.80	1.54
30-50% Pellet	5.27	2.32	1.77
30-50% Supernatant	1.87	1.79	1.67
	FOLD STIMULATION IN THE UNTREATED EFFUSION[b]		
	4.53	3.07	0.00

[a] Fold stimulation in the samples with respect to the PBS controls. [b] With respect to Ham's F12 control.

Analysis of the results in Table 3.1.10.1b indicate that the greatest fold increase over the respective dilution controls were in the precipitates at the 20-30% and 30-50% salt cuts. The magnitude of stimulation over the dilution controls was in the same order for both of these precipitates over the range of dilutions assayed. However, the relative concentration of proteins in each of these precipitates was at least 2x after fractionation and resuspension (with respect to the untreated fluid). Comparing the fold stimulation in the precipitates at 20-30% and 30-50% salt cuts to the fold stimulation in the untreated sample, only the precipitate at 20-30% salt diluted 1/10 shows a significant increase in fold stimulation (with respect to the untreated fluid at this dilution).

The activity in this fluid therefore appears to be comprised of more than one species the majority of which (but not all) precipitates at 20-30% ammonium sulphate concentration.

3.1.10.2 Growth stimulatory activity in malignant effusions compared to that in serum and plasma from autologus donors

Effusions associated with human disease are classified clinically as either **transudates** or **exudates** on the basis of total protein concentration. Samples with protein concentrations < 30 g/L are considered transudates and those with total protein concentrations > 30 g/L are classified as exudates. Transudates are normally associated with changes in membrane permeability, but exudates are associated with infection and malignancy. Pleural fluid can have total protein concentrations from 1 g/L up to concentrations in the normal range for plasma, 66-87 g/L. On this basis the majority of effusions received were classified as exudates. Two samples had total protein concentrations in the transudate range. One of these was from a patient with non-Hodgkins lymphoma and the other from a patient with a non-malignant proliferative disorder of the peritoneal cavity (leiomyosarcoma). No direct correlation between total protein and growth stimulatory activity in the HEp-2 assay was apparent with the samples assayed, and the detection of comparable growth stimulatory activity in the fluid associated with the proliderative dissorder indicated that this activity was not restricted to malignant effusions.

In an effort to understand the nature of the growth factor activity in these effusions, the possibility that this species was derived from systemic factors, carried in the serum or plasma, was investigated. Unfortunately samples of autologus serum and/or plasma were available in only two of the cases studied in this work. In the first example a serum and plasma sample from the patient designated 'Breast 3' were assayed over the same range of dilutions in the HEp-2 assay. The plasma sample was collected in a Li-heparin tube. The serum was collected from clotted blood after centrifugation. The results of this preliminary experiment are presented in Table 3.1.10.2(i).

Table 3.1.10.2(i) Comparison of growth-promoting activity in a malignant effusion with that in autologus serum and plasma from a patient with breast carcinoma

Sample	EFFUSION		SERUM		PLASMA	
Dilution	Colony ± S.E.M. Area mm^2 mm^2	Fold[a]	Colony ± S.E.M. Area mm^2 mm^2	Fold	Colony ± S.E.M. Area mm^2 mm^2	Fold
None	78.33 ± 0.31	18.65	78.08 (n=1)	18.59	N.D.[b]	12.46
1/5	52.86 ± 4.08	12.59	72.08 ± 2.65	17.16	52.35 ± 2.87	N.D.
1/10	38.83 ± 1.43	9.25	69.00 ± 4.42	16.43	77.03 ± 16.45	18.34
1/20	25.11 ± 1.53	5.98	55.88 ± 2.42	13.30	27.44 ± 1.40	6.53
1/40	18.41 ± 1.61	4.38	41.08 ± 2.33	9.78	12.89 ± 3.15	3.07
1/80	15.19 ± 1.05	3.62	29.23 ± 1.42	6.96	12.58 ± 11.02	2.99
1/100	10.47 ± 1.09	2.49	24.93 ± 1.73	5.93	13.54 ± 7.35	3.22
Experiment Control Growth[c] (n=9)					4.20 ± 0.73	

n=3 for all determinations unless otherwise stated. [a] Fold stimulation with respect to the control. [b] Not determined. [c] Ham's 12.

The degree of stimulation in the serum sample was higher at all concentrations assayed than in the corresponding fluid at these dilutions, but the levels of stimulation supported by the plasma sample were in the same order as the effusion over the entire range of concentrations tested (the value for the 1/10 dilution of plasma was an exception, but the standard error on this determination was also very high). Although no value for the plasma protein concentration was established, that of the associated effusion from the same donor was 31 g/L which is at least half the concentration expected for normal plasma. If the same mitogenic species is active in both samples then this would imply that the specific activity in the effusion sample was higher.

In the second example a peritoneal fluid from a patient with mixed mesodermal ovarian carcinoma ('Ovarian 4') was compared to an autologus plasma sample (collected using EDTA as an anticoagulant) for growth-promoting activity. The results are shown in Table 3.1.10.2(ii).

Table 3.1.10.2(ii) Comparison of growth-promoting activity in an ovarian ascitic fluid with that in plasma from the same donor

Sample	ASCITES	PLASMA
Dilutions	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
None	68.01 ± 2.53	N.D.[a]
1/5	40.59 ± 3.27	0.07 ± 0.07
1/10	34.02 ± 8.92	2.72 ± 0.52
1/30	19.38 ± 3.22	9.90 ± 1.25
1/50	16.98 ± 1.04	20.26 ± 2.67
1/100	12.74 ± 1.77	12.41 ± 1.89
1/200	9.62 ± 1.07	11.17 ± 0.72
Experiment Control Growth[b]		5.77 ± 0.67

n=3 unless otherwise stated. [a] Not determined. [b] Ham's F12.

Inhibition was detected at low dilutions of the plasma sample. This was most likely explained by the clotting which was observed in this sample. At dilutions below 1/30 the same order of stimulation was seen in both fluid and plasma sample in agreement with the results of the previous experiment.

Although it was possible to compare growth-promoting activity in autologus fluid and plasma from only two donors, the indications from this preliminary work were that a species of equivalent potency exists in the plasma of these patients. A greater number of samples need to be assayed together with normal plasma and/or serum samples to determine whether this activity is a normal systemically produced factor, or a factor produced by cells associated with diseased states such as inflamation or malignancy.

3.1.11 ESTABLISHMENT OF A CONTINUOUS HUMAN BREAST ADENOCARCINOMA CELL LINE - BAC

3.1.11.1 Clinical History of BAC

A pleural effusion taken for palliative treatment from a patient post bilateral mastectomy, radiotherapy, and chemotherapy (Novantrone) was processed as described Section 2.5.7. Two rounds of ficoll treatment removed red blood cells from the initial cell pellet and a dense layer of tumour cells remained at the ficoll/medium interface after centrifugation. Microscopic examination revealed that cells in this 'buffy' layer comprised almost exclusively of large cells (too large to be macrophages) existing in clumps with tubular shaped morphology. A portion of this original tissue was stored in liquid N_2 for DNA fingerprint analysis. The remainder was cultured in DMEM/Ham's F12 supplemented with 10F6182-NCS. A synopsis of the primary history of this cell line *in vitro* is given here. Stocks were refed at 2-7 day intervals as necessary.

Day 1. Cells in suspension were plated in 75 cm^2 tissue culture flasks.

Day 10. All non-attached cells were replated in fresh flasks. No signs of fibroblast growth in the original flasks.

Day 44. First passage of this breast adenocarcinoma cell line (designated BAC). Cells were found difficult to trypsinise and, after detaching from the monolayer in clumps, were refractory to forming a single cell suspension — even after 15 minutes in standard trypsin (0.25% w/v)/ EDTA (0.02% w/v) solution. Cells were replated as clumps.

Day 68. Similar difficulty was encountered in achieving detachment of monolayer and single cell suspension at the second passage of this line.

Day 93. Passage 3 of BAC.

Day 109. Passage 4.

Day 121. Passage 5.

The growth rate of BAC was relatively slow. Multiple nucleoli were clearly visible in many cells of this cell population at passage 2 (figure 3.1.10). Actively dividing cells (characterised by a rounded up morphology and highlighted by light refraction around cell doublets), are also visible in this culture. Cell migration at the leading edge of these colonies was slow, forming tightly-packed colonies which contacted to form confluent monolayers that began to stratify, i.e. growing upward to from double layers (see figure 3.1.11). Stratification was accompanied by a change in the appearance of cells on the 'visible' upper layer. The main feature of this different morphology was a more clearly discernable and what appeared to be a wider inter-cell junction (compare photographs in figures 3.1.10 and 3.1.11 taken at the same magnification). A subconfluent monolayer displaying the characteristic rounded and discreet nature of BAC colonies in monolayer is shown in figure 3.1.12.

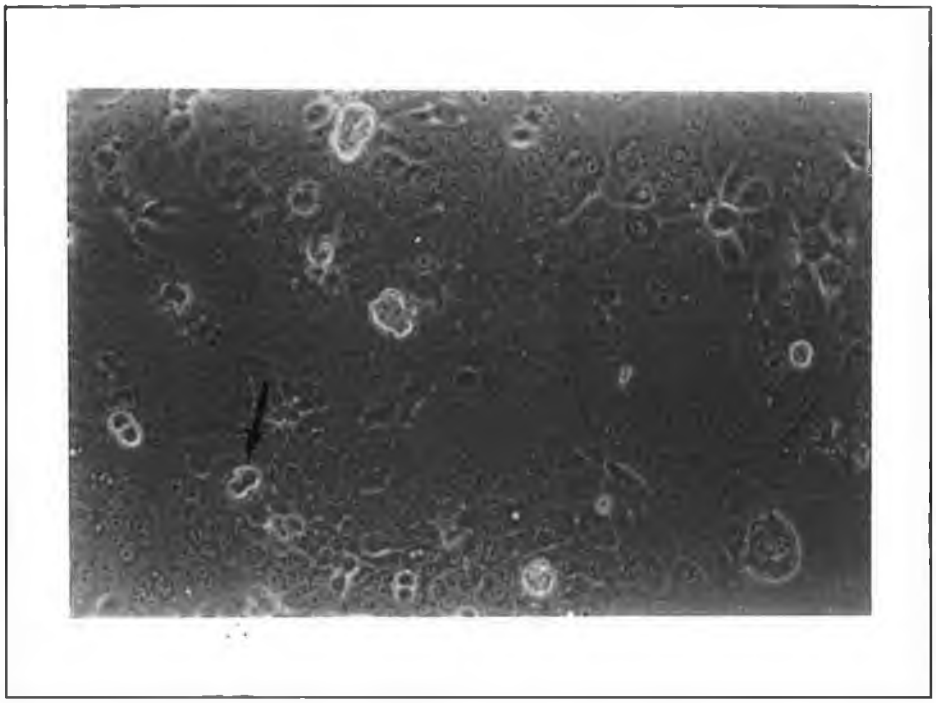


Figure 3.1.10 A 150X magnification of a tightly-packed monolayer colony of passage 2 BAC cells. Actively dividing cells are visible and indicated by arrows. Cells with multiple nuceoli can also be seen.

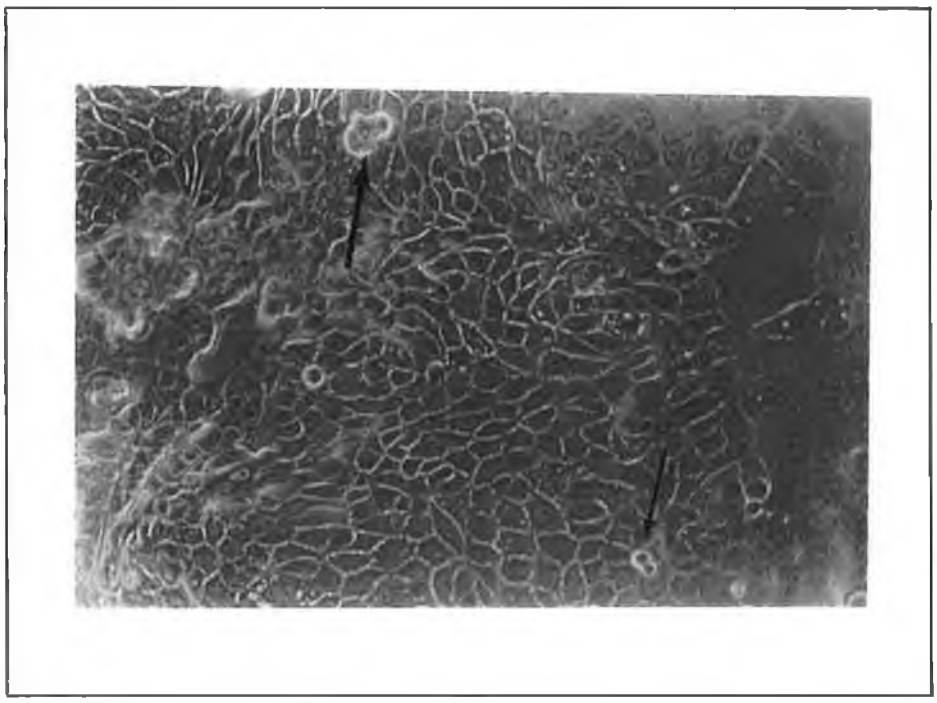


Figure 3.1.11 Stratification of passage 3 BAC cells at confluency in monolayer. Dividing cells are still visible and indicated by arrows (150X magnification).

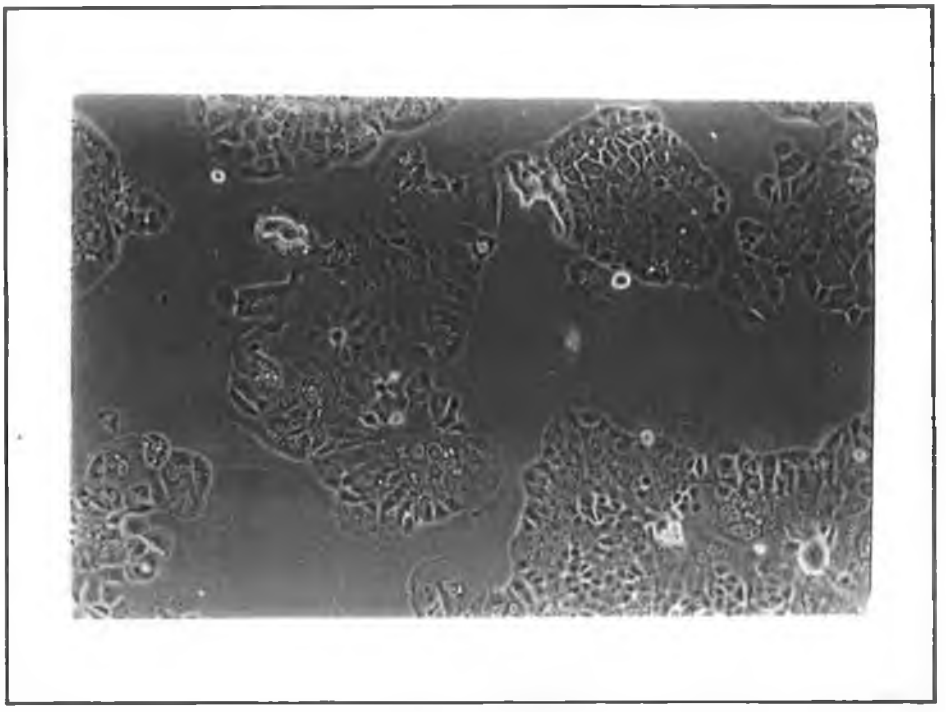


Figure 3.1.12 Colony morphology of passage 3 BAC cells in monolayer culture at passage 3. (60X magnification).

3.1.12 CHARACTERISATION OF NEW HUMAN CARCINOMA CELL LINES

It is important to determine the biological and genetic character of newly established cell lines for a number of reasons. Principally, the risk of cross-contamination of cell lines would appear to a more common occurance than acknowledged. Precautions taken in this laboratory to avoid cross-contamination of cell lines are outlined in Section 2.4.3.1. The expression of a tissue-specific differentiated phenotype or some other special property may be required for particular applications and therefore both the origin of the cell line, and expression of the specific determinant, should be validated.

Several methods have been used to distinguish cell lines from different species and to discriminate between lines from the same species. These include chromosomal, immunological, and enzymological (isoenzyme) analysis of cell line or species specific markers. Commercial kits are available for measuring polymorphic enzyme loci. Although the measurement of these unique isoenzyme phenotypes can be useful in distinguishing a small number of cell lines, when larger numbers of cell lines need to be distinguished the technique is limited by the low frequency of unique phenotypes. A number of such isoenzymes were analysed for the cell lines established here but these were not found to be useful for discriminating between human cell lines (data not shown).

A detailed cytogenetic comparison of the cell lines DLRP and DLKP was undertaken by Elizabeth Law in this laboratory {Law *et al.*, (1992)}. This technique can detect inter-species cross-contamination, but it is a highly specialised and time consuming procedure.

A recently developed alternative method for cell line characterisation is the DNA 'fingerprint' techology, based on the discovery by Jeffreys *et al.*, (1985a) of hypervariable sequences in the human genome, can be used to prepare individual-specfic 'fingerprints' of human DNA {Jeffreys *et al.*, (1985b)}. In the same way the origin of cell lines can be determined using tiny quantities of DNA from the original tissue donor (where available) and the resulting cell line. In the absence of original tissue this technique can discriminate between different cell lines on the basis of their unique band pattern {Thacker *et al.*, (1988); Gilbert *et al.*, (1990)}. The uniqueness of an individual's DNA ensures that the chances of discovering an identical banding between differnt cell lines is negligible. It is therefore a powerful technique for detecting cross-contamination.

3.1.12.1 DNA fingerprint analysis of the cell lines DLRP and BAC

At the time of establishment of the cell line DLRP DNA fingerprinting was not a commercially available service. None of the original tumour tissue (from which the line was established) was available for analysis therefore. In order to distinguish DLRP using this technique, all the cell lines in culture at the time of its inception were included in the analysis. These were the human cell lines SCC-9, RPMI-2650, SW900, A-549, and HEp-2. Other cell lines used later in this work were also included in this analysis. These were the cell lines A-431, SK-MES-1, SK-LU-1 and HTB 120. Stocks of these cell lines together with DLRP were shipped as live cultures to Cellmark Diagnostics for analysis (Section 2.6.2)

Human-specific single locus probes were used to produce the banding profiles in figure 3.1.13. The profile of DLRP was completely unique with respect to those of the other cell lines. In the case of BAC two separate samples of the original tissue stored at -196°C in liquid N_2 were sent for analysis together with a live culture of the cell line. The profile obtained after radiography with a multi locus probe is shown in figure 3.1.14. The band patterns of the tumour tissue and cell line DNA are identical.

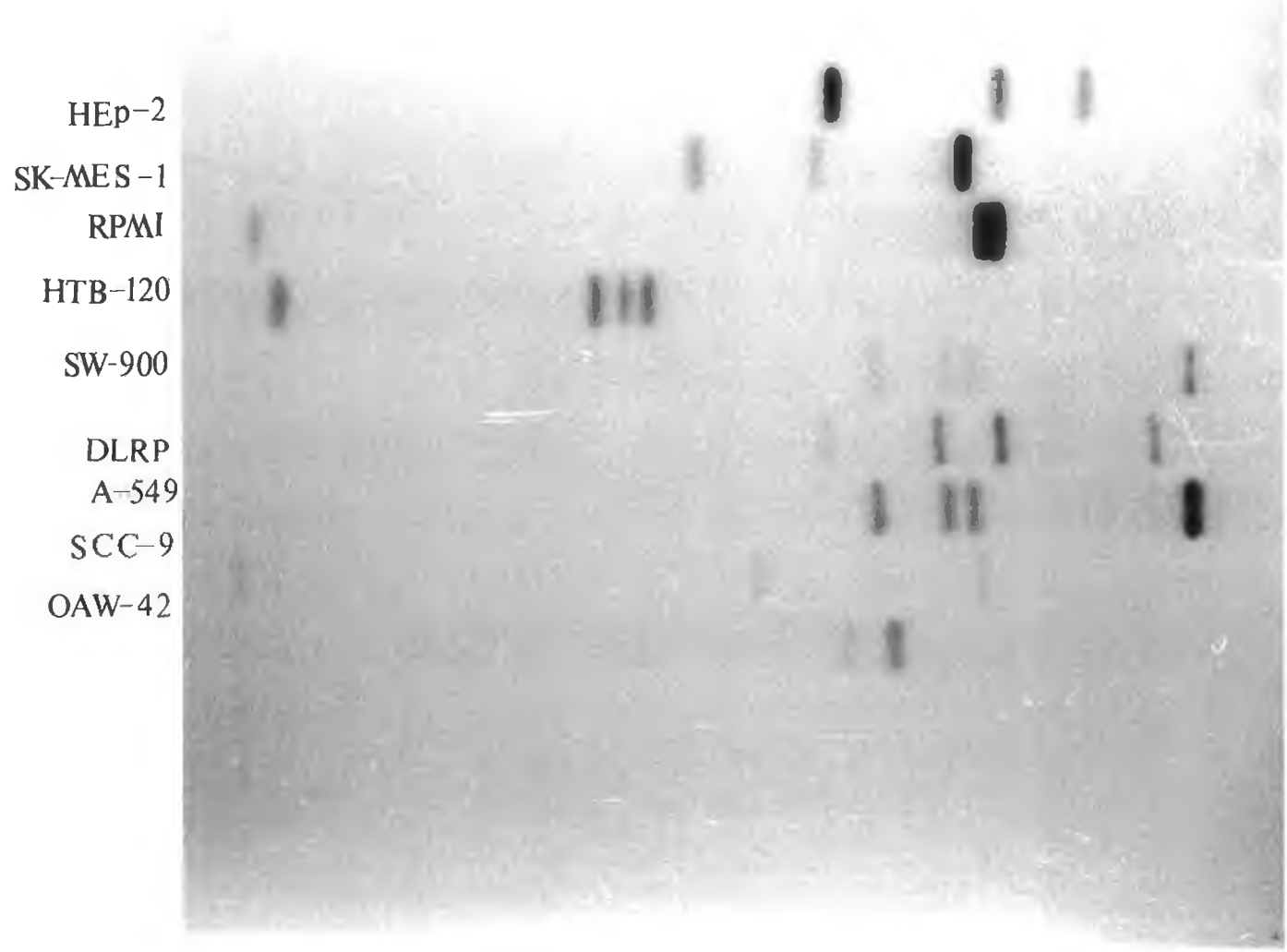


figure 3.1.13 DNA fingerprint of DLRP and several other cell lines in use at the time of its inception. The profile for DLRP is distinct compared to that of the other cell lines analysed.

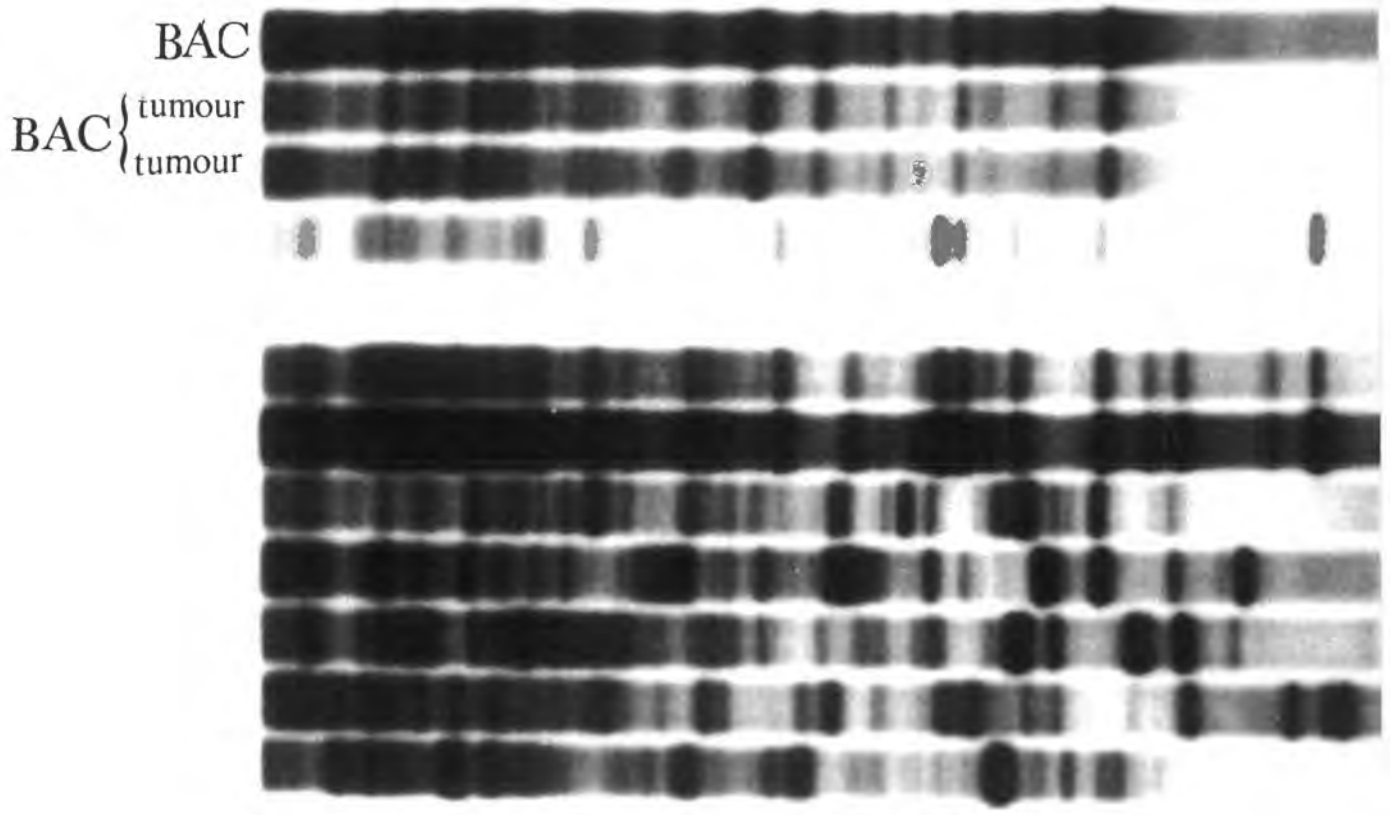


figure 3.1.14 DNA fingerprint obtained with a multi-locus probe. The banding pattern for BAC and two samples of the original tumour DNA are identical.

3.1.12.2 Histopathology

Patholigists use a number of criteria to characterise malignant cells in tissue biopsy sections. One such feature is abnormal mitotic figures in the dividing cells. These abnormalities include tripolar and multipolar mitoses and are characterisitc of the malignant phenotype. Studies by Gilvarry *et al.*, (1990) indicated that this criterion may also be used to characterise malignant cells *in vitro*. One such abnormal mitotic figure in a population of DLRP cells is shown in figure 3.1.15 indicating the malignant character of these cells.

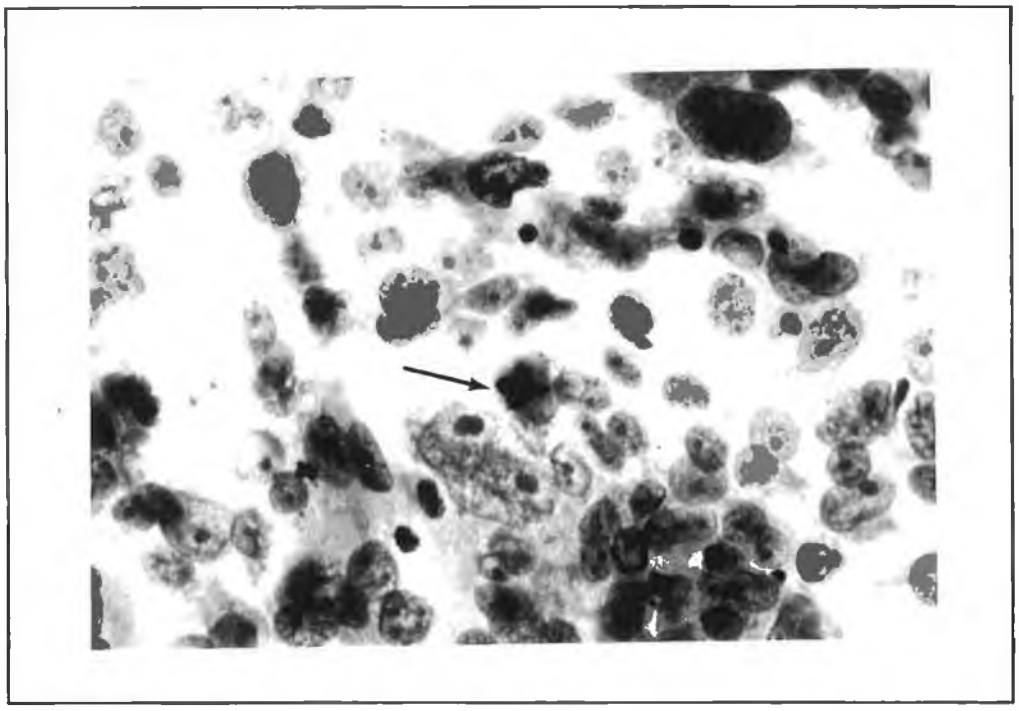


Figure 3.1.15 A multipolar mitoses in a DLRP cell (indicated by the arrow), stained with Carazzi's hematoxylin and 0.5% eosin stain.

3.1.12.3 Immunohistochemical characterisation of the cell lines DLRP and BAC

The availability of monoclonal antibodies which recognise cell type-specific antigens permits the classification of cells on this basis. Fluorescently labelled antibodies were used in this work to visualise the binding of monoclonals specific to epithelial- (Ep-16 and cytokeratin Nº 18), and NSCLC-specific antigens.

Epithelial-specific antigens

The mouse hybridoma cell line Ep-16 isolated by Hamburger *et al.*, (1985) produces an IgMκ monoclonal antibody which is directed against an antigen present on the plasma membrane of human foreskin keratinocytes, but does not react with mesodermal cells, lymphocytes, or internal epithelia. SCC-9, a carcinoma cell line derived from an external epithelium, was used as a positive control for staining with this antibody and MRC-5, a human lung embryonic fibroblast cell line, was used as negative control. The staining patterns of these positive and negative controls are compared in figures 3.1.16 & 3.1.17. The staining pattern of DLRP with this antibody is shown in figure 3.1.18.

Cytokeratin Nº 18

The filamentous protein keratin is a major component of the network of intermediate filaments which constitute the cytoskeleton of epithelial cells. The fact that it is absent from fibroblast-like cells makes it useful diagnostically for distinguishing these two cell types. DLRP and BAC were examined for the presence of intracellular keratin expression and compared to the staining patterns for the negative control, MRC-5, and the characterised epithelial cell line SCC-9 which was used as a positive control. The results of these positive and negative staining patterns with anti-cytokeratin antibody are shown in figures 3.1.19 & 3.1.20 respectively. The presence of cytokeratin in DLRP is shown in figures 3.1.21 & 3.1.22. BAC staining with anti-cytokeratin antibody was very strong as seen in figures 3.1.23 & 3.1.24. The definition in the black and white photo in figure 3.1.24 demonstrates a well-defined cytokeratin network.

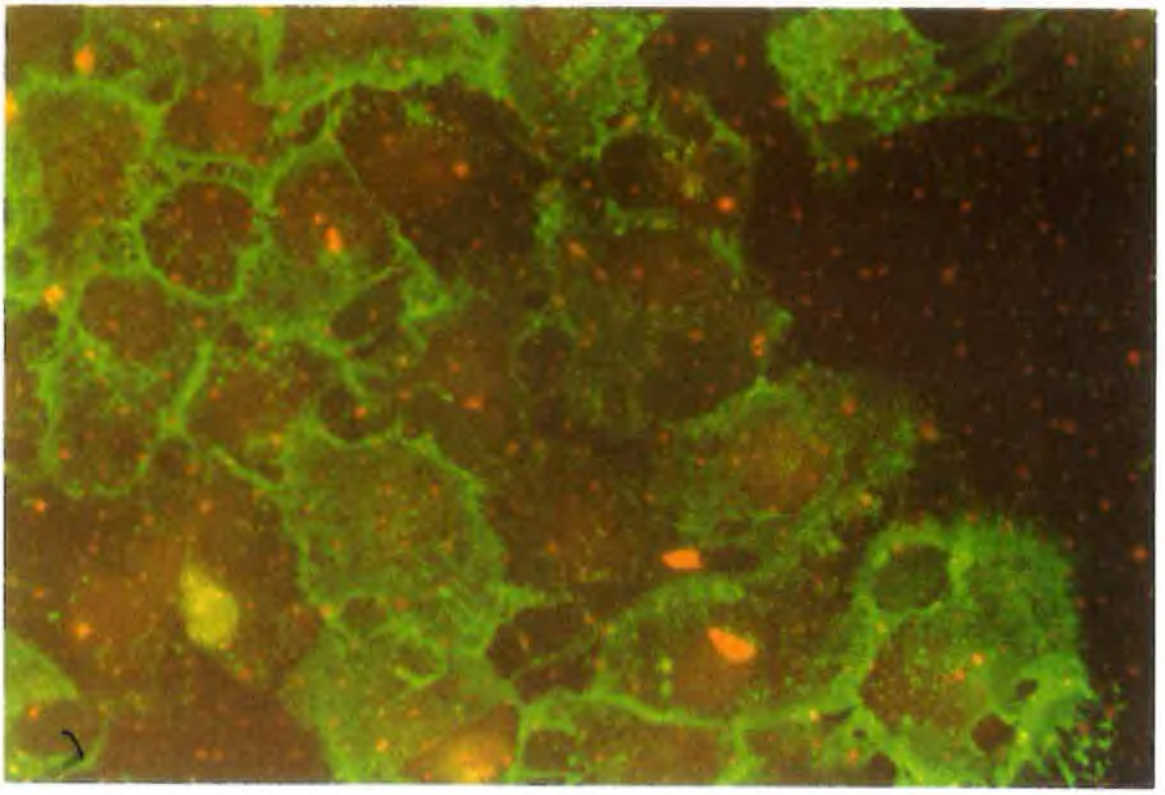


Figure 3.1.16 SCC-9 cells are positive for the surface antigen recognised by Ep-16. (Magnification, 40X).

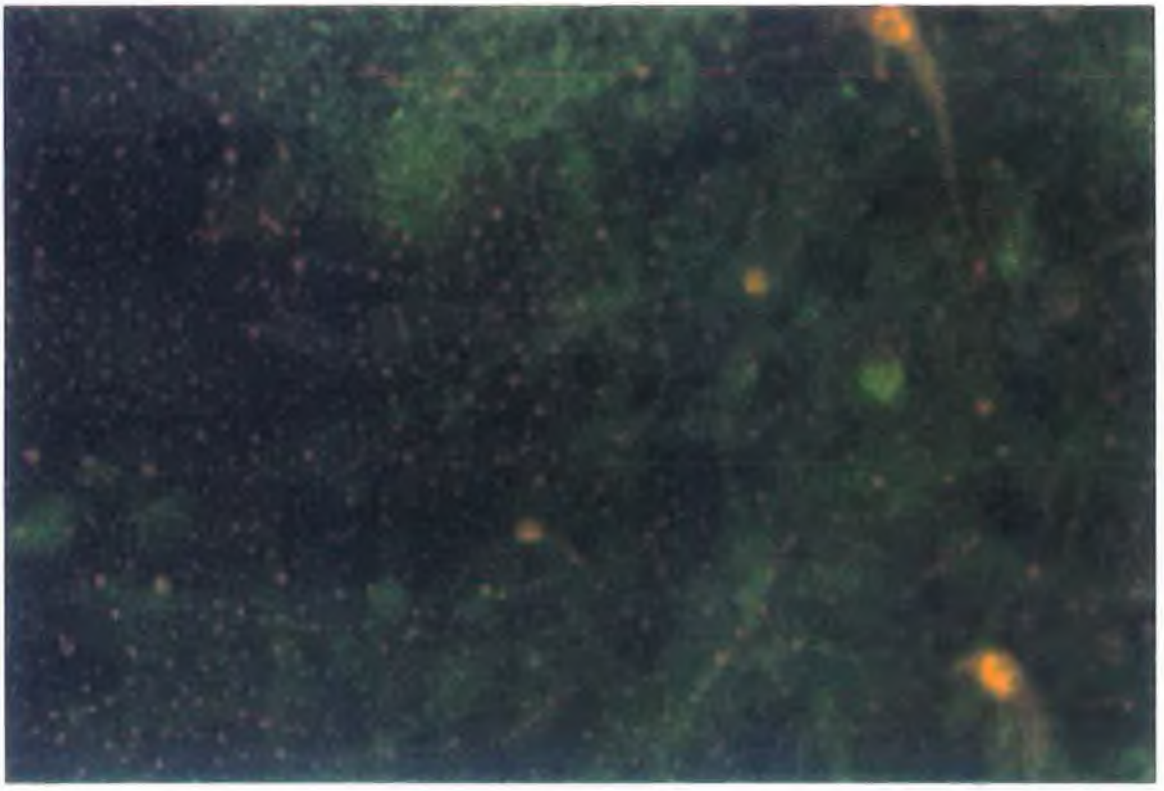


Figure 3.1.17 The negative staining pattern of MRC-5 cells obtained with the Ep-16 epithelial-specific antibody. (Magnification, 40X).

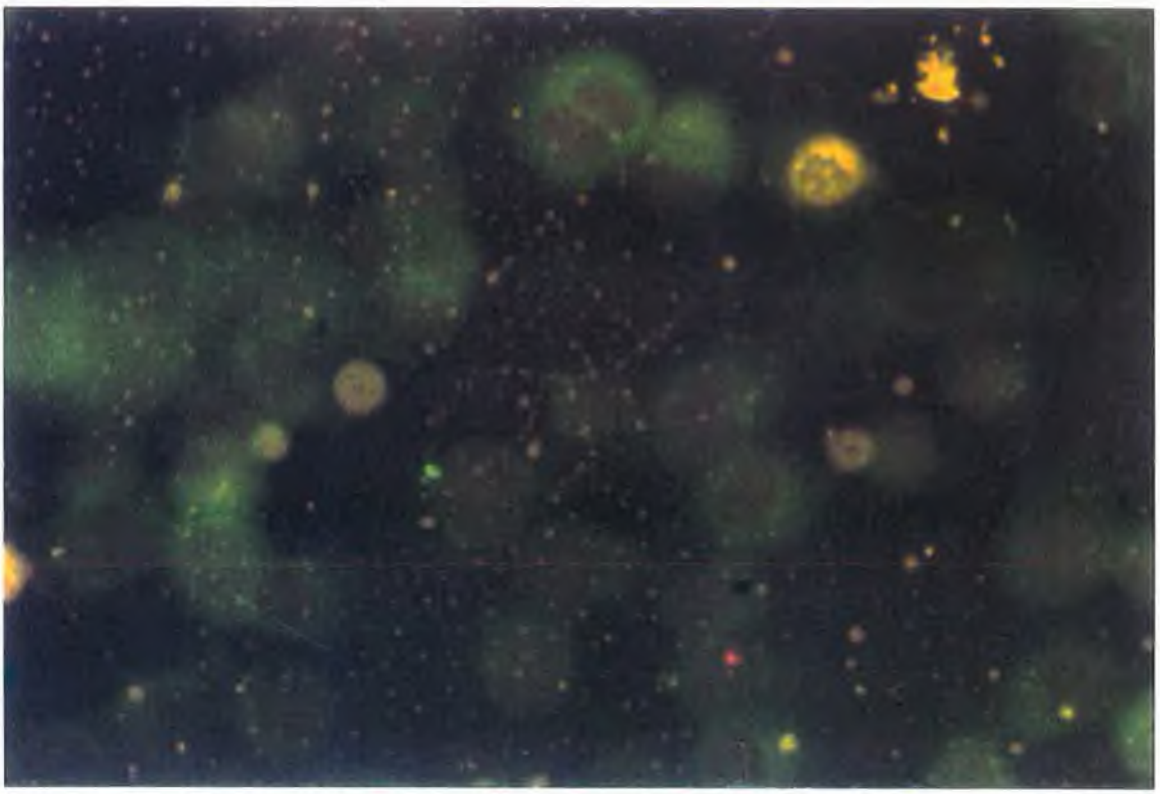


Figure 3.1.18 DLRP stains very weakly with the Ep-16 antibody (Magnification, 40X).

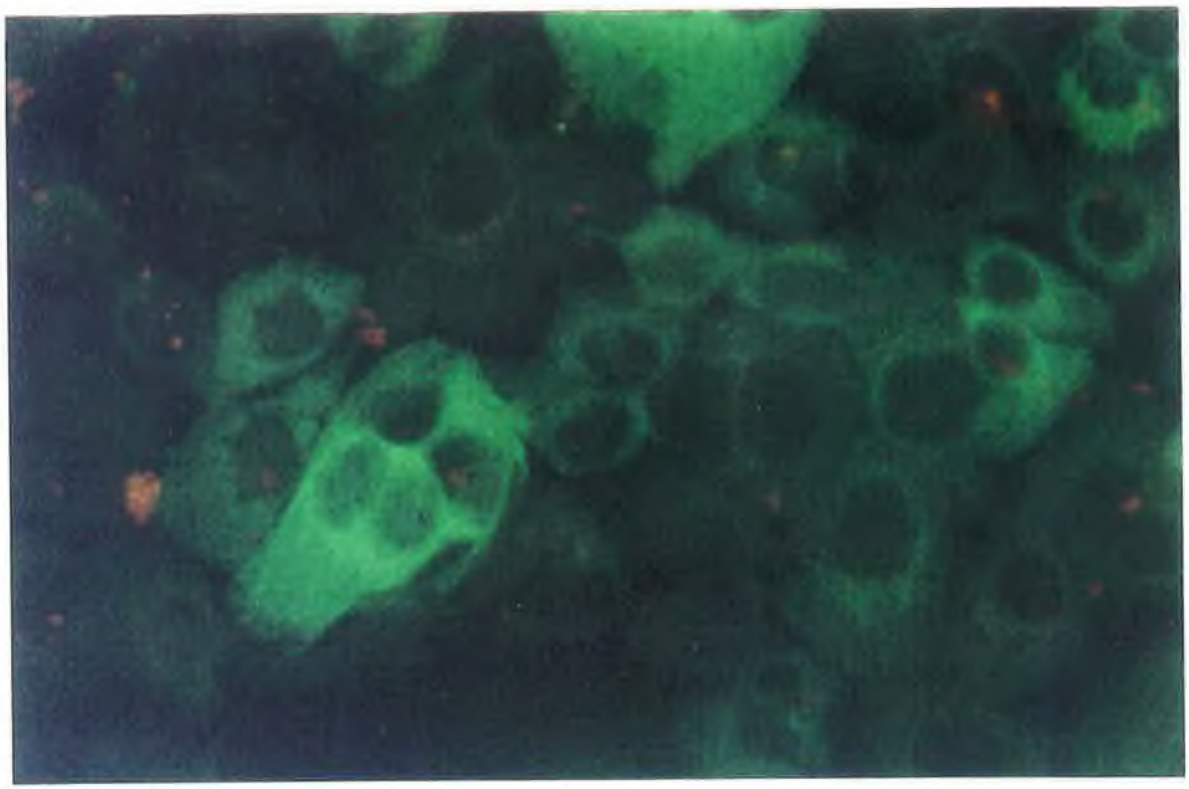


Figure 3.1.19 Positive staining of the epithelial cell line SCC-9 with anti-cytokeratin № 18 antibody. (Magnification, 40X).

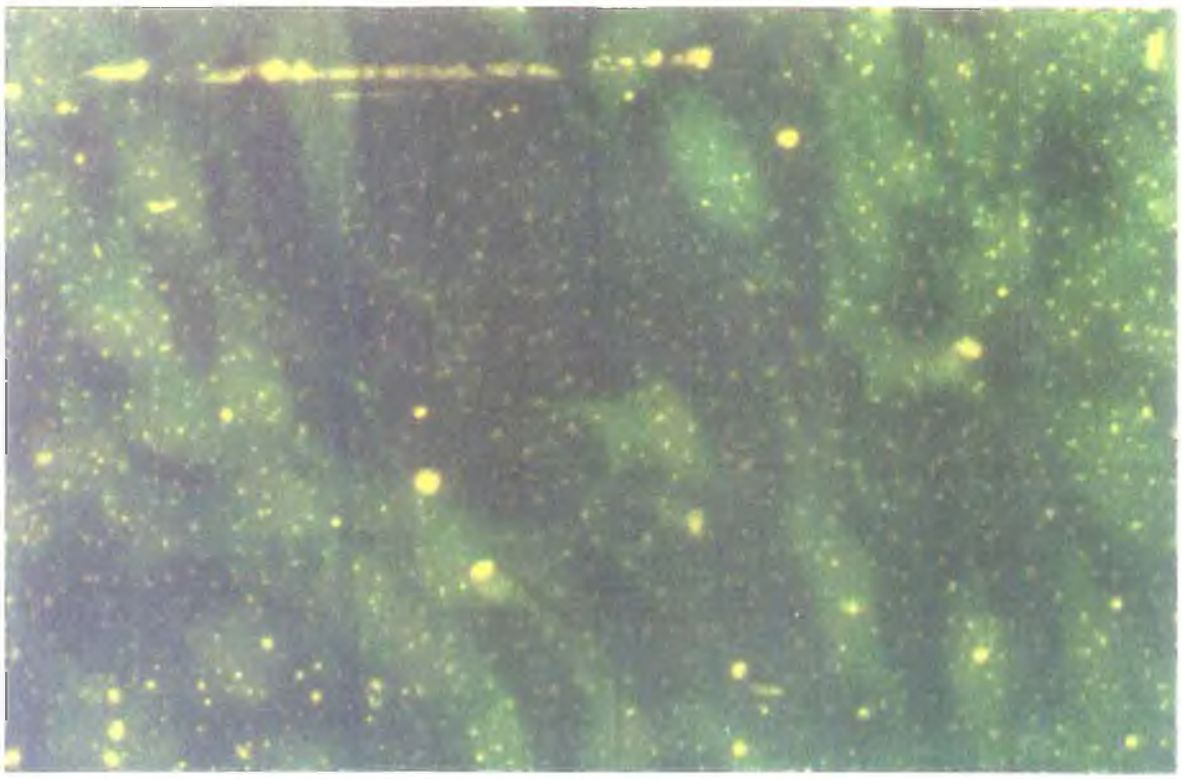


Figure 3.1.20 MRC-5 human embryonic fibroblasts are negative for cytokeratin expression (Magnification, 40X).

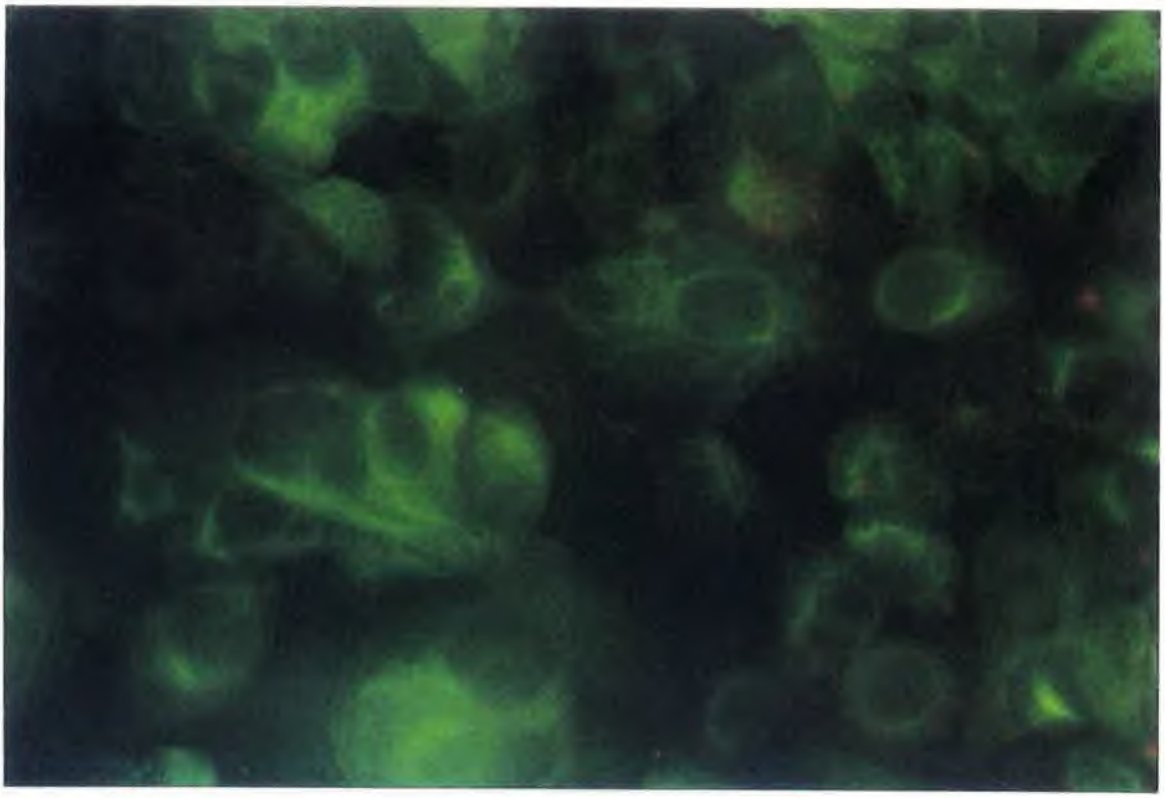


Figure 3.1.21 DLRP staining positive for cytokeratin, viewed at 40X magnification.

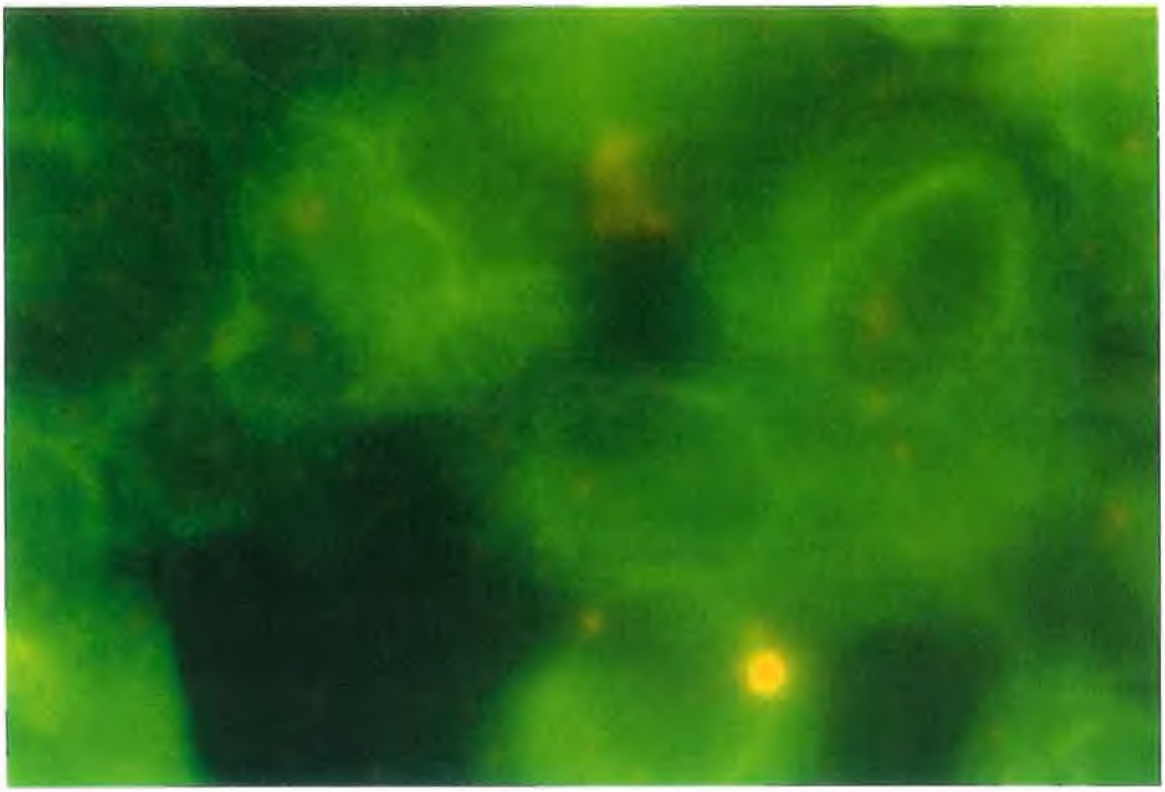


Figure 3.1.22 Positive cytokeratin staining of DLRP, viewed at a higher power (160X). Cytokeration-stained filaments are more clearly visible at this magnification.

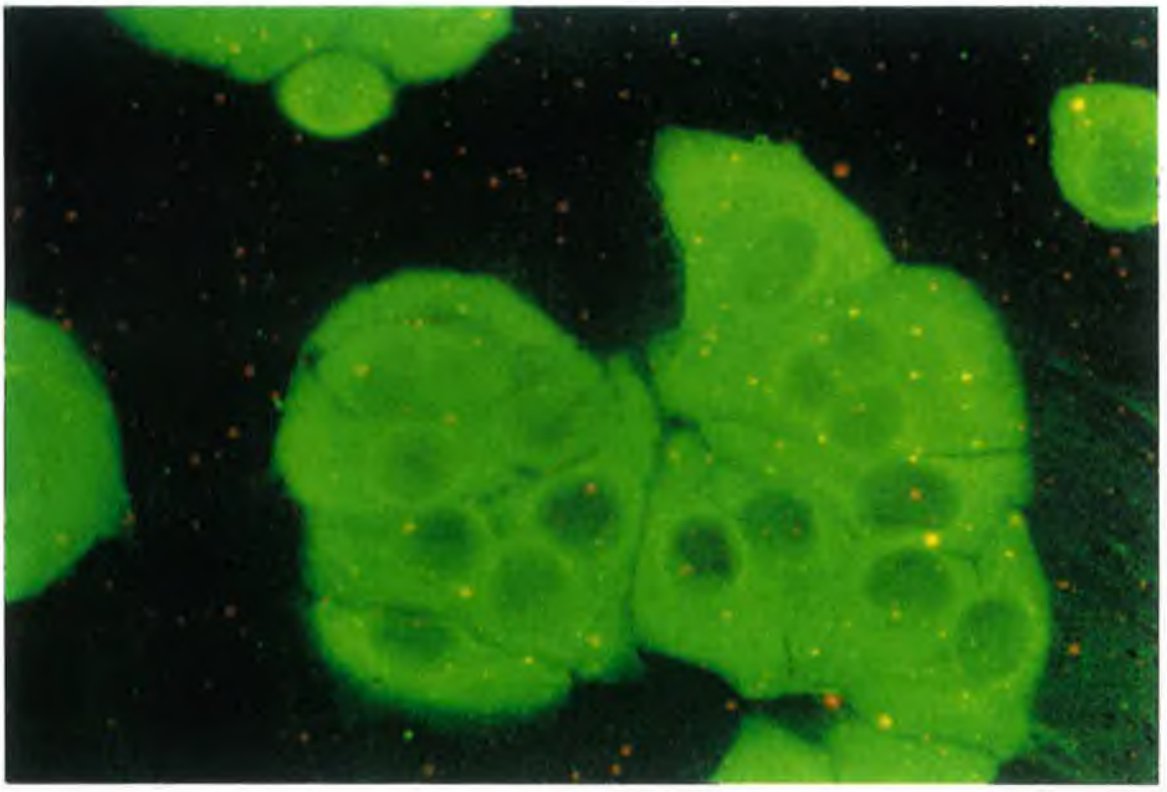


Figure 3.1.23 The pattern of BAC viewed at 40X reveals intense cytokeratin staining with little definition of filament structure, possibly due to the tight packing of these cells in monolayer culture.

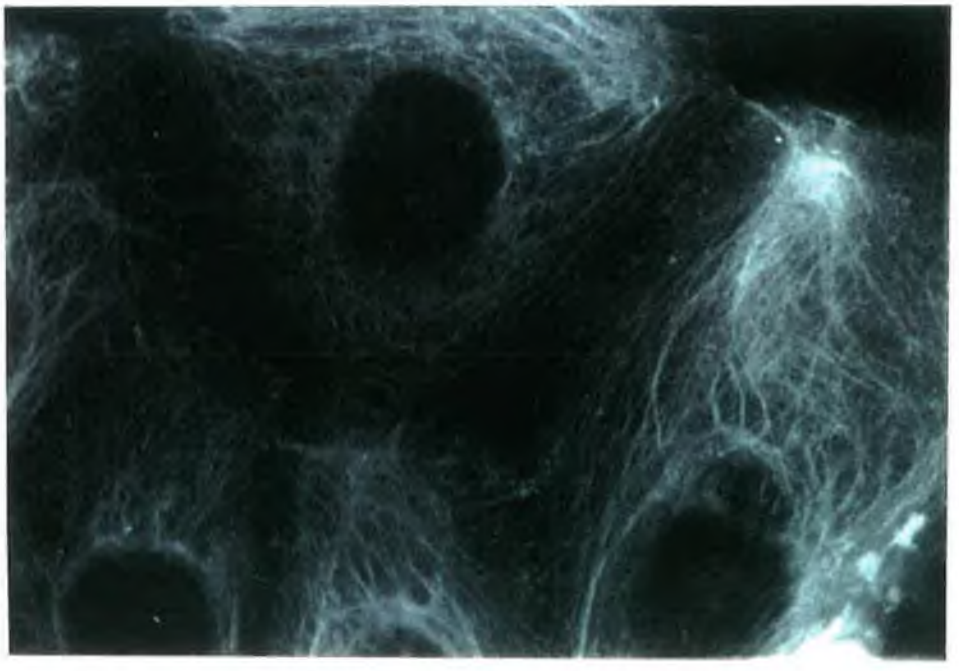


Figure 3.1.24 BAC stained for cytokeratin and viewed at a higher power. The cytoskeletal network is more clearly defined in this field. (Magnification 160X).

NSCLC-specific antibodies

Two murine monoclonal antibodies 703D4 and 704A1 raised against a human large cell lung cancer line (NCI-H157) were reported to bind to non-small cell lung carcinomas but not small cell carcinomas {Mulshine *et al.*, (1983)}. The epitopes recognised by these antibodies were also expressed on melanomas and on a osteogenic sarcoma and renal cell carcinoma. The antibodies did not recognise a variety of normal tissue types, or carcinomas of lymphoid, neuroblastoma, colon and breast origin. The staining pattern of both antibodies suggested that the epitopes were associated with cytoskeletal elements in squamous cell carcinomas.

In this work the staining patterns of the newly established NSCLC cell lines DLRP and DLKP and the breast carcinoma cell line BAC were compared to those of a human small cell carcinoma cell line, HTB 120, as well as a human embryonic lung fibroblast cell line, MRC-5. Two other NSCLC (not established in this laboratory), SK-MES-1 (a squamous cell carcinoma) and SK-LU-1 (an adenocarcinoma), were included as controls. All cell lines were maintained in culture, plated, fixed and stained as described in Section 2.6.1.

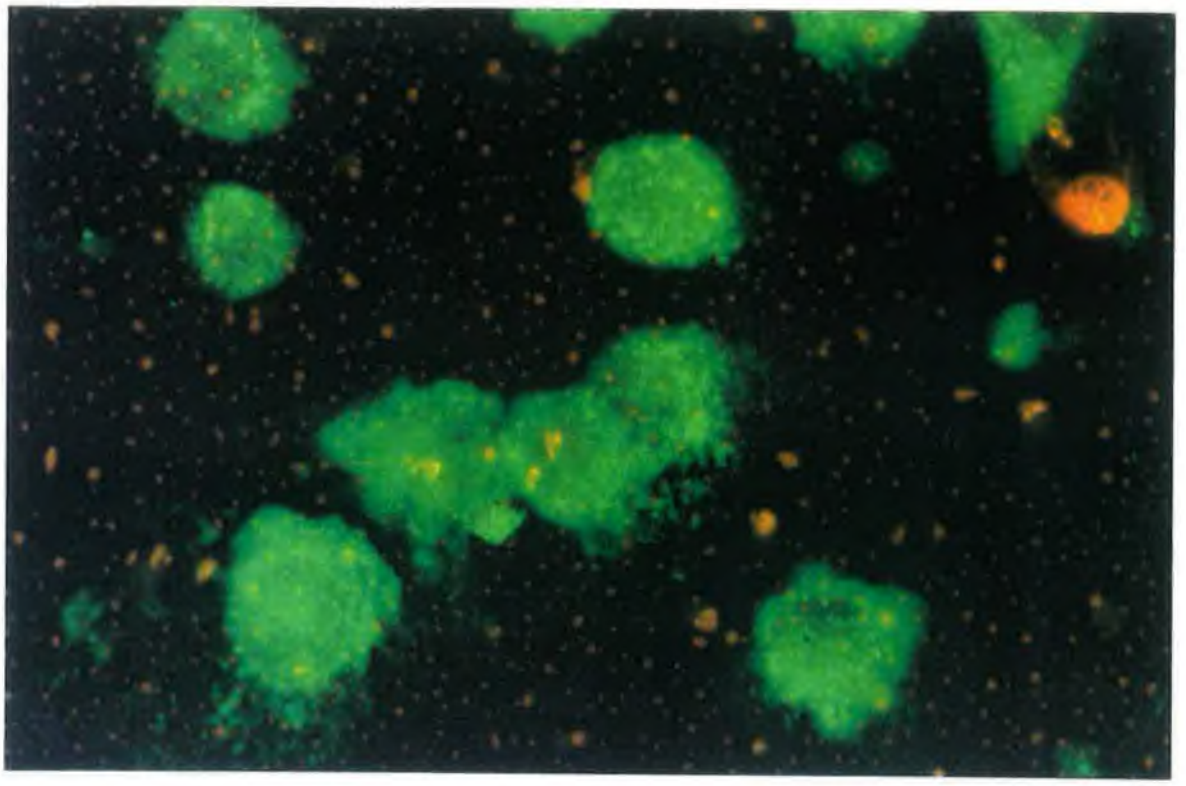


Figure 3.1.25 Positive staining of the NSCLC cell line SK-LU-1 with antibody 703D4. (Magnification 40X).

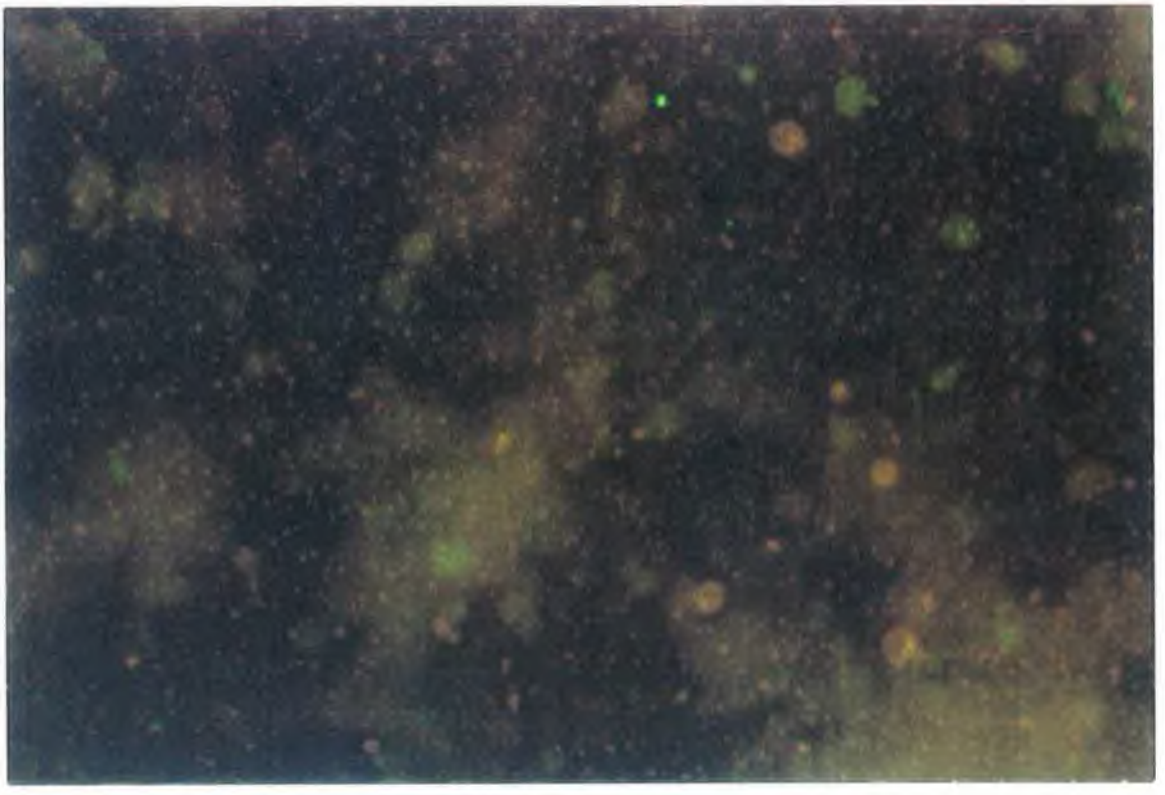


Figure 3.1.26 The SCLC cell line HTB 120 does not expresses the 703D4 NSCLC-specific antigen. (Magnification 40X).

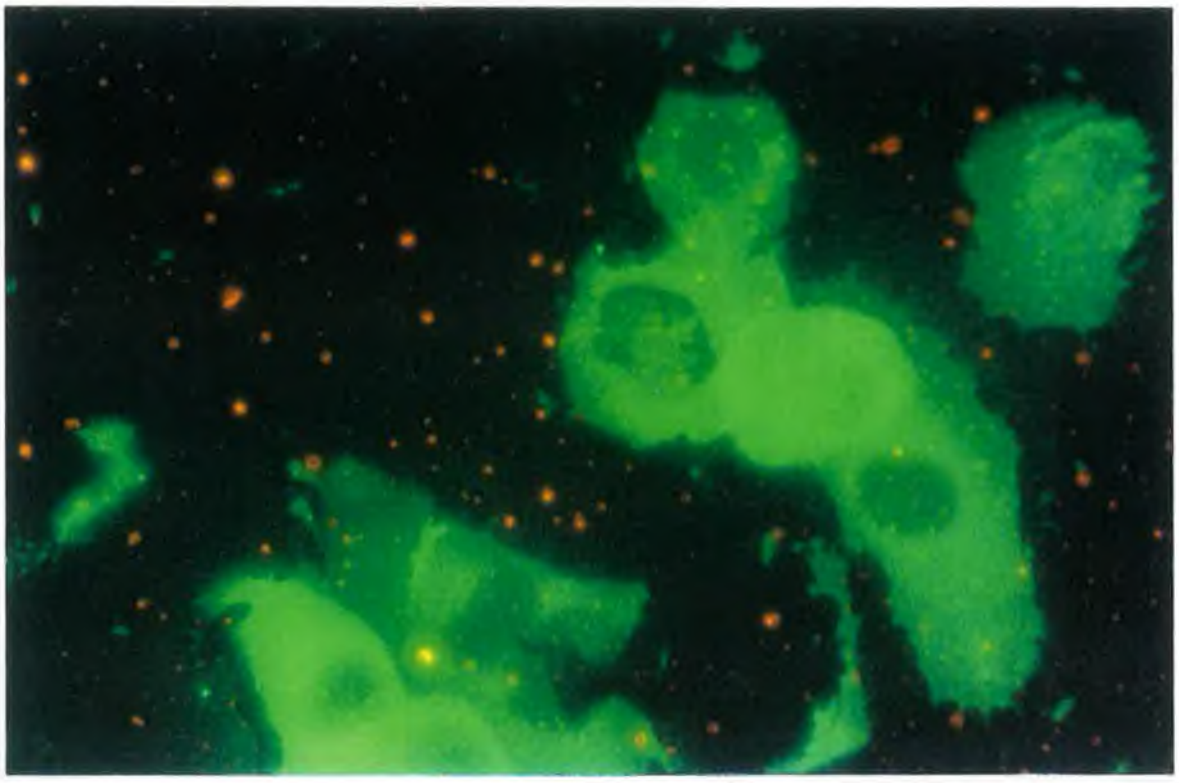


Figure 3.1.27 DLRP demonstrates a positive staining pattern with the 703D4 antibody. (Magnification 40X)

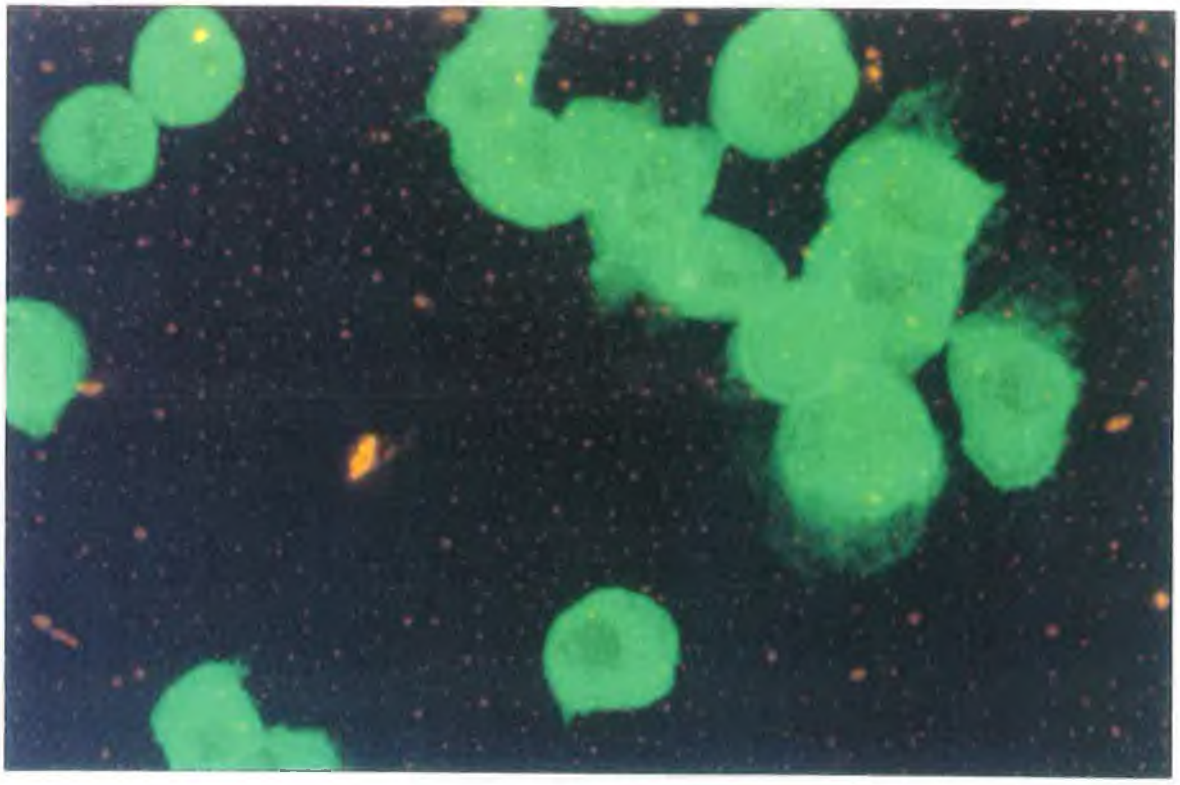


Figure 3.1.28 Positive staining pattern with 703D4 in the human lung squamous cell carcinoma line, SK-MES-1. (Magnification 40X).

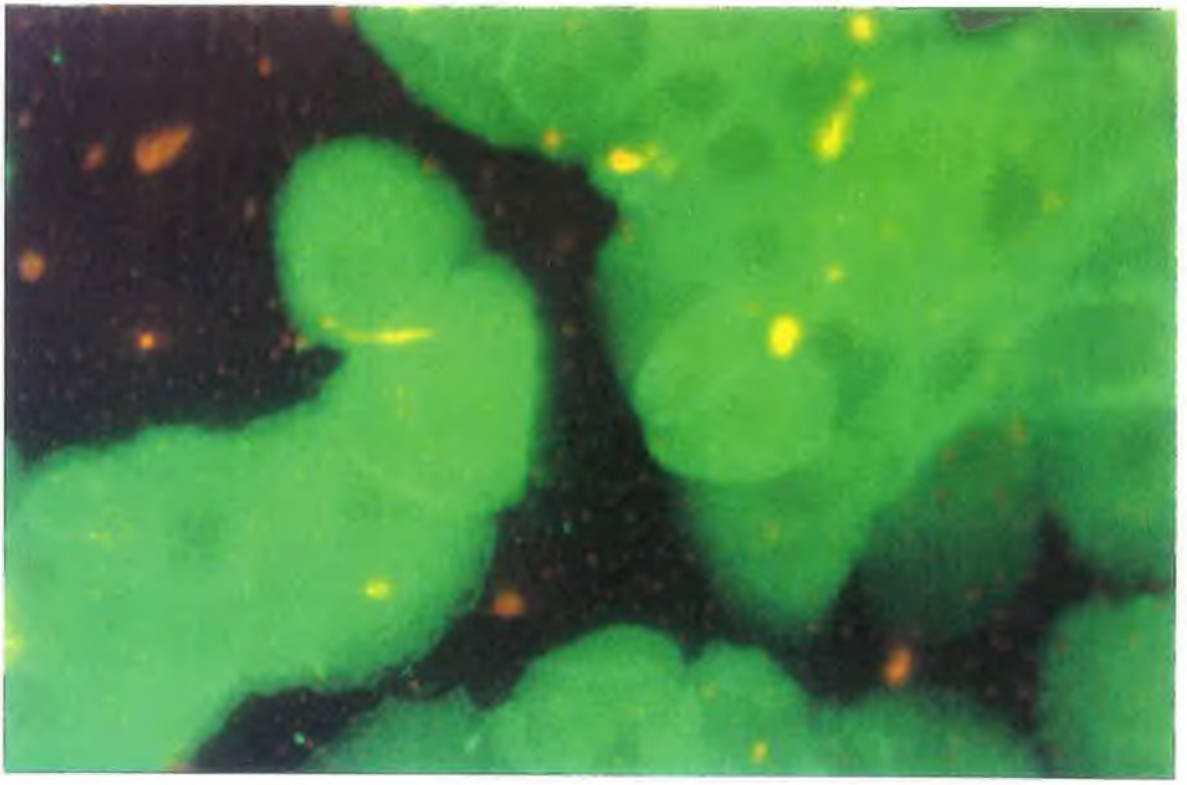


Figure 3.1.29 The human breast adenocarcinoma cell line, BAC, also demonstrates positive staining with the anti-NSCLC antibody 703D4. (Magnification 40X).

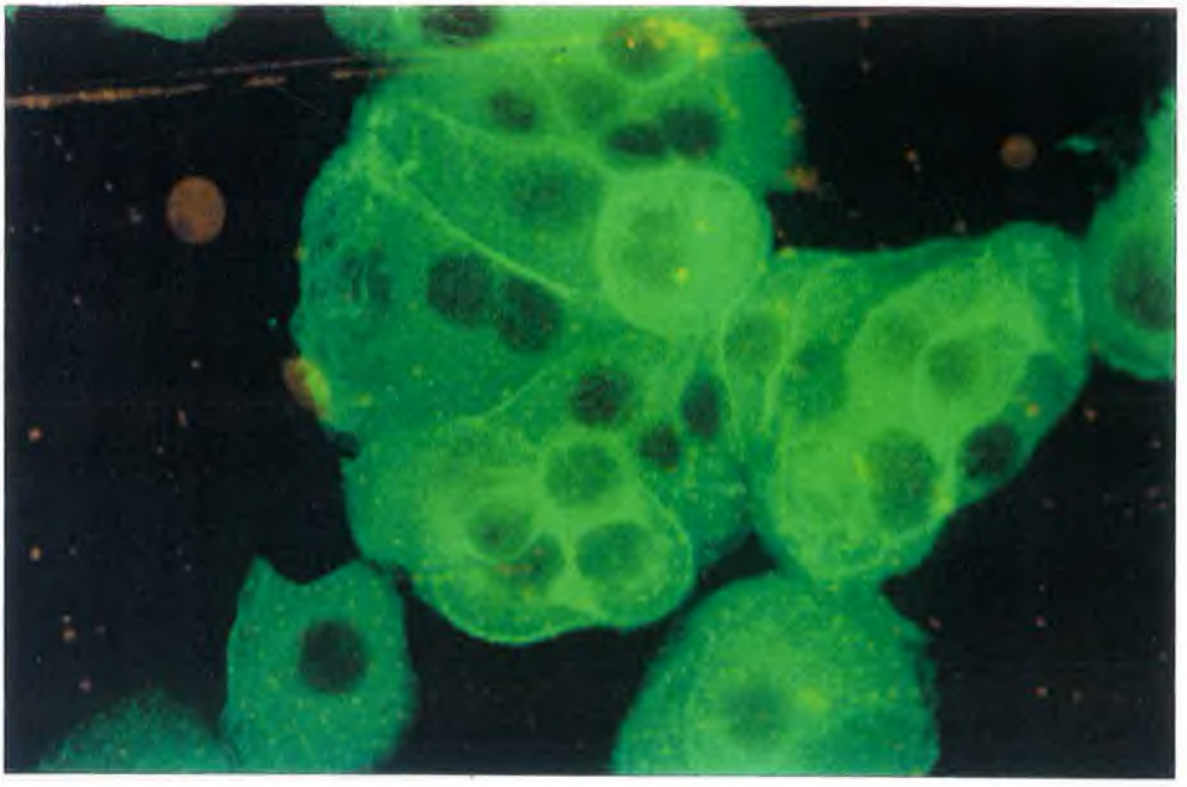


Figure 3.1.30 The second anti-NSCLC antibody, 704A1, also reacts with an antigen expressed on BAC. (Magnification, 40X).

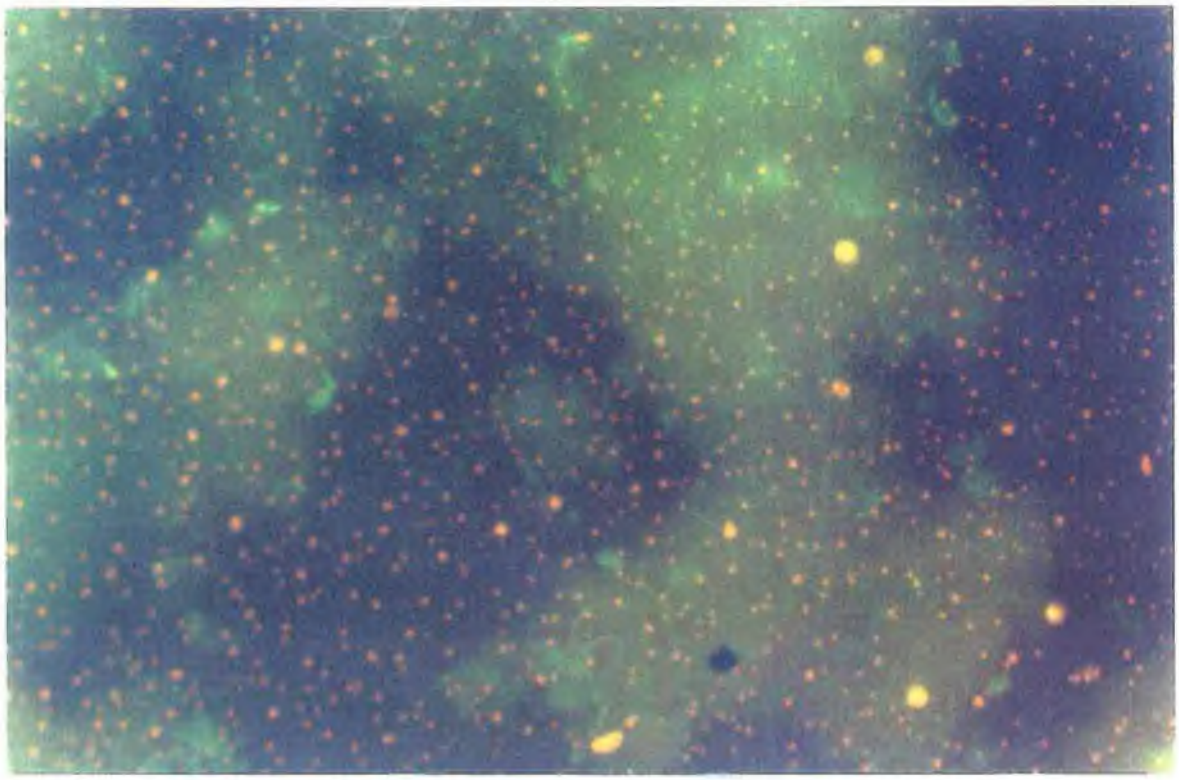


Figure 3.1.31 HTB 120 also demonstrates a negative staining pattern for 704A1. (Magnification, 40X).

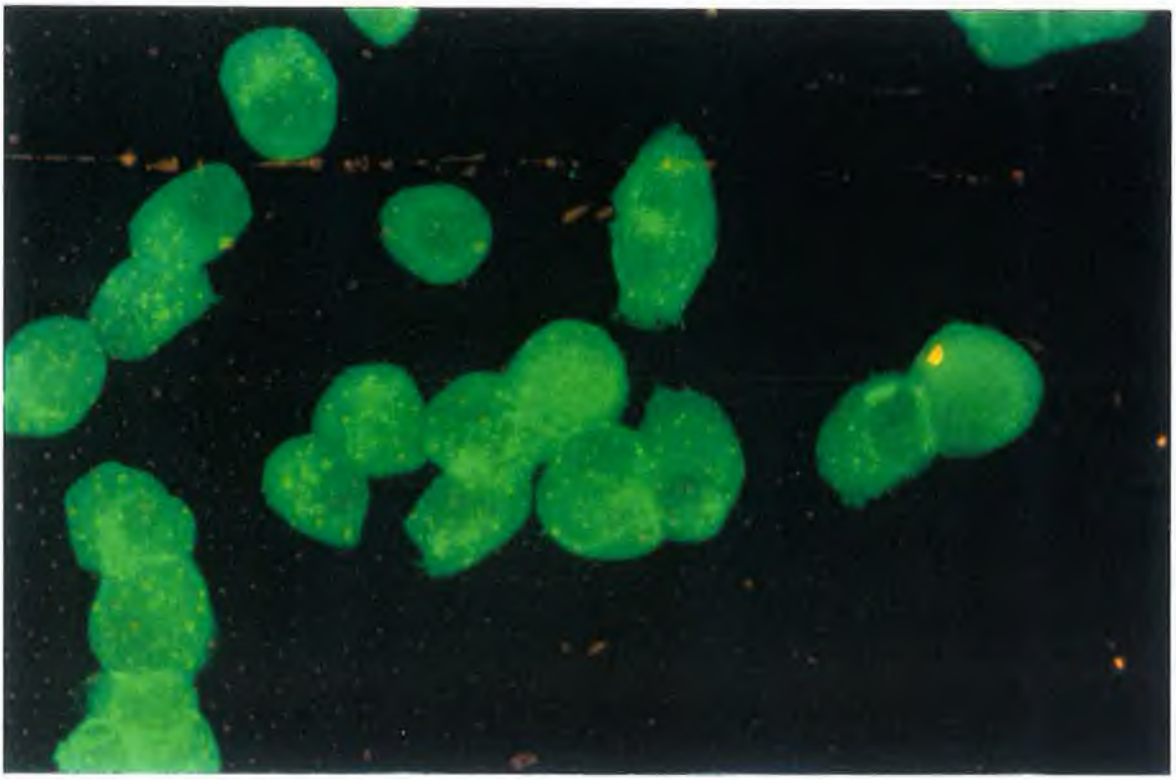


Figure 3.1.32 Positive staining pattern with 704A1 antibody in SK-MES-1 cells. (Magnification, 40X).

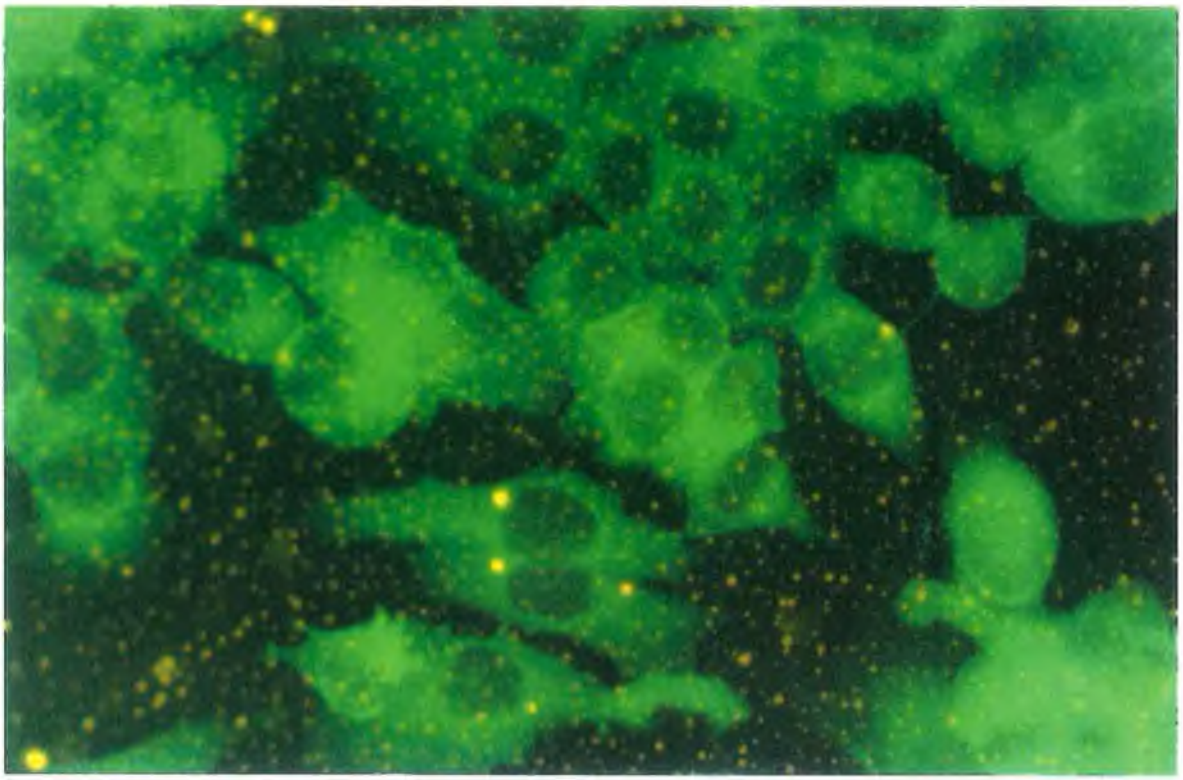


Figure 3.1.33 DLRP gives a positive staining pattern with the anti-NSCLC antibody, 704A1 (Magnification, 40X).

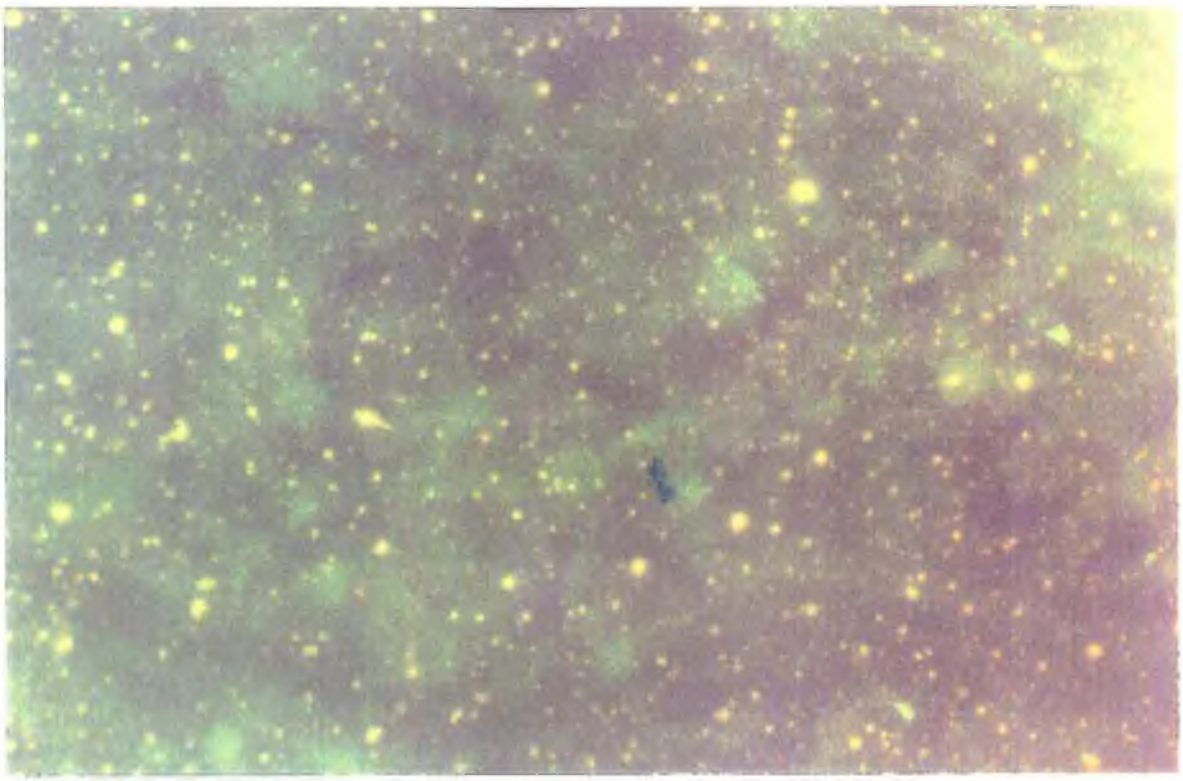


Figure 3.1.34 MRC-5 cells demonstrate negative staining for the 704A1 antibody (Magnification, 40X).

3.2 DEVELOPMENT OF A BIOASSAY FOR THE DETECTION OF AUTOCRINE GROWTH FACTORS PRODUCED BY THE HUMAN LARYNGEAL CARCINOMA CELL LINE HEP-2

A 1:1 volume/volume mixture of DMEM/Ham's F12 was used for routine maintenance of HEp-2 cell stocks. This formulation is widely used as a basal medium in the development of serum-free media and in the maintenance of many established cell lines {Maurer (1992)}. Ideally the growth medium used in an autocrine assay should be completely defined (in terms of salts, carbohydrate source, amino acids, vitamins, hormones, polypeptide growth modulators, attachment factors etc.), in order to discriminate the contribution made by cell-secreted factors to the overall growth control.

While it has been possible to develop serum-free media for a limited number of cell types, the fastidious growth requirements of animal cells *in vitro* usually necessitate the optimisation of such media to suit a particular cell type. An alternative approach is to design an assay where the minimum concentration of serum is used. The latter approach was taken in the development of the HEp-2 autocrine assay.

For animal cells grown *in vitro* in a particular concentration of serum, a minimum plating density exists. Plating densities less than this 'cut-off' point will not support cell growth. This value is dependent on other parameters in the system, such as basal medium and serum types, as well as growth surface to media volume ratios (for adherent cell types). In addition, for any given set of growth paramenters, the minimum plating density will ultimately depend on the particular cell type (cell line) under investigation.

3.2.1 Preliminary determination of cell plating density for HEp-2 in 24-well plates

Due to the reduced scale, a 24-well assay plate system was chosen initially for development with HEp-2. Reduced quantities of costly cell culture materials can therefore be used. In addition, the potential number of determinations which can practically be performed in a single assay is greater than with larger plate sizes.

Experience with the handling of HEp-2 cells suggested that the minimum plating density for this cell line was very low compared to more recently established cell lines. This was confirmed in a preliminary experiment shown in Table 3.2.1 which was designed to explore the minimum plating density for this cell line in the 24-well plate system. HEp-2 were plated in 5% serum down to a concentration of 1×10^2 cells/well.

Table 3.2.1 Determination of the minimum plating density for HEp-2 at 5% serum

Plating Density Cells/well	Colony ± S.E.M. Area mm^2 mm^2
5×10^3	26.51 ± 8.57
1×10^3	21.11 ± 6.85
5×10^2	6.55 ± 0.98
1×10^2	2.24 ± 1.17

n=4 for all determinations.

The results of this experiment shown in Table 3.2.1 indicated that plating densities as low as 1×10^2 cells/well could still give detectable levels of growth after a seven day incubation. The amount of growth was determined by measuring cell colony area (Section 2.12.4). The data in this experiment formed the basis for the choice of plating density used in Section 3.1.6.4.

The next experiment was performed to define the minimum plating densities at low concentrations of serum. HEp-2 were plated at two densities, 4×10^2 and 2×10^2 cells per well in four different batches of calf serum. Three concentrations of serum (1, 2, and 3%) were used in each case. The combination of low serum and plating densities was designed to determine the most stringent conditions that would still support HEp-2 cell growth in 1 ml of medium on the 24-well plate.

Cells were trypsinised and counted as described (Sections 2.2.4 and 2.2.5). Cell suspensions were diluted to 2x the desired final concentration per ml in serum-free DMEM. Test sera were prepared in Ham's F12 at 2x the desired final concentration plus 2x L-glutamine. 0.5 ml of the test sera (three foetal and one newborn calf serum) were plated in 0.5 ml of cell suspension on 24-well plates. Each determination was performed in quadruplicate. After incubating for 7 days at 37°C in an atmosphere of 5% CO_2 the plates were washed and stained with 0.25% crystal violet stain (Section 2.12.4.1) and the colony area of each well was measured by image analysis (Section 2.12.4.2). The mean and the standard error on each determination was calculated. The results are presented in Table 3.2.1.(i).

Table 3.2.1.(i) Determination of the minimum plating density for HEp-2 at low concentrations of newborn and foetal calf serum

Plating Density 2×10^2 Cells/well			
Percentage Serum	1%	2%	3%
Serum Type:Batch №	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
FCS:801017	0.29 ± 0.03	0.17 ± 0.06	0.12 ± 0.03
NCS:10F6182	0.29 ± 0.14	0.90 ± 0.16	1.89 ± 0.31
FCS:10F	0.80 ± 0.21	2.04 ± 0.33	1.85 ± 0.29
FCS:701113	0.56 ± 0.15	1.35 ± 0.30	1.60 ± 0.40
Plating density 4×10^2 Cells/well			
FCS:801017	0.66 ± 0.26	0.90 ± 0.17	1.27 ± 0.18
NCS:10F6182	0.96 ± 0.11	3.18 ± 0.17	5.80 ± 0.45
FCS:10F	2.00 ± 0.28	4.70 ± 0.22	5.00 ± 0.44
FCS:701113	2.31 ± 0.20	4.09 ± 0.76	5.26 ± 0.46

n=4 for all determinations. Abbreviations: FCS, foetal calf serum; NCS, newborn calf serum; S.E.M, standard error of the mean.

The results indicated that growth of HEp-2 was negligible with all batches of serum tested under the most stringent conditions used (1% serum and 2 × 10^2 cells per well). Given the inherent variability between replicate bioassays performed under identical conditions, such low readings could translate into zero growth in subsequent experiments.

The difference in growth supported by different batches of serum at the same concentrations and cell plating densities illustrated the importance of serum batch selection. Foetal calf serum (FCS) batch Nº 801017 was a poor stimulator of HEp-2 growth in these low serum concentrations at both plating densities consistent with previous experimental results (Section 3.1.6.4). Newborn calf serum (NCS) batch Nº 10F6182 was as good as the more expensive FCS batch Nºs 10F and 701113 at high concentrations, but a linear increase in growth in response to increasing serum concentration was obtained with NCS batch 10F6182 at both plating densities. Batches 10F (FCS) and 10F6182 (NCS) were chosen as candidate batches in a preliminary autocrine experiment with conditioned medium (CM).

3.2.1.1 Preliminary evidence for autocrine stimulation in the HEp-2 system

This experiment was designed to look for an autocrine stimulator(s) in medium conditioned by HEp-2. A number of parameters were examined. HEp-2 were assayed at two plating densities with two different batches of serum. Cell suspensions were prepared in DMEM as described (Section 2.12.4.2) to give 1 and 2% background levels at both plating densities. HEp-2 conditioned medium (CM2, i.e the second collection as described in Section 2.7.2) was assayed neat or diluted in serum-free Ham's F12. Non-conditioned Ham's F12 was included as a control. The assay was incubated as described in Section 3.2.1 above and the colony area measured by image analysis as before. The results are presented in Table 3.2.1.1a.

Table 3.2.1.1a Preliminary detection of autocrine stimulatory activity in HEp-2 conditioned medium

Plating Density 2 × 10^2 cells/well				
Serum Type	NCS:10F6182		FCS:10F	
Percentage Serum	1%	2%	1%	2%
SAMPLES	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
CM2 undiluted	0.55 ± 0.11	1.79 ± 0.35	0.76 ± 0.13	1.61 ± 0.18
CM2 diluted ½	0.52 ± 0.05	1.41 ± 0.29	0.33 ± 0.19	0.96 ± 0.13
Control	0.36 ± 0.18	0.68 ± 0.15	0.34 ± 0.05	0.92 ± 0.37
Plating Density 4 × 10^2 cells/well				
CM2 undiluted	1.82 ± 0.23	3.48 ± 0.41	2.36 ± 0.46	4.29 ± 0.48
CM2 diluted ½	1.37 ± 0.33	2.51 ± 0.47	0.90 ± 0.30	1.87 ± 0.36
Control	0.77 ± 0.02	2.05 ± 0.34	0.54 ± 0.20	2.29 ± 0.27

n=4 for all determinations. Abbreviations: CM2, 2nd Collection of HEp-2 conditioned medium.

Significant stimulation above the control level of growth was measured in the undiluted CM2 sample with all combinations of the assay parameters, with the exception of 2×10^2 cells/well in 1% NCS. When the CM2 was diluted ½, stimulation over control was detected only under two sets of conditions both incorporating the NCS batch, (1) at 2% NCS and 2×10^2 cells/well; and (2) at 1% NCS and 4×10^2 cells/well.

Because the background level of growth in the controls varied between the different assay conditions, the degree of stimulation in the undiluted CM2 samples were expressed as *fold stimulation over control* (i.e. sample mean ÷ control mean). This data is presented in Table 3.2.1.1b.

Table 3.2.1.1b Fold stimulation detected in HEp-2 CM under different assay conditions

PLATING DENSITY	2×10^2 cells/well		4×10^2 cells/well	
PERCENTAGE SERUM	1%	2%	1%	2%
SERUM TYPE	FOLD STIMULATION WITH RESPECT TO CONTROL			
NCS:10F6182	N.S.	2.63	2.36	1.70
FCS:10F	2.24	1.75	4.37	1.87

n=4 for all determinations. N.S; Not significant allowing for standard errors.

Analysis of the above data indicate that although autocrine stimulation can be detected in the HEp-2 CM2 under all but one set of assay parameters tested. When the degree of stimulation is expressed as ***fold stimulation with respect (w.r.t.) to the control*** (for the sake of comparison) it becomes apparent that the magnitude of stimulation measured w.r.t. the control is greater for some combinations of serum and cell plating density. Increasing the plating density in 1% serum from 2×10^2 to 4×10^2 cells/well resulted in an increase in the fold stimulation detected. At 2% serum, such an increase in sensitivity could only be achieved by reducing the cell plating density in medium (substituted with 10F6182-NCS). Due to the technical limitations associated with the measurement of low densities of HEp-2 in some circumstances, a combination of 4×10^2 cells/well with 1% serum (10F6182) were the preferred parameters for detecting autocrine activity in subsequent experiments. The degree of stimulation is greater (with respect to the control) at the lower serum concentration. Reduction of the plating density appeared to reduce the fold stimulation detected, except for cells plated in 2% NCS.

In subsequent HEp-2 autocrine assays in the 24-well system, a plating density of 4×10^2 cells/well was used in medium with a final serum concentration of 1% (v/v).

3.2.1.2 Endpoint measurement in the HEp-2 autocrine assay - comparison of colony area and colony number

The following experiment was designed to determine the range of stimulation that might be detected with a plating density of 4×10^2 cells/well and to compare the linearity of the proliferative response as measured by colony area and colony number.

HEp-2 proliferation was stimulated with increasing concentrations of serum in the range 1-20% (v/v) under the assay conditions outlined above (Section 3.2.1). Three different serum batches and one serum substitute (which contained serum at 0.25% v/v) were used. The plates were stained after seven days and the colony area and colony number determined by image analysis. The mean and standard errors were calculated and colony area and colony number were plotted against percentage serum in figure 3.2.1.2.

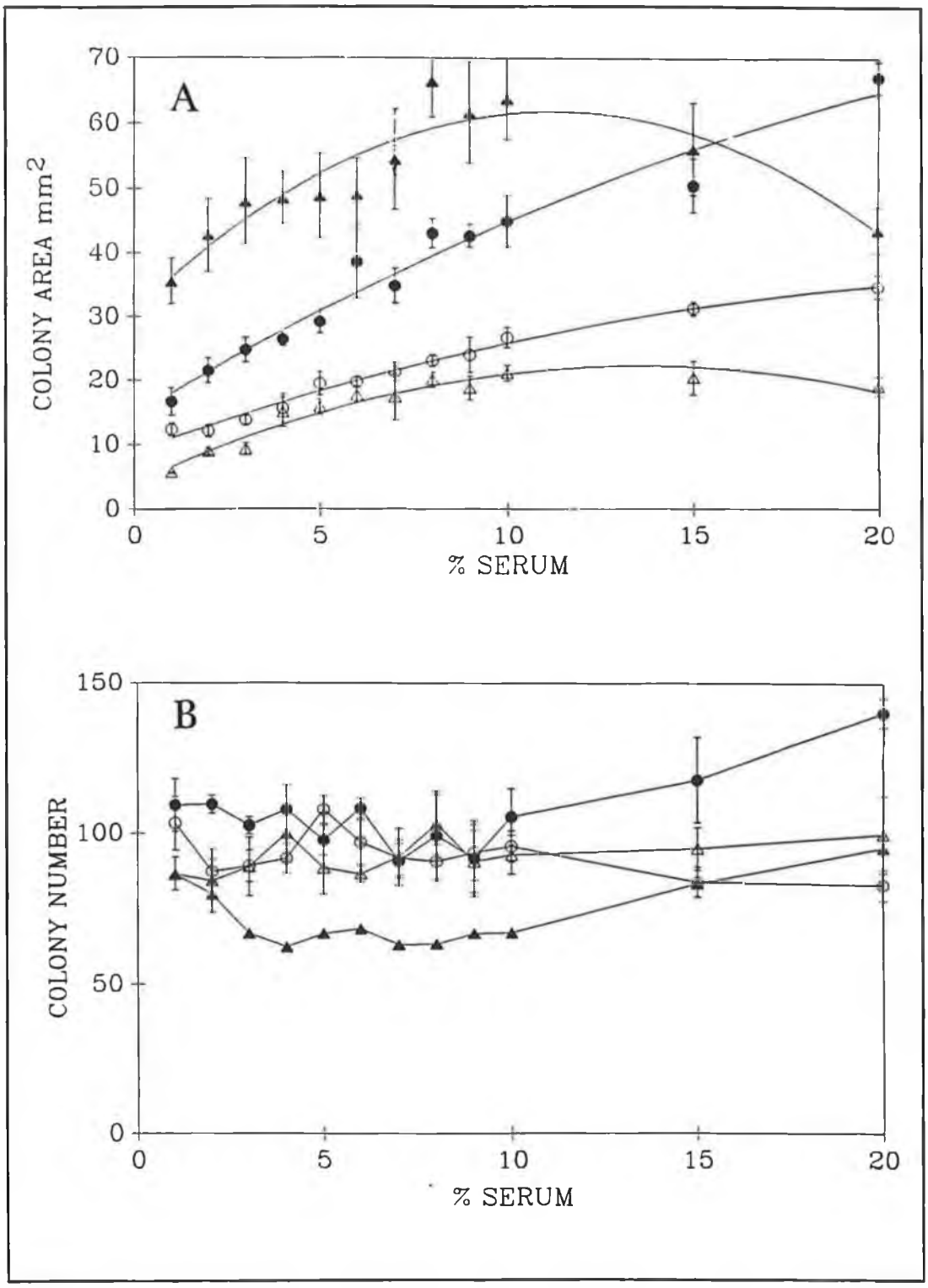


Figure 3.2.1.2 The growth of HEp-2 in monolayer in response to increasing concentrations of three bovine sera and a serum substitute: growth response measured by colony area, A; and colony number, B. (▲—▲) FCS 701051; (Δ—Δ) Serum substitute NuSerum IV; (●—●) FCS 701113; (○—○) NCS 10F6182.

The plot of colony area versus percentage serum concentration (figure 3.2.1.2,A) clearly demonstrates a dose response relationship between increasing serum concentration and HEp-2 growth. Using colony area as an endpoint for quantification also distinguishes batches of serum which are 'good' stimulators of HEp-2 growth from poorer ones. In addition, the response to three of the samples (NCS-10F6182, FCS-701113, and the serum substitute NuSerumIV) tested, demonstrated good linearity up to 10%.

Colony number measured on the same plates did not demonstrate any dose response to serum stimulation over the concentration range tested (figure 3.2.1.2,B). Colony number was therefore not considered a useful parameter for quantifying cell growth. Colony area was used instead for all subsequent experiments using the 24-well plate system.

3.2.1.3 Choice of basal medium type for the HEp-2 autocrine assay

Ham's F12 was the initial choice for use in the collection of conditioned medium (CM) from HEp-2 for a number of reasons. This medium contains a rich nutrient mixture compared to other formulations (e.g.DMEM) and it was used in combination with DMEM for routine maintenance of cell stocks.

The following experiment was devised to investigate the effect of other basal media combinations in the HEp-2 autocrine assay. Briefly, cells were plated under HEp-2 autocrine assay conditions (Section 2.12.4.2) in DMEM and then one of four conditioned media prepared from different basal media (in 75 cm^2 flasks, see Section 2.7.1) was tested for stimulatory activity. Two collections of CM prepared after 48 hours and 72 hours respectively in DMEM, Ham's F12, MEM, and RPMI-1650 respectively were assayed. The results of this experiment are presented in Table 3.2.1.3. Fold stimulation was calculated with respect to the relevant medium control (as in Section 3.2.1.1 above).

Table 3.2.1.3 Comparison of fold stimulation over control detected in different basal media conditioned by HEp-2

SAMPLE	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation with respect to the control
MEM CM1	13.25 ± 0.64	2.17
MEM CM2	13.74 ± 2.04	2.25
MEM Control	**6.10 ± 3.05**	-
DMEM CM1	16.76 ± 1.69	2.71
DMEM CM2	17.98 ± 2.59	2.90
DMEM Control	**6.19 ± 0.41**	-
RPMI-1640 CM1	14.37 ± 2.44	1.95
RPMI-1640 CM2	14.32 ± 0.66	1.94
RPMI-1640 Control	**7.37 ± 0.87**	-
Ham's F12 CM1	17.97 ± 0.91	2.98
Ham's F12 CM2	18.35 ± 1.30	3.05
Ham's F12 Control	**6.02 ± 0.24**	-

n=3 for all determinations.

The results indicated that the highest apparent fold stimulation was detected in conditioned Ham's F12. Although the level of stimulation was only slightly lower in DMEM, collection of CM using Ham's F12 was continued because this conditioned medium displayed neligible amounts of protease activity when the four different CMs were assayed for a battery of serine proteinases (see Appendix D for table of activities) in collaboration with Rhona O'Leary {O'Leary *et al.*, (1991),}. In that study high protease activity correlated with a high calcium concentration in the basal medium. Ham's F12 contains the lowest calcium concentration of the

four media assayed.

3.2.1.4 Growth of HEp-2 cells during conditioned medium production

During the collection of CM in 75 cm^2 flasks it was observed that HEp-2 continued to proliferate to confluency after transfer to serum-free medium. Thus cells which were semiconfluent (i.e. 70-80% confluent) at the start of CM collection reached confluency after five days in serum-free medium. This was true for all the basal media used. The survival of viable cells in serum-free medium was pertinent to the question of CM collection.

The next experiment was designed to monitor the proliferation and viability of HEp-2 in serum-free medium with respect to time. The effect of regular medium change was examined and the pH was measured at the time of collection. Serum-free MEM was used as the basal medium for CM collection (supplemented with 2 mM L-glutamine).

On day 1, twelve 75 cm^2 flasks were each seeded with 1.36×10^6 cells in DMEM/Ham's F12 supplemented with 5% FCS and 2 mM L-glutamine. The following day, one flask was trypsinised and cell number counted by haemcytometer (Sections 2.2.4 & 2.2.5 respectively). On day 5 the flasks had reached 70-80% confluency (as judged by eye) and another flask was harvested and the total cell number determined. All the remaining flasks were washed with sterile phosphate buffered saline (PBS) and 20 mls of serum-free MEM added for CM collection per flask (Section 2.7.1). In six of these flasks the medium was changed every day for six days. Each day one of the flasks was trypsinised (after CM collection) and the total cell number and the percentage viability determined by trypan blue dye exclusion (Section 2.2.5). Longer incubation periods for CM collection (i.e. without medium change) were examined in the three remaining flasks and cell number and viability determined as before.

Table 3.2.1.4 contains the results of the measurements in this experiment. In figure 3.2.1.4, total cell number per flask is plotted versus the number of days in culture.

Table 3.2.1.4 Proliferation and percentage viability of HEp-2 cultures in serum-free medium during the collection of conditioned medium

CULTURES IN GROWTH MEDIUM				
DAYS IN CULTURE	CELL Nº Per Flask	% VIABILITY	CM pH	Incubation Time for CM
0	1.36×10^6	N.D.	N.D.	-
2	2.00×10^6	N.D.	N.D.	-
5	8.08×10^6	N.D.	N.D.	-
CULTURES IN SERUM-FREE MEDIUM CHANGED DAILY				
6	1.60×10^7	98.40	7.50	1
7	2.23×10^7	95.85	7.46	1
8	2.64×10^7	95.93	7.50	1
9	3.57×10^7	98.50	7.42	1
10	4.15×10^7	95.18	7.20	1
11	4.28×10^7	92.50	7.22	1
12	3.42×10^7	95.03	7.30	1
CULTURES IN SERUM-FREE MEDIUM NOT CHANGED DAILY				
6	2.01×10^7	94.00	7.38	2
7	2.31×10^7	93.00	7.31	3
8	2.27×10^7	94.50	7.31	4

n=3 for all determinations. N.D; Not determined.

These results indicate that HEp-2 cells could be maintained in exponential growth if they are refed every 24 hours with fresh serum-free medium. Under these conditions cultures of HEp-2 continued to proliferate for up to six days after transfer into serum-free medium while maintaning a relatively high percentage viability at 95.5% on average. If the medium was not changed, these cultures ceased to divide after three days, but the viability was only slightly lower at 94%. This data would indicate that HEp-2 are capable of sustained cell proliferation in the absence of serum stimulation, suggesting that they produce self-stimulating growth factors. The inclusion of 20 mM HEPES buffer ensured that the pH of the CM did not fall below 7.2, even after four days incubation with medium change.

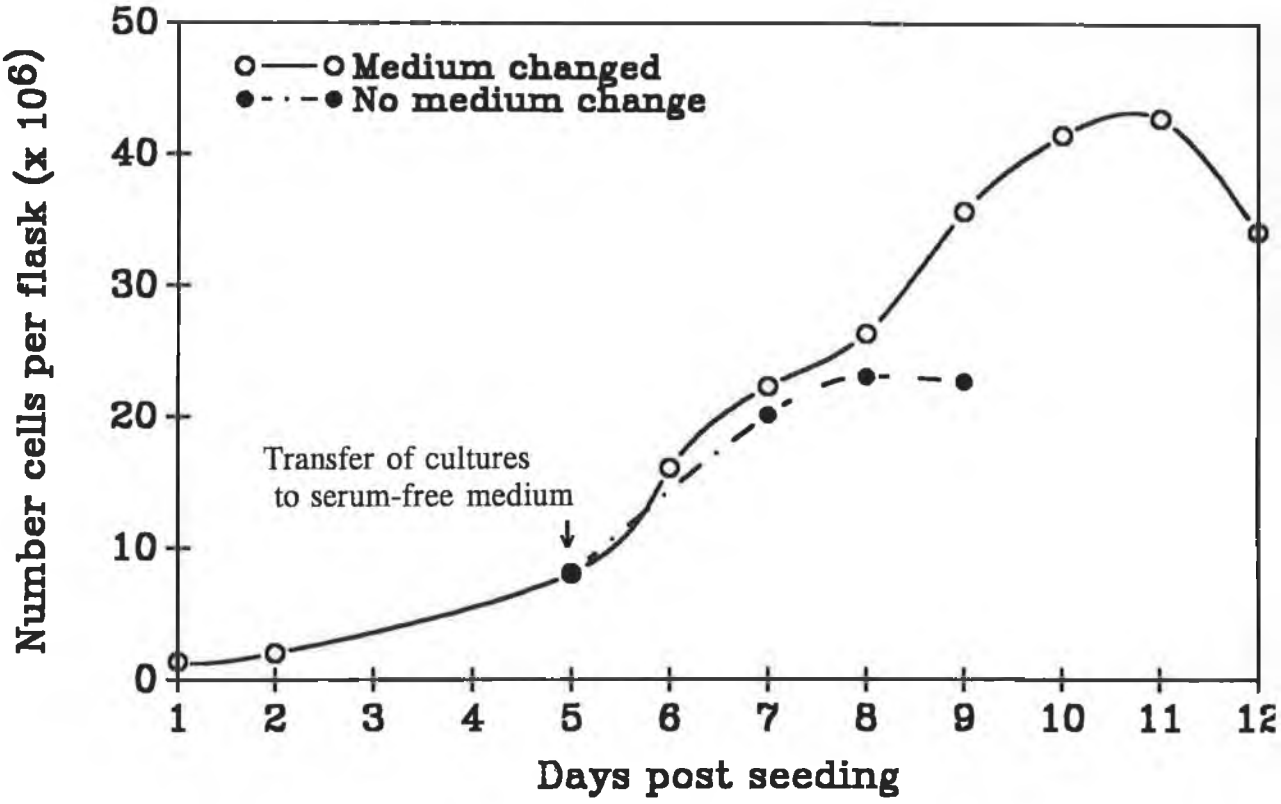


Figure 3.2.1.4. The growth of Hep-2 cells in serum-free MEM following 5 days growth in 5% serum-supplemented DMEM/Ham's F12 (1:1).

3.2.1.5 Reproducibility of the autocrine effect in HEp-2 CM

In order to determine how reproducible the autocrine effect was, a series of lots of HEp-2 CM were prepared (in Ham'sF12) over a two month period and assayed for autocrine activity. The results are presented in Table 3.2.1.5.

Table 3.2.1.5 Autocrine stimulatory activity in a several lots of HEp-2 CM

CM Batch	Colony ± S.E.M Area mm^2 mm^2	Fold Stimulation over experiment control mean
A	16.41 ± 1.20	2.70
B	17.39 ± 1.98	2.86
C	20.94 ± 2.58	3.45
D	13.72 ± 2.42	2.26
E	14.37 ± 1.17	2.37
F	12.28 ± 0.97	2.02
G	16.70 ± 1.16	2.75
H	26.44 ± 3.08	4.36
I	19.86 ± 0.63	3.27
J	23.27 ± 0.83	3.83
K	15.27 ± 1.80	2.52
L	15.31 ± 1.98	2.52
M	16.02 ± 2.76	2.64
N	21.92 ± 1.41	3.61
O	22.62 ± 2.14	3.73
P	17.62 ± 2.35	2.90
Q	17.68 ± 1.46	2.91
Ham's F12	6.07 ± 0.62	-

The autocrine effect was measure in 17 separate lots of the unconcentrated (crude) CM assayed in this experiment. An average 2.98 fold increase in growth over control was demonstrated for all the CM lots assayed. There was no correlation between the degree of stimulation and the time in storage before assay (maximum time in storage was 2 months). Similarly, differences in the level of autocrine activity observed could not be attributed to the collection system used (e.g roller bottle, tissue culture flask, cell factory, or spinner culture).

3.2.2 USE OF ACID PHOSPHATASE AS AN ENDPOINT FOR THE MEASUREMENT OF AUTOCRINE GROWTH STIMULATION IN HEp-2 CELLS

Measurement of lysosomal acid phosphatase activity has been used as an indicator of cell number {Connolly *et al.*, (1986)}. This technique has been applied to the quantification of cell number in this laboratory in a 96-well plate system {Martin & Clynes (1991)} and the method also demonstrated good correlation with viable cell number (determined by colony forming efficiency) {Martin & Clynes (1993)}.

The advantages of the 96-well system over the 24-well assay are firstly one of scale. The maximum volume used per well on the 96-well plate is 0.2 ml compared to the 1 ml used in the 24-well plate HEp-2 assay. Therefore smaller quantities of materials are required for experimentation, an important consideration where limiting quantities of high cost materials are used. The number of replicates and/or samples which can practically be handled in a typical 96-well assay experiment is also greater and the assay can be semi-automated. Another reason for using acid phosphatase was to provide an additional verification of the autocrine effect observed in the 24-well plate assay. In this case, a qualitatively different endpoint was used to measure cell proliferation.

In order to apply this method to the measurement of the HEp-2 autocrine activity, it was first necessary to define the optimum assay parameters for detecting HEp-2 autocrine stimulatory activity in the 96-well plate. To this end, the parameters used for the 24-well system were scaled down to a final volume of 0.2 ml. All the other procedures were the same as for the 24-well assay except that the cell densities were adjusted.

The following experiment measured the growth of HEp-2 plated over a range of plating densities from 2×10^2 to 7×10^2 cells per well. Two lots of HEp-2 CM, shown to be active in the 24-well autocrine assay, were added to the same range of cell concentrations up to 6×10^2 cells/well in separate experiments. Acid phosphatase activity was measured after a seven day incubation period as described in Section 2.12.5. The results of this experiment are shown in Table 3.2.2.

Table 3.2.2 Detection of autocrine stimulation in Hep-2 using Acid Phosphatase as an endpoint for growth measurement

CELL N° PER WELL	CONTROL MEDIUM ABS ± S.D.[a]	CONDITIONED MEDIUM ABS ± S.D.[a]	Fold Stimulation w.r.t[b] the Control
Experiment 1		**Lot 3 Unconcentrated CM**	
2×10^2	0.084 ± 0.016	0.193 ± 0.028	**2.29**
3×10^2	0.088 ± 0.016	0.238 ± 0.046	**2.70**
4×10^2	0.198 ± 0.016	0.368 ± 0.155	**1.86**
5×10^2	0.214 ± 0.025	0.449 ± 0.088	**2.10**
6×10^2	0.275 ± 0.117	0.443 ± 0.274	**1.61**
7×10^2	0.336 ± 0.034	N.D.[c]	
Experiment 2		**Lot 9 Unconcentrated CM**	
2×10^2	0.088 ± 0.006	0.192 ± 0.083	**2.18**
3×10^2	0.141 ± 0.013	0.313 ± 0.099	**2.22**
4×10^2	0.230 ± 0.021	0.450 ± 0.048	**1.96**
5×10^2	0.291 ± 0.024	0.547 ± 0.050	**1.88**
6×10^2	0.346 ± 0.042	0.711 ± 0.066	**2.05**
7×10^2	0.426 ± 0.015	N.D.	N.D.

n=8 for all determinations. [a] Absorbance ± standard deviation measured at 405 nm. [b] with respect to. N.D; not determined.

These results demonstrated that an autocrine effect could be measured over the range of plating densities assayed here. The fold stimulation over the medium control was in the same order at every plating density examined (two-fold on average). A plot of initial plating density versus absorbance at 405 nm for control and CM-stimulated growth (i.e. acid phosphatase activity measured after 7 days) was linear, as seen in figure 3.2.2.

The application of acid phosphatase as a method of endpoint determination was employed in the latter part of this project for experiments which required the use of expensive components (e.g. the oligodeoxynucleotides).

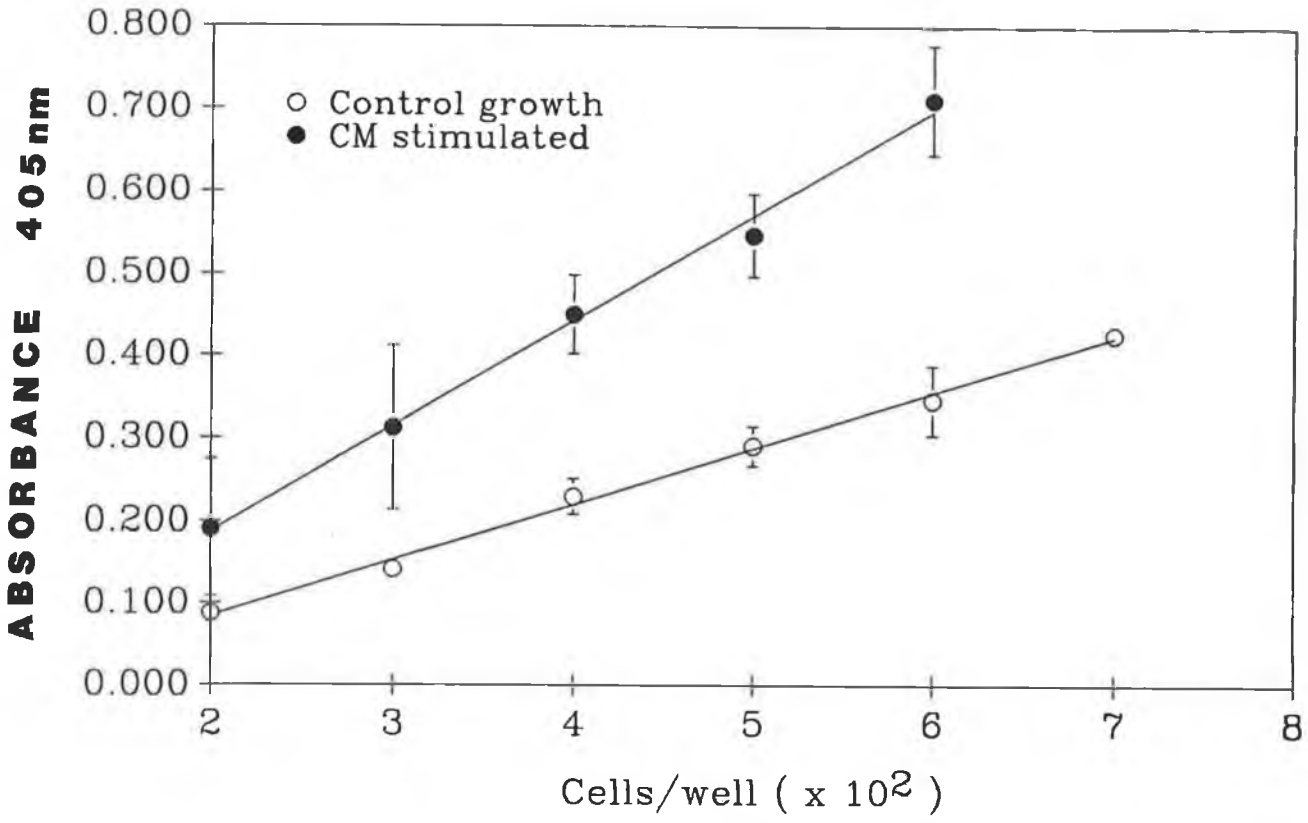


Figure 3.2.2 **A plot of HEp-2 cell inoculum plating densities in 96-well plates versus acid phosphatase activity measured after seven days in CM-stimulated and unstimulated wells.**

3.2.3 ULTRAFILTRATION OF HEp-2 CONDITIONED MEDIUM

3.2.3.1 Concentration of conditioned medium by ultrafiltration

The nature of the HEp-2 autocrine effect was investigated further by attempting to concentrate the mitogenic species in CM using ultrafiltration. The aim of this experiment was to rule out the involvement of small molecules such as the metabolic waste products lactate and ammonia which are toxic to animal cells *in vitro* (i.e. < 1000 daltons which are removed in the ultrafiltration process) and to look for a concentration dependance with autocrine activity. Two lots of HEp-2 CM were combined 1:1 (v/v) and concentrated 10x with a 1000 dalton MW cut-off membrane (described in Section 2.8.1). Separate aliquots of the resulting concentrate {R1(10x)} were dialysed in dialysis tubing (Section 2.8.2) with respective MW cut-off points at 1,200 daltons and 10,000 daltons (aproximately). Dialysis was performed after ultrafiltration to ensure the complete removal of small molecules not removed by ultrafiltration. The ultrafiltration filtrate (F1) was also retained for assay. Samples were filter-sterilised (Section 2.1.3) and then assayed. The results are shown in Table 3.2.3.1.

Table 3.2.3.1 Autocrine activity in HEp-2 CM concentrated by ultrafiltration

Sample	Dilution	Colony ± S.E.M. Area mm^2 mm^2	Sample	Dilution	Colony ± S.E.M. Area mm^2 mm^2
CM Lot 1	None	10.87 ± 0.54	CM Lot 2	None	11.41 ± 0.40
	1/2	8.77 ± 0.77		1/2	6.99 ± 0.39
	1/4	8.16 ± 0.70		1/4	4.05 (n=1)
	1/8	4.93 ± 0.47		1/8	2.96 ± 0.77
R1 (10x) Not Dialysed	None	1.13 ± 0.56	Filtrate (R1)	None	2.82 ± 0.25
	1/2	2.14 ± 0.36		1/2	2.18 ± 0.23
	1/4	3.02 ± 0.45		1/4	1.23 ± 0.44
	1/8	0.13 ± 0.08		1/8	2.10 ± 0.19
R1 (10x) Dialysed (1200 MW)	None	0.00 ± 0.00	R1 (10x) Dialysed (12000 MW)	None	0.01 ± 0.01
	1/2	0.29 ± 0.13		1/2	0.70 ± 0.34
	1/4	5.89 ± 0.41		1/4	4.45 ± 0.92
	1/8	6.15 ± 0.93		1/8	4.73 ± 0.61
Experiment Control Mean (Ham's F12) **2.25 ± 0.26**					

n=4 for all determinations.

The results of this experiment indicated that there was autocrine stimulatory activity in both lots of CM before they were combined and concentrated. However, the undiluted 10x concentrate of CM (R1) assayed without dialysis was inhibitory (i.e. resulting in growth below the Ham's F12 control value). Further dilution of this sample brought the level of growth back to that in

the control range (at dilutions of ¾, and ½) but it did not restore the autocrine effect. The almost negligible level of growth at the ¼ dilution was not consistent with this trend and may be a consequence of some other factor such as a plating error (see Section 3.2.3.2). Growth in the filtrate sample was in the same order as that of the control.

Dialysis of the R1(10x) concentrate (retentate) with either of the dialysis tubings increased the apparent inhibition observed in the undiluted sample. However, subsequent dilution of these dialysed retentates resulted in the restoration of the autocrine stimulatory activity. It was necessary to dilute both of the dialysates by at least ¼ to remove the inhibition. The restoration of stimulatory activity was maximal at higher dilution (1/8 was the highest dilution assayed). More stimulatory activity appeared to be restored in the samples dialysed with low MW cut-off tubing (comparing the mean values at the 1/8 dilution) but this difference was not significant when standard errors are taken into account.

It was concluded from these results that the autocrine stimulatory species was retained by an ultrafiltration membrane with a MW cut-off of 1 kDa. Concentration with this membrane resulted in apparent inhibition (when the concentrate was assayed without dilution), suggesting the concentration of an inhibitor. This inhibitory species was not removed by dialysis, but after dilution of the dialysed retentate by a factor of ¼ it was demonstrated that autocrine stimulatory activity was still detectable after these treatments.

The origin of the concentratable inhibitory species was unclear. It may have been present in the CM to begin with, but the possibility remained that it resulted from the ultrafiltration and/or the dialysis steps employed. In order to better understand the composition of HEp-2 CM, it was fractionated further by ultrafiltration and the respective fractions were assayed in the following experiment.

3.2.3.2 Size fractionation of HEp-2 conditioned medium using ultrafiltration

Ten fold concentrations of HEp-2 CM were prepared by ultrafiltration (described in Section 2.8.1.1). By using membranes with progressively lower nominal molecular weight cut-offs it was possible to generate CM retentates that concentrated molecules within defined (approximately) molecular weight ranges.

A single lot of CM (Lot A) was used to produce concentrated fractions (retentates) with molecules of molecular weights between 1 and 5 kDa, 5 and 10 kDa and 10 and 30 kDa (designated R1-5, R5-10 & R10-30 respectively). In addition, concentrates with all molecules above the cut-off points 1, 5, 10, and 30 kDa (designated R1, R5, R10 & R30 respectively) were also prepared. F1(F5) is the filtrate from the passage of CM through a 5 kDa cut-off membrane followed by filtration through a 1 kDa membrane. Similarily F5(F10) and F10(F30) represent double filtrates.

The retention of autocrine activity after dialysis (Section 3.2.3.1 above) in tubing with a cut-off of 12 kDa suggested that autocrine activity should be retained by the 10 kDa ultrafiltration membrane. Therefore, an additional R10-30 concentrate was made with a separate lot of Hep-2 CM (Lot B) and included as a control in this experiment together with three other unconcentrated (crude) lots of HEp-2 CM (Lots C, D & E).

The activity of these samples was measured in the 24-well image analysis assay as described in Section 2.12.4.2. In this experiment a medium control on each plate was included to check for

inter-plate variation in growth (arising from plating errors). The results are presented in Table 3.2.3.2(i).

Table 3.2.3.2(i) Autocrine activity of HEp-2 CM fractionated on the basis of molecular size using ultrafiltration

Conditioned Medium Lot A 10x Retentates	Colony ± S.E.M. Area mm² mm²	Fold Stimulation w.r.t Experiment Control Mean[a]	Fold Stimulation w.r.t Plate Control Mean[b]
Unconcentrated	10.41 ± 1.03	3.29	3.23
R1	0.02 ± 0.01	0.01	0.01
R1-5	0.65 ± 0.17	0.21	0.26
R5	0.01 ± 0.02	0.00	0.00
R5-10	4.06 ± 0.72	1.28	1.61
R10	1.19 ± 1.04	0.38	0.47
R10-30	14.11 ± 1.63	4.46	3.15[c]
R30	0.07 ± 0.06	3.29	3.23
Lot A Filtrates			
F1(F5)	5.42 ± 1.81	1.72	1.20[c]
F5(F10)	5.23 ± 0.90	1.66	1.17[c]
F10(F30)	5.81 ± 1.08	1.84	1.30[c]
Conditioned Medium Controls			
Lot B Unconcentrated	14.19 ± 1.63	4.49	5.92
Lot B R10-30 (10x)	10.65 ± 1.33	3.37	3.31
Lot C Unconcentrated	12.06 ± 1.80	3.82	3.74
Lot D Unconcentrated	17.33 ± 1.32	5.48	5.38
Lot E Unconcentrated	11.95 ± 0.69	3.78	3.71
Experiment Control Mean (Ham's F12) = 3.16 ± 0.83 mm²			

[a] Fold stimulation with respect to the experiment control mean (i.e. = 3.16). [b] Fold stimulation with respect to the mean value for growth in Ham's F12 on the same plate as the sample determination.

[c] Indicates a high control value on this plate relative to the overall experiment control mean.

Autocrine activity was detected in all lots of crude HEp-2 CM assayed but the degree of stimulation over the control varied between lots. Significant autocrine stimulatory activity was detected in only the R10-30 retentate. The absence of inhibitory activity was noted in the R10-30 concentrate, even at 10x. However, neither of R10-30 fractions assayed demonstrated a ten fold increase in stimulatory activity at 10x compared to the respective crude CM (unconcentrated CM from which they had been concentrated).

The appearance of inhibition in all but the R5-10 fraction of 10x concentrates was consistent with the preliminary results in Section 3.2.3.1 above. Two results were informative. Inhibition detected in the R30 fraction suggested the existence of a large molecular weight inhibitory species which would be expected to be retained by all the single-membrane concentrates prepared with lower MW cut-off membranes. Masking of the 10-30 kDa autocrine stimulatory species by such a large MW concentratable inhibitor would explain the absence of stimulatory activities expected in the R1, R5 and R10 retentates, due to the R10-30 stimulatory species. However, such an inhibitor could not account for the detection of inhibition which manifested in the R1-5 fraction. This suggested a separate low molecular weight species.

The low levels of stimulatory activity in the double filtrates F5(F10) and F10(F30) (see Section 2.8.1.1 for details) are only significant with respect to the overall experiment control. If the relatively high control on the same plate was used to score activity in these samples ($= 4.5 \pm 0.74$ mm^2) then no stimulatory activity could be detected in these fractions. Such anomalies in plating densities are more likely to occur in larger experiments due to technical reasons. A number of factors which become more prevalent with time after preparation of single cell suspensions may contribute to this problem. These include cell clumping, adhesion to vessel surfaces, or sedimentation, all of which may lead to changes in cell density. This was in spite of all efforts to the contrary to minimise cell density changes such as regular mixing during plating etc. Plate controls were included in all subsequent experiments to check for such inter-plate variation.

The same 10x concentrates (Lot A) were assayed again in the following experiment. In this case the effect of dilution was examined. All dilutions were made in serum-free Ham's F12. The results are shown in Table 3.2.3.2(ii).

Table 3.2.3.2(ii) The effect of dilution on the detection of autocrine activity in ultrafiltrated Hep-2 conditioned medium concentrates

LOT A CONDITIONED MEDIUM CONCENTRATES					
Fraction	Dilution	Colony ± S.E.M. Area mm^2 mm^2	Fraction	Dilution	Colony ± S.E.M. Area mm^2 mm^2
R1	10.0x	0.00 ± 0.00	R1-5	10.0x	0.29 ± 0.28
	7.5x	0.00 ± 0.00		7.5x	0.36 ± 0.41
	5.0x	5.11 ± 0.14		5.0x	0.24 ± 0.14
	2.5x	12.16 ± 2.57		2.5x	1.47 ± 0.38
	1.0x	14.53 ± 1.75		1.0x	6.14 ± 0.77
R5	10.0x	0.00 ± 0.00	R5-10	10.0x	5.66 ± 0.55
	7.5x	0.59 ± 0.19		7.5x	6.07 ± 1.07
	5.0x	15.71 ± 2.46		5.0x	6.52 ± 1.22
	2.5x	13.11 ± 0.82		2.5x	7.79 ± 0.91
	1.0x	13.01 ± 1.91		1.0x	6.07 ± 1.84
R10	10.0x	1.05 ± 0.58	R10-30	10.0x	15.43 ± 0.65
	7.5x	19.09 ± 2.33		7.5x	14.99 ± 1.10
	5.0x	18.76 ± 1.09		5.0x	15.24 ± 0.44
	2.5x	16.84 ± 1.49		2.5x	8.90 ± 0.87
	1.0x	13.90 ± 2.11		1.0x	8.26 ± 1.09
R30	10.0x	0.00 ± 0.00	Unconc[a] CM Lot A	None	10.57 ± 1.19
	7.5x	12.26 ± 1.35		3/4	7.10 ± 0.53
	5.0x	17.21 ± 0.87		1/2	7.90 ± 1.14
	2.5x	16.86 ± 2.74		1/4	7.48 ± 1.09
	1.0x	13.68 ± 2.94		1/10	5.73 ± 0.41
EXPERIMENT CONTROL MEAN (Ham's F12) = 7.41 ± 1.08 mm^2					

Table 3.2.3.2(ii) Continued overleaf.

Table 3.2.3.2(ii) continued.

LOT A FILTRATES	F1(F5)	F5(F10)	F10(F30)
Dilutions	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
None	9.71 ± 1.10	4.34 ± 1.29	6.21 ± 0.54
3/4	9.67 ± 1.68	3.33 ± 0.50	5.40 ± 0.81
1/2	9.27 ± 0.44	5.80 ± 1.61	9.64 ± 1.14
1/4	9.76 ± 0.51	7.81 ± 2.64	8.16 ± 1.67
1/10	9.90 ± 1.28	5.65 ± 1.58	8.54 ± 1.55
EXPERIMENT CONTROL MEAN (Ham's F12) = 7.41 ± 1.08 mm^2			

n=4 unless otherwise stated.

The overall growth level was higher in this than in the previous experiment. This was attributable to the longer incubation period used (eight days instead of seven). The data in Table 3.2.3.2(ii) are presented graphically in figure 3.2.3.2,(i),(ii), & (iii).

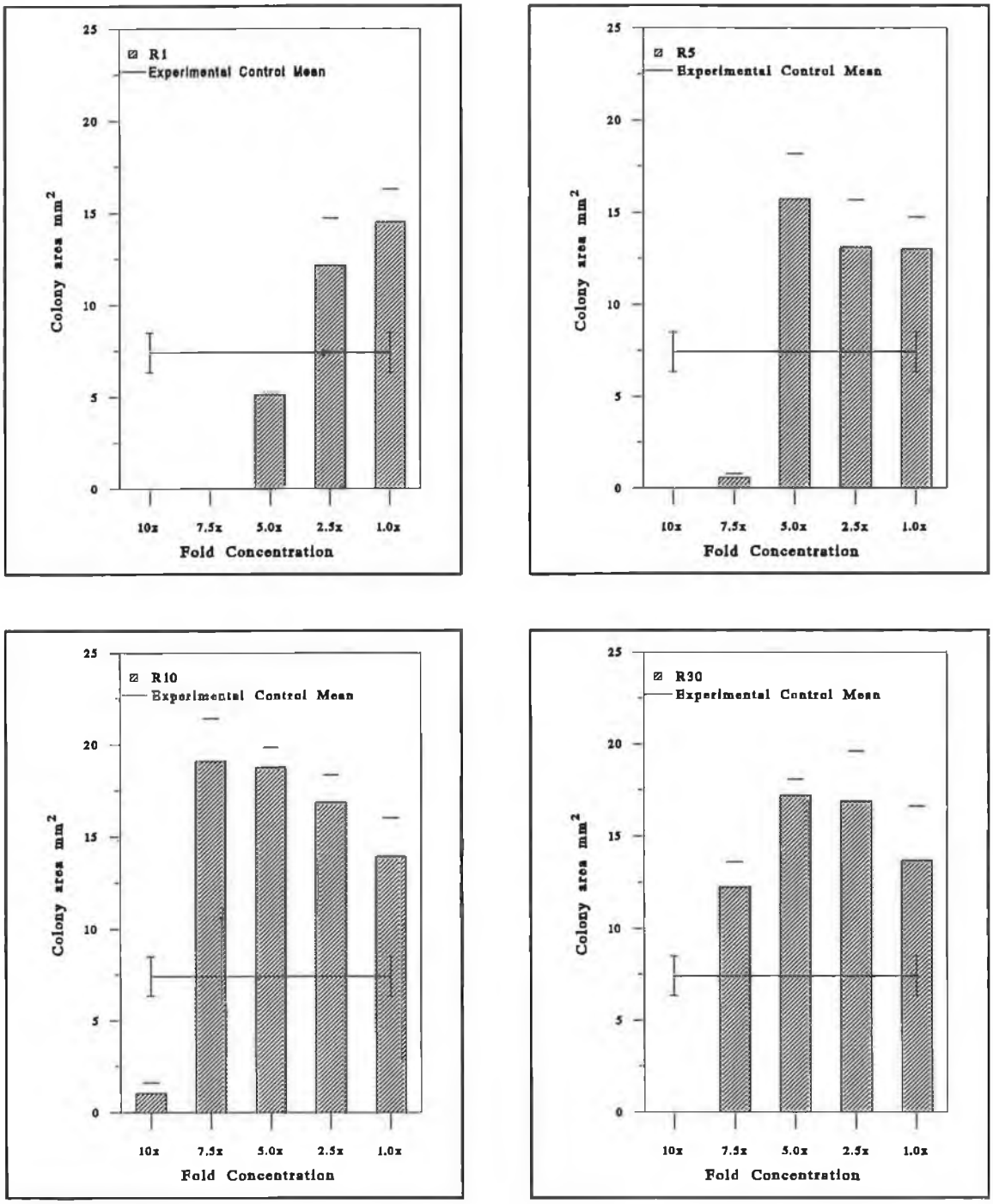


Figure 3.2.3.2,(i) The effect of dilution on the autocrine stimulatory activity retained in different molecular weight fractions of HEp-2 CM separated, by ultrafiltration. A, >1 kDa; B, >5 kDa; C, >10 kDa, D, >30 kDa.

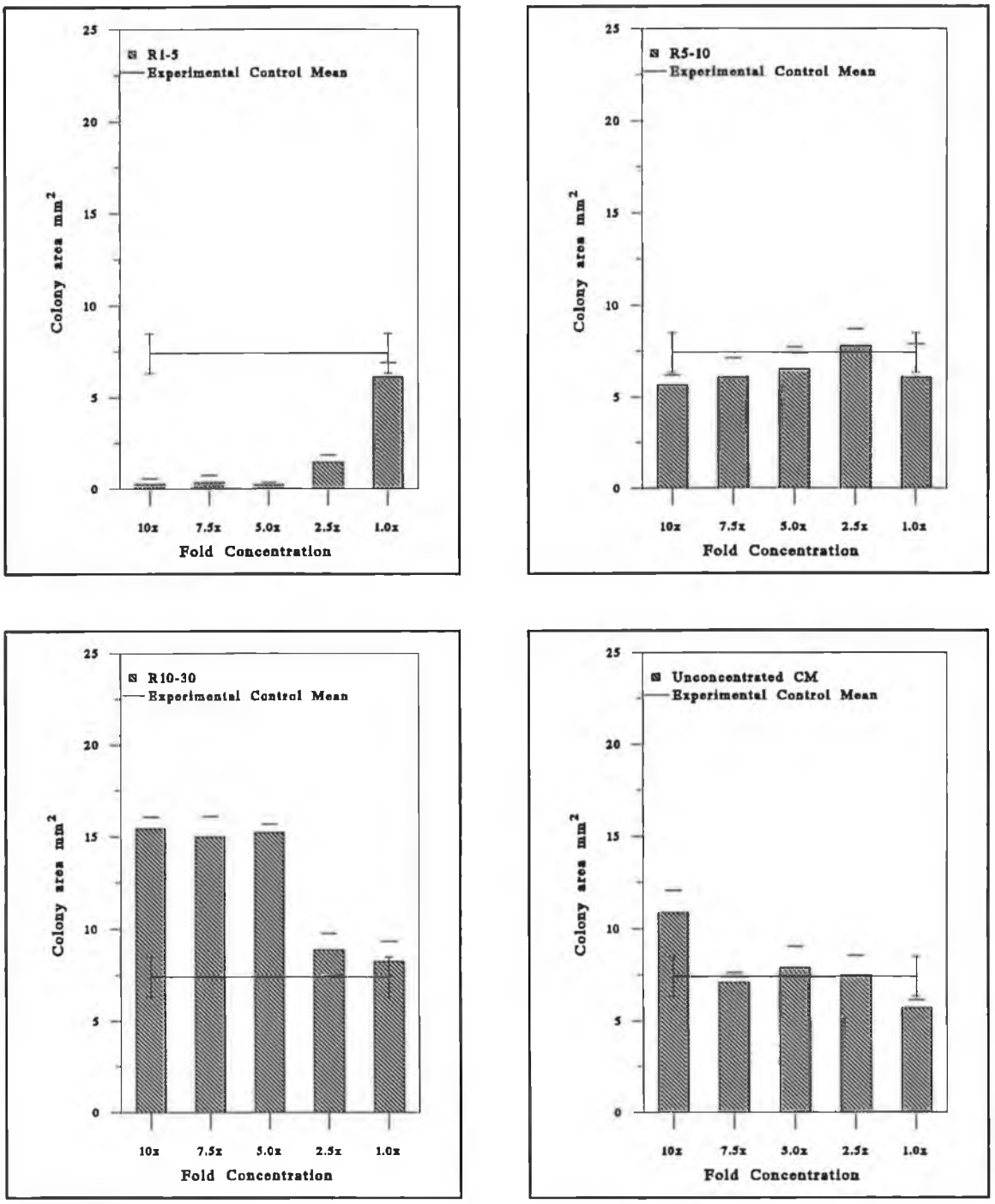


Figure 3.2.3.2,(ii) **The effect of dilution on the autocrine growth stimulatory activity in different molecular weight fractions of HEp-2 CM, separated by ultrafiltration. E, 1-5 kDa; F, 5-10 kDa; G, 10-30 kDa; F, crude CM.**

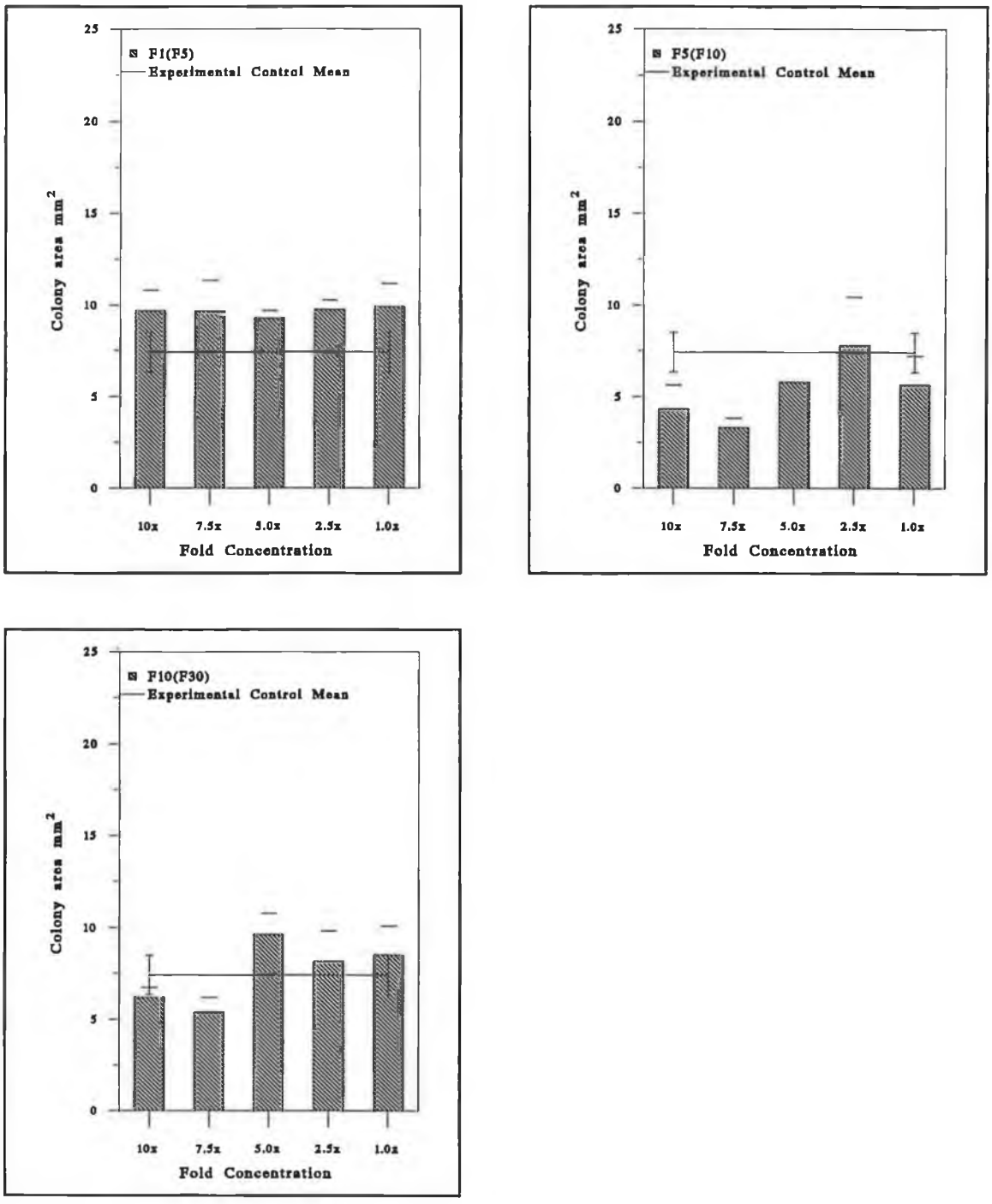


Figure 3.2.3.2,(iii) **The effect of double filtrates of HEp-2 CM (from MW fractionation of CM) on HEp-2 proliferation. I, R1-5 filtrate; J, R5-10 filtrate; K, filtrate from the R10-30 fraction.**

These results confirmed the presence of autocrine stimulatory activity and lack of inhibitory activity in the 10-30 kDa CM fraction. Again stimulatory activity appeared to be concentrated in this 10x fraction, but the growth response of HEp-2 was not linear. This R10-30 stimulatory activity was dilutable, but at dilutions less than 5x growth was the same as in the control.

Inhibition was detected again in the R30 at 10x, but with further dilution of this fraction autocrine stimulatory activity appeared at 7.5x, and was maximal at concentrations between 2.5 and 5x. The R30 autocrine stimulatory activity was also dilutable, but in this case at a 1x dilution it was in the same order as that in the crude CM.

A similar pattern was seen with dilution of the retentates R1, R5 and R10. Inhibition was detected in all 10x concentrates of these fractions but was abolished with progressively higher dilutions thereof. Autocrine stimulation became detectable with increasing dilution of these samples. The magnitude of dilution required to abolish inhibition and/or manifest stimulation in any of these concentrates was inversely related to the nominal MW cut-off of the membrane used to concentrate the CM. This suggested the retention of increasingly greater amounts of a lower MW inhibitory species with lower nominal MW cut-off membranes. It was also noted that the largest degree of stimulation over control was detected in the R10 fraction. Like the R30 fraction this activity was dilutable, but stimulation of the same order as that in the crude CM was still present at 1x. Taken together with the result of the R30 fraction, this suggested that the stimulator active in crude HEp-2 CM was larger than 30 kDa.

Evidence for a low molecular weight inhibitor in HEp-2 CM was given by the results of the R1-5 fraction. Inhibition was detected in all dilutions of this sample down to 1x, although the trend in growth with increasing dilution was upwards towards the control level, suggesting that this species was not inhibitory at the expected concentration in crude CM.

Only control levels of growth were detected in the R5-10 fraction at 10x and all dilutions thereof.

Similarily in the double filtrates F10(F30) and F1(F5) only control growth was detected at all dilutions. Dilutable inhibitory activity was detected in the F5(F10) filtrate, consistent with the presence of a low MW species detected in the R1-5 fraction.

Autocrine stimulatory activity detected in crude CM was lost after dilution.

3.2.3.3 Determination of autocrine activity in relation to protein concentration in conditioned medium fractionated and concentrated by ultrafiltration

The BioRad protein (macro) assay described in Section 2.11.6 was used to measure the protein concentration of the CM concentrates assayed in the previous section. The data in table 3.2.3.2(ii) representing 'active' fractions were expressed as *fold stimulation over the experiment control* (= sample mean ÷ control mean) in Table 3.2.3.3(i). The data for the BioRad assay are presented in Table 3.2.3.3(ii) and a plot of the standard curve for this assay is shown in Appendix C.

Table 3.2.3.3(i) Fold stimulation of 10x HEp-2 conditioned medium concentrates in the autocrine assay

Conditioned Medium Lot A	Fold Stimulation with respect to Control Growth				
CM Fractions	10.0x	7.5x	5.0x	2.5x	1.0x
Unconcentrated CM	1.43	N.S.	N.S.	N.S.	N.S.
R1	0.00	0.00	0.69	1.64	1.96
R5	0.00	0.08	2.12	1.77	1.76
R10	0.14	2.58	2.53	2.27	1.88
R10-30	2.08	2.02	2.06	N.S.	N.S.
R30	0.00	1.65	2.32	2.28	1.85

N.S; Not significant with respect to standard errors.

Table 3.2.3.3(ii) Determination of protein concentration in HEp-2 CM using the BioRad macro assay

Protein Standard [BSA] mg/ml	Absorbance @ 595nm	Lot A CM Samples[a]	Absorbance @ 595 nm
0.0	0.000	Unconcentrated	0.026
0.2	0.288	R1	0.234
0.4	0.505	R1-5	0.008
0.6	0.691	R5	0.365
0.8	0.908	R5-10	0.001
1.0	1.073	R10	0.350
1.2	1.177	R10-30	0.108
1.4	1.258	R30	0.307

[a] All retentates were concentrated 10x with respect to the unconcentrated conditioned medium.

Definition of a unit of autocrine activity

For the purpose of comparing specific autocrine stimulatory activity in different fractions of HEp-2 CM, a unit of autocrine activity was arbitrarily defined as **'that activity which gives a single fold increase in growth over the experiment control mean'**.

Autocrine stimulatory activites were expressed as 'units' per milligram of protein (mg/ml) for the different fractions and compared in Table 3.2.3.3(iii) below. The protein concentrations of the 10x concentrates were determined from the stanard curve (Appendix C). Protein concentrations of diluted retentates were deduced by extrapolation from the respective 10x concentrate (by dividing by the relevant dilution factor).

Table 3.2.3.3(iii) Comparison of specific autocrine activity in different HEp-2 ultrafiltration concentrates

Sample	[Protein] mg/ml	Autocrine activity of retentates per microgram of protein Units/mg Protein				
Concentrations		10.0x	7.5x	5.0x	2.5x	1.0x
UC	0.025	N.D.	N.D.	N.D.	N.D.	57.20
R1	0.200	N.D.	N.D.	N.D.	32.80	98.00
R5	0.326	N.D.	N.D.	13.00	21.85	55.00
R10	0.305	N.D.	11.27	16.54	29.87	62.67
R10-30	0.100	20.80	26.93	41.20	N.D.	N.D.
R30	0.265	N.D.	8.29	17.44	34.55	71.15

Abbreviations: UC, Unconcentrated conditioned medium; N.D, Not determined.

In Table 3.2.3.3(ii) the stimulatory activities in all 'active' fractions, with the exception of R1, were maximal at a 5x dilution. Comparing the activities per mg of protein at 5x, more activity was detected in the R10-30 fraction per mg protein than for any of the other fractions. On this basis the activities in the R10 and R30 fractions were in same order. However, at a dilution equivalent to that in crude CM (i.e 1x) the R10-30 species is no longer mitogenic. This does not rule out the involvement of the 10-30 kDa activity in the autocrine effect measured in unconcentrated (crude) CM but the activity of the R30 species at the same concentration supports the involvement of a large molecular species (i.e > 30 kDa) in this effect. The loss of the 10-30 kDa species may reflect its stability, or its denaturation at low concentrations. If this is the case, then perhaps the larger MW species may act as a stabilising factor for the 10-30 kDa species. The question of stability of autocrine activities in crude and fractionated CM is addressed in Section 3.2.4 below. It is noteworthy that all the other singly filtered concentrates (R1, R5 and R10) remained active at 1x.

3.2.3.4 Measurement of autocrine activity in the R10-30 fraction of HEp-2 conditioned medium using acid phosphatase as an endpoint

The following experiment was performed to confirm that the autocrine effect measured in fractionated CM using the 24-well assay could also be measured using a qualitatively different method for endpoint determination. Using the acid phosphatase assay described in Section 2.12.5 the stimulatory activity in two R10-30 (10x) retentates (prepared from different lots of active HEp-2 CM) was assayed.

HEp-2 were plated at a number of cell densities in control medium. The same range of plating densities were used here as in the preliminary autocrine experiment using acid phosphatase (Section 3.2.2 above). The CM concentrates were assayed at every plating density in the range and the fold increase over the respective control growth was deteremined. The results are presented in Table 3.2.3.4.

Table 3.2.3.4 Measurement of HEp-2 autocrine activity in the R10-30 fraction of CM using acid phosphatase

Plating Density per well	CONTROL	Lot 3 R10-30 (10x)	Fold stimulation w.r.t.c[a]	Lot 9 R10-30 (10x)	Fold stimulation w.r.t.c[b]
	ABS ± S.D.[a]	ABS ± S.D.[a]		ABS ± S.D.[a]	
2×10^2	0.048 ± 0.012	0.140 ± 0.012	2.92	0.169 ± 0.016	3.52
3×10^2	0.121 ± 0.016	0.228 ± 0.011	1.88	0.298 ± 0.015	2.46
4×10^2	0.133 ± 0.022	0.302 ± 0.026	2.27	0.359 ± 0.021	2.69
5×10^2	0.182 ± 0.019	0.335 ± 0.007	1.84	0.440 ± 0.027	2.42
6×10^2	0.273 ± 0.112	0.434 ± 0.014	1.59	0.461 ± 0.037	1.69
7×10^2	0.365 ± 0.054	N.D.[c]	-	N.D.[c]	-

n=8 unless otherwise stated. [a] Absorbance ± standard deviation measured at 405 nm. [b] Fold stimulation with respect to control. [c] Not determined.

This experiment showed that autocrine stimulatory activity in fractionated CM could also be measured using the acid phosphatase assay. The apparent fold increase in cell growth over control was detected at the lowest plating density, 2×10^2 cells/well. Increasing the plating density reduced the sensitivity of the assay.

3.2.4 PHYSICOCHEMICAL STABILITY PROPERTIES OF THE HEp-2 AUTOCRINE ACTIVITY

In order to characterise the nature of the factor(s) responsible for autocrine growth factor activity in HEp-2 CM, a series of physicochemical treatments were performed on crude (unconcentrated) CM as well as two ultrafiltration fractions of HEp-2 CM, designated R10-30 and R30 respectively. All three CM had previously been shown to be active in the HEp-2 assay. Serum-free cell culture medium (Ham's F12) was used as control medium. Samples of Ham's F12 were exposed to the same treatments as CM samples and therefore acted as treatment controls.

Some treatments of Ham's F12 had a negative effect on growth, that is, the level of growth in the 'treated' control was lower than that in the untreated Ham's F12. To facilitate the interpretation of the stability data in such cases, the measured growth of HEp-2 in response to treated and untreated CM was expressed as the growth supported by the respective CM sample *in excess of growth in the corresponding treated medium* (Ham's F12). In this analysis it is assumed that any negative effect on the control medium is independent of the observed effects on the autocrine species.

Where treatments had a negative effect on control growth (e.g. experiments with pH, heat, and protease treatments), the data are presented in two separate tables. The first table presents the original results, together with the standard errors on those mean determinations. The second table presents the same data expressed as the *growth in excess of control* for every CM treatment. 'Growth in excess of control' is calculated by subtracting the respective 'treated control' (Ham's F12) value from the correspondingly treated CM sample value. The resulting data are then expressed as a percentage of the growth in untreated CM.

Because the physicochemical treatments did not necessarily reduce autocrine activity (and in some cases potentiated it), the data for *growth in excess of control* were expressed as *percentage activity remaining* after treatment. This was calculated as a percentage of the untreated CM value.

3.2.4.1 pH stability

In a preliminary experiment crude HEp-2 CM was exposed to two extremes of pH for 1 hour at 4°C. The pH of the CM and control Ham's F12 was reduced to pH 3.6 and raised to pH 11.0 in separate aliquots, using 1M HCl and 1.5 M NaOH respectively (Section 2.9.2). After treatment the pH was restored to pH 7.5 and the samples were freeze-dried (Section 2.8.3), reconstituted in their starting volume, and filter-sterilised before assay. The results are presented in Table 3.2.4.1(i)a.

Table 3.2.4.1(i)a pH stability of HEp-2 autocrine activity

Untreated Samples		Colony Area mm² ± S.E.M. mm²
Conditioned medium		17.31 ± 0.89
Ham's F12		4.64 ± 1.12
pH Treatments		
3.6	CM	3.35 ± 0.87
3.6	Ham's F12	3.80 ± 2.21
11.0	CM	9.66 ± 2.34
11.0	Ham's F12	3.32 ± 0.46

n=4 for all determinations

The results from this pilot experiment with pH stability are presented as 'growth in excess of the respective controls' and the activity remaining after pH treatment is shown as a percentage of the untreated samples in Table 3.2.4.1(i)b.

Table 3.2.4.1(i)b Percentage autocrine activity remaining after pH treatment

Sample Treatment	Absolute Excess Growth in CM over Control Medium[a]	% Autocrine Activity Remaining After Treatment
None	12.67	100
pH 3.6	-0.45[b]	0
pH 11.0	6.34	50

[a] Sample (CM) mean minus control (Ham's F12) mean. [b] The level of growth in conditioned medium was less than that in the control medium.

The data indicated that the autocrine activity in crude HEp-2 CM was acid labile at pH 3.6 and aproximately 50% was base stable at pH 11.0.

In order to define the pH stability more precisely a range of pH tests from 2.5 to 11.5 were performed in the next experiment. The pH exposure time at 4°C was extended from 1 to 2 hours. The stability of the autocrine stimulatory activity in two 10x ultrafiltration concentrates, R10-30 and R30, was also determined in this experiment. To avoid significant volume changes during pH adjustment (which then calls for a freeze-drying step), 5M HCl and 5M NaOH were used. The

results of this experiment are shown in Table 3.2.4.1(ii).

Table 3.2.4.1(ii) The stability of crude and ultrafiltrated HEp-2 CM exposed to a range of pHs

pH	Lot 4 CM	R10-30 (10x)	R30 (10x)	Ham's F12
	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
Untreated	34.39 ± 1.69	20.11 ± 3.69	20.42 ± 2.02	13.08 ± 3.66
ACID TREATMENT				
2.50	4.96 ± 0.66	3.42 ± 0.86	0.91 ± 0.32	4.50 ± 1.03
3.50	9.50 ± 0.32	2.80 ± 0.48	0.00 ± 0.00	N.D.
5.00	16.57 ± 2.63	4.60 ± 1.20	0.00 ± 0.00	N.D.
6.50	8.83 ± 0.78	3.71 ± 0.86	0.00 ± 0.00	N.D.
BASE TREATMENT				
8.55	21.27 ± 2.37	11.42 ± 0.96	0.00 ± 0.00	N.D.
9.50	18.52 ± 1.04	17.69 ± 1.80	0.00 ± 0.00	N.D.
10.50	6.66 ± 1.58	17.34 ± 1.93	3.79 ± 0.94	N.D.
11.50	0.00 ± 0.00	12.68 ± 1.05	0.00 ± 0.00	0.00 ± 0.00

n=3 for all determinations. N.D; Not determined.

This experiment indicated that adjusting pH with 5M acid and base significantly reduced growth in the control medium. Acid treatment reduced control growth by 66% and no growth was seen in the control exposed to base. It was thought that a possible explanation for this effect might have been an increase in osmotic pressure, resulting from the greater quantities of acid and base needed to adjust pH at these extremes (pH 2.5 and 11.5).

Due to this treatment effect, it was not possible to draw definitive conclusions about sample stability in the alkaline range. However, when growth in these samples is compared to the respective untreated CM and untreated Ham's F12 control, the indications were that autocrine stimulatory activity appears to be stable in crude CM up to pH 9.5, and as high as pH 10.5 in the R10-30 fraction. Zero growth in this pH range was seen with the R30 fraction, except at pH 10.5. The apparently complete loss of autocrine activity in the R30 fraction at alkaline pH appears to distinguish this activity from that in the R10-30 fraction, but this experiment needs to be repeated.

Exposure of crude CM and the R10-30 fraction to pH 2.5 abolished autocrine activity in these fractions. However, in the R30 fraction exposed to pH 2.5, autocrine stimulation was not only

abolished but complete growth inhibition was seen.

Acid treatment of Ham's F12 alone reduced growth in the only pH control assayed. As there was no independent control for the effect of pH adjustment on Ham's F12 exposed to other acid pHs, definitive conclusions about stability in acid pH could only be made at pH 2.5. However, comparing the level of growth in these samples with their untreated counterparts, no evidence for acid resistant autocrine activity in the R10-30 fraction was apparent. A single sample of crude CM exposed to pH 5.0 shows evidence of acid-resistant stimulatory activity, but the lack of such activity at pH 6.5 cannot easily be explained. Complete growth inhibition was seen for the R30 fraction at all acid pHs.

The next experiment was performed on a single lot of crude HEp-2 CM. The effect of possible changes in osmotic pressure (particularily at extremes of pH) on cell growth was examined by measuring osmolarity after pH adjustment. In this case a separate pH control was performed (on Ham's F12) for every pH treatment of CM. The results of this experiment are presented in Table 3.2.4.1(iii)a.

Table 3.2.4.1(iii)a The effect of osmolarity and pH changes on HEp-2 autocrine activity

pH	Lot C2/3 Conditioned Medium		Ham's F12 Control Medium	
	Colony ± S.E.M. Area mm^2 mm^2	Osmolarity[a] OsMol/Kg	Colony ± S.E.M. Area mm^2 mm^2	Osmolarity[a] OsMol/Kg
ACID TREATMENT				
2.5	1.68 ± 0.51	N.D.	0.37 ± 0.06	0.409
3.5	4.60 ± 0.26	0.478	1.14 ± 0.22	0.456
5.0	5.11 ± 1.01	0.467	1.93 ± 0.53	0.381
6.5	3.70 ± 0.47	0.311	0.76 ± 0.23	N.D.
BASE TREATMENT				
8.5	11.24 ± 1.19	0.368	3.22 ± 1.04	0.354
9.5	12.95 ± 1.18	N.D.	3.97 ± 0.53	N.D.
10.5	10.77 ± 0.28	0.367	4.30 ± 0.43	0.404
11.5	0.96 ± 0.27	0.419	3.25 ± 0.63	0.456
NO TREATMENT				
	15.90 ± 2.65	N.D.	**4.44 ± 1.52**	N.D.

n=3 for all determinations. [a] Osmolarity was determined before dilution (½) in assay. N.D; Not determined.

This experiment demonstrated that pH adjustment using 5M HCl and 5M NaOH raises the osmolarity of CM and control medium above the normal range for animal cell culture media which is 0.280-0.320 OsMol/Kg. However, higher osmolarity values did not correlate with the growth inhibition (observed here in acid treated control medium). This implied that the reduction in growth due to acid treatment of the control medium was independent of osmolarity changes. The cause of this inhibition is not known, but a possible explanation might be the appearance of toxins produced by the chemical reaction of media components in acid pH.

Table 3.2.4.1(iii)b presents the results of this experiment {Table 3.2.4.1(iii)a} in terms of 'growth in excess of the respective control'. These values are also expressed as 'percentage autocrine activity remaining'.

Table 3.2.4.1(iii)b Percentage autocrine activity remaining in HEp-2 CM after pH treatment

SAMPLE TREATMENT	CM Growth in Excess of that in Control Medium	% Autocrine Activity Remaining
None	11.46	100
pH	**Acid**	
2.5	1.30	11
3.5	3.46	30
5.0	3.18	28
6.5	2.94	26
pH	**Alkali**	
8.5	8.02	70
9.5	7.07	62
10.5	6.47	56
11.5	-2.29[a]	0

[a] Level of growth in CM sample was less than the level of growth in the control medium at pH 11.5.

The data indicated that up to 30% of the autocrine activity in crude CM is stable down to pH 3.5. At pH 2.5 there was an apparent 11% residual activity. However, this result was contrary to indications from the previous experiment {Table 3.2.4.1(i)} which showed a complete loss of autocrine stimulatory activity after only 1 hour at pH 3.6. The use of a freeze-drying step employed in that experiment may account for the discrepancy between the two results. In the latter case, all autocrine activity was abolished after exposure to pH 11.5, but approximately 60-70% remained active between pH 8.5 and 10.5.

3.2.4.2 The effect of heparin on the pH stability of HEp-2 autocrine activity

The following experiment was performed in conjunction with the previous one {Table 3.2.4.1(iii)a} and the same lot of CM was used (Lot C2/3). Samples were exposed to acid and base exactly as before, except for the inclusion here of heparin at 10 μg/ml in all cases (see Section 2.9.5). Sample osmolarity was also measured after pH treatment.

The experiment was designed to assess the potential of heparin as a stabilising agent for autocrine activity exposed to acid and base treatments. Heparin had been reported to stabilise acidic and basic fibroblast growth factors to inactivation by heat and acid {Gospodarowicz & Cheng (1986)}. The results are shown in Table 3.2.4.2.a.

Table 3.2.4.2a pH stability of HEp-2 autocrine activity in the presence of heparin

pH	Lot C2/3 Conditioned Medium		Ham's F12 Control Medium	
	Colony ± S.E.M. Area mm² mm²	Osmolarity* OsMol/Kg	Colony ± S.E.M. Area mm² mm²	Osmolarity* OsMol/Kg
ACID TREATMENT				
2.5	1.76 ± 0.03	1.546	0.12 ± 0.02	0.384
3.5	2.43 ± 0.13	0.464	0.18 ± 0.04	0.489
5.0	0.00 ± 0.00	0.429	0.19 ± 0.08	0.362
6.5	2.50 ± 0.31	0.383	0.42 ± 0.09	0.367
BASE TREATMENT				
8.5	11.51 ± 1.34	0.375	4.06 ± 0.48	0.351
9.5	9.34 ± 0.52	0.409	2.48 ± 0.23	0.397
10.5	7.27 ± 1.91	0.367	4.30 ± 0.43	0.404
11.5	1.36 ± 0.62	0.419	3.25 ± 0.63	0.456
NO TREATMENT				
	10.12 ± 1.85	N.D.	**3.26 ± 0.88**	N.D.[b]

n= 3 for all determinations. [a] Osmolarity was determined before dilution (½) in assay. N.D; Not determined.

These results demonstrated that added heparin alone reduces overall growth in the HEp-2 autocrine assay. When compared to the levels in the absence of heparin {Table 3.2.4.1(iii)a} the growth in untreated CM, and untreated control samples plus heparin, was 30-40% lower. The same percentage reduction was seen in the base-treated samples. High osmolarity values did not correlate with growth reduction.

Addition of heparin to the acid treatments appeared to augment the effect of acid exposure already noted. The cumulative effect of heparin and acid treatment reduced control growth to a barely

detectable level. The linearity of the assay in this range cannot be assured and therefore no definitive conclusions could be drawn for autocrine stability with heparin in acid pH.

The data for this experiment are presented in Table 3.2.4.2b as percentage autocrine activity remaining.

Table 3.2.4.2b Percentage autocrine activity remaining after pH treatment in the presence of heparin

SAMPLE TREATMENT	CM Growth in Excess of that in Control Medium	% Autocrine Activity Remaining
None	6.86	100
pH	Acid	
2.5	1.64	24
3.5	2.25	33
5.0	N.D.	N.D.
6.5	2.08	30
pH	Alkali	
8.5	7.45	109
9.5	6.86	100
10.5	4.32	63
11.5	-0.81[a]	0

N.D; Not determined (Due to zero growth in CM sample). [a] Level of growth in CM sample was less than the level of growth in the control medium at pH 11.5.

An extra 30-40% of the autocrine activity in crude CM was stabilised against pH inactivation by heparin in the range pH 8.5-9.5, resulting in 100% stability up to pH 9.5. At pH 10.5 the percentage activity remaining was not significantly changed (60% aproximately) in the presence or absence of heparin {ref. Table 3.2.4.1(iii)b}. Complete loss of autocrine activity at pH 11.5 was not prevented by added heparin. No significant protective role was implied for heparin in the stability of the autocrine stimulatory activity to acid exposure. However, for the reasons outlined above this observation was not conclusive.

3.2.4.3 Proteinase sensitivity of the HEp-2 autocrine activity

The HEp-2 autocrine species was characterised further by exposing crude CM to the protease trypsin in the presence and absence of heparin. The stability of the active stimulatory species in CM was measured thereafter in the HEp-2 autocrine assay.

CM and control medium samples were mixed with purified bovine trypsin (Section 2.9.4) to a final concentration of 10 μg/ml, with and without added heparin at 10 μg/ml. After 2 hours at 37°C the samples, with heparin were each split into two aliquots and one of these set aside for assay directly. The action of trypsin was inhibited by the addition of soyabean trypsin inhibitor (TI) to all other samples to a final concentration of 20 μg/ml.The following controls were included in this experiment: (1) 20 μg/ml soyabean trypsin inhibitor only: (2) Premixed trypsin (10 μg/ml) and TI (20 μg/ml); (3) Untreated samples of CM and Ham's F12. Heparin only was added to CM and control medium to act as a separate heparin control. The samples were assayed in the HEp-2 autocrine assay and the results are presented in Table 3.2.4.3(i).

Table 3.2.4.3(i) The sensitivity of the HEp-2 autocrine activity to trypsin in the presence and absence of heparin

TREATMENT	Lot C2/3 Conditioned Medium		Ham's F12 Control Medium	
	Colony ± S.E.M. Area mm^2 mm^2	% Change in Abs Growth over Ctrl w.r.t Untreated[a]	Colony ± S.E.M. Area mm^2 mm^2	% Change in Control Growth w.r.t. UT Ctrl[b]
MINUS HEPARIN				
None	26.88 ± 1.58	0	11.00 ± 0.31	0
Trypsin, 2 hrs, +TI post 2 hrs.	23.12 ± 1.83	-24	10.84 ± 1.75	N.S.
Premix TI + Trypsin, 2 hrs.	23.75 ± 1.07	N.S.	9.15 ± 1.99	N.S.
TI only, 2 hrs.	32.28 ± 4.34	N.S.[c]	13.06 ± 0.63	+19
PLUS HEPARIN				
None	20.01 ± 2.67	-43	10.31 ± 1.03	N.S.
Trypsin, 2 hrs, +TI post 2 hrs.	17.89 ± 2.24	-43	5.92 ± 1.66	-37
Trypsin, 2 hrs, No TI	16.39 ± 2.67	-66	2.99 ± 0.19	-73

n=3 for all determinations. N.S; Not significant. TI; Soyabean trypsin inhibitor. [a] % Change in absolute growth over control with respect to that in the untreated CM sample. [b] With respect to the level of growth in untreated Ham's F12. [c] Ignoring the standard errors on this determination the % change was +20% (i.e. the same as the control).

Heparin alone reduced growth in untreated CM by 43% (with respect to untreated control). This level of reduction was consistent with that observed in the previous section (at 30-40%). However, there was no significant reduction in control growth (i.e. Ham's F12) with added heparin as noted above (Section 3.2.4.2). This experiment indicated that trypsin treatment abolished 24% of the autocrine stimulatory activity in crude CM in the absence of heparin.

The separate negative effects of heparin and trypsin on HEp-2 growth were additive when these agents were combined. Therefore, heparin does not appear to protect autocrine stimulatory activity from proteolytic degradation. Using this combination in the absence of TI, the levels of reduction in control and CM-stimulated growth were quantitatively similar at 73% and 66% respectively. The addition of TI to the 'trypsin and heparin' sample after treatment brought growth in the CM-stimulated sample back up to the same level as the 'heparin alone' sample (i.e. to a 43% reduction). This suggests that the extra 23% of activity saved here was due to the interaction of TI with a negative regulatory factor produced by HEp-2 whose activity is mediated by heparin. In the control, the percentage reduction was also lessened in the presence of TI (from 73% to 37%). This might imply that a trypsin-like enzyme is active in the presence of heparin under the conditions of the HEp-2 assay. A modest increase in control growth by 19% in the presence of TI would seem to support this hypothesis, however, in subsequent experiments TI alone did not support such an increase in HEp-2 growth. Premixing trypsin with TI effectively blocked the protease effect on CM-stimulated growth in the absence of heparin.

The next experiment was performed to determine the trypsin sensitivity of the autocrine species in the R10-30 and R30 ultrafiltration fractions, and to compare their stability profiles to that of the autocrine stimulatory activity in crude CM. The R10-30 and R30 fractions at 10x concentration were the same as those used in the pH stability determination (Section 3.2.4.1), but a different lot of crude CM was used here (Lot C4) than in the preliminary experiment above. Sample treatments and reagents were the same as those described in the preliminary experiment {3.2.4.3(i)}. The results are presented in Table 3.2.4.3(ii)a.

Table 3.2.4.3(ii)a The sensitivity of HEp-2 autocrine activity in crude and ultrafiltrated CM to trypsin exposure

SAMPLE	Lot C4 CM	R10-30 (10x)	R30 (10x)	Control[a]
TREATMENT	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
Untreated[b]	34.39 ± 1.69	20.11 ± 3.69	20.42 ± 2.02	13.08 ± 3.66
Trypsin, 2 hrs, +TI post 2 hrs.	14.71 ± 1.72	13.42 ± 2.08	14.20 ± 0.36	6.59 ± 0.62
Premix TI + Trypsin, 2 hrs.	30.77 ± 4.06	13.42 ± 2.08	10.96 ± 2.25	14.65 ± 2.57
TI only	31.09 ± 4.96	12.74 ± 1.40	17.36 ± 1.16	14.76 ± 1.34

n=3 for all determinations; TI :- Soyabean trypsin inhibitor. [a] Ham's F12. [b] This sample was also used for pH stability studies in Section 3.2.5.1.

These results indicated that the 2 hour incubation with trypsin followed by TI addition had a negative effect on the control growth level. Other treatments did not display a significant 'treatment effect'. A reduction in control growth in this case is difficult to explain given that the premixed trypsin and TI had no effect on the control growth. The reduction in growth may be explained by ineffective mixing of TI after the trypsin treatment, thereby leaving residual tryptic activity in the control, or perhaps the low reading is explained by plating errors. It was assumed that this was a real effect on control growth and autocrine growth stimulation was therefore calculated with respect to the corresponding treatment control. If such is not the case then the data presented in Table 3.2.4.3(ii)b will overestimate the *percentage activity remaining* after trypsin treatment. Trypsin inhibitor did not alter control growth as observed in the previous experiment.
In Table 3.2.4.3(ii)b the percentage reduction in autocrine activity was calculated with respect to untreated CM as follows:

$$\% \text{ Decrease in Autocrine Activity} = \frac{\text{Treated CM - Treated Ham's F12}}{\text{Untreated CM - Untreated Ham's F12}} - 1 \times 100$$

The degree to which a particular treatment affects control growth was determined by calculating the percentage reduction in control growth. This is given by:

$$\% \text{ Reduction in control growth} = \frac{\text{Treated Ham's F12}}{\text{Untreated Ham's F12}} - 1 \times 100$$

Table 3.2.4.3(ii)b Percentage autocrine activity remaining in crude and ultrafiltrated HEp-2 CM after exposure to trypsin

SAMPLE TREATMENT	CM[a]	% ACTIVITY REMAINING	R10-30 (10x)	% ACTIVITY REMAINING	R30 (10x)	% ACTIVITY REMAINING
None	21.31	100	7.03	100	7.34	100
Trypsin-2 hrs +TI	8.12	38	1.72	25	7.61	104
Premix TI + Trypsin	16.12	76[b]	-1.23[c]	0	-3.69[c]	0
TI only	16.33	78[b]	-2.02[c]	0	2.60	35

[a] Unconcentrated conditioned medium. [b] These values are equivalent to 100% activity remaining allowing for standard errors. [c] Conditioned medium growth levels were less than the corresponding control levels.

In this lot of crude CM (Lot C4) at least 62% of the autocrine activity was abolished after exposure to trypsin. This is more than double the amount that was sensitive to trypsin in Lot C2/3 CM {used in the earlier experiment, Table 3.2.4.3(i)}. The level of growth in this sample after treatment was in the same order as the untreated control and all other 'treatment controls' suggesting that in fact all the autocrine stimulatory activity in this lot of CM was abolished by exposure to trypsin. While the premixed trypsin plus TI control and TI alone appeared to reduce activity slightly in crude CM. This effect was not significant in terms of standard errors.

Trypsin abolished 75% of the R10-30 autocrine activity, but the R30 autocrine stimulatory activity was apparently stable to tryptic digestion.

The premixed trypsin and TI however, were able to abolish all autocrine stimulatory activity in both ultrafiltration fractions. Similarly TI alone had this effect on the R10-30 fraction and reduced the activity in R30 by 65%.

3.2.4.4 Comparative sensitivities to trypsin of autocrine activity in early versus late collections of HEp-2 conditioned medium

This experiment was performed to confirm the indications (Section 3.2.4.3.) that the autocrine stimulatory activity in early collections of HEp-2 CM (i.e. Lot C2/3, a pool of a 2nd and 3rd collection) were less sensitive to trypsin inactivation than later collections (Lot C4, a fourth collection). Using the same stocks of purified trypsin and TI as before, CM samples were exposed for 3 hours to trypsin (10μg/ml) before the addition of TI (20 μg/ml). An extra sample of each CM was exposed to crude trypsin (GIBCO at 250 μg/ml, total protein). Separate treatment controls were not performed in this case. The change in autocrine activity in the two CM samples was expressed as the *percentage decrease in autocrine activity* with respect to the untreated CM. The results are presented in Table 3.2.4.4.

Table 3.2.4.4 A comparison of trypsin sensitivity of autocrine activities in different lots of HEp-2 conditioned media

SAMPLE	Lot C2/3 Conditioned Medium		Lot C4 Conditioned Medium	
TREATMENT	Colony Area $\pm$ S.E.M. mm^2 mm^2	%Reduction in the Absolute Growth over the Control	Colony Area $\pm$ S.E.M. mm^2 mm^2	%Reduction in the Absolute Growth over the Control
Untreated	15.97 $\pm$ 0.75	0 (=100%)	16.81 $\pm$ 2.11	0 (=100%)
Trypsin (p), 3 hrs + TI post 3 hrs	15.71 $\pm$ 0.75	N.S.	9.92 $\pm$ 0.66	47
Premixed TI and Trypsin (p), 3 hrs	17.82 $\pm$ 3.09	N.S.	13.40 $\pm$ 1.52	N.S.
Trypsin (p), 3 hrs No TI	14.20 $\pm$ 0.42	13	10.22 $\pm$ 0.55	45
Trypsin (c), 3 hrs + TI post 3 hrs	6.68 $\pm$ 1.71	67	3.29 $\pm$ 0.83	91
Premixed TI and Trypsin (c), 3 hrs	7.83 $\pm$ 0.60	58	3.79 $\pm$ 0.60	88
TI only	16.97 $\pm$ 2.07	N.S.	16.18 $\pm$ 1.06	N.S.
Control Growth (Ham's F12) = 2.03 $\pm$ 0.33 mm^2				

n=3 for all determinations. N.S; Not significant.

This experiment confirmed that the later collection (C4) of CM was more sensitive to trypsin inactivation than the earlier lot (C2/3). No stimulatory activity was lost in Lot C2/3 after exposure to pure trypsin for three hours, compared to the 47% lost in Lot C4. Soyabean trypsin inhibitor (TI) effectively blocked the action of purified trypsin while TI alone had no effect on growth. However if TI was omitted after treatment, then 13% of the stimulatory activity in lot C2/3 was abolished. The omission of TI from Lot C4 did not significantly alter the percentage activity remaining.

Crude trypsin more effectively inactivated autocrine stimulatory activity in both lots of CM. This probably reflects the higher concentration of total preparation (crude trypsin) used, but the contribution of other degredative enzymes in this crude enzyme cannot be excluded. 67% of the autocrine stimulatory activity in the early collection CM lot C2/3 was abolished by exposure to crude trypsin compared to the 91% lost in lot C4. The concentration of TI used was insufficient to completely block the action of the crude trypsin preparation.

3.2.4.5 Heat stability of the HEp-2 autocrine activity

The stability of the autocrine activity in crude HEp-2 CM was addressed first. A single lot of CM (Lot C2/3) was heated in separate aliquots at 65°C for 10, 20, 30 and 60 minute intervals. The same CM was also boiled for 5 minutes. Aliquots of Ham's F12 were exposed to the same treatment. All CM and control treatments were duplicated with added heparin at 10 μg/ml to determine its potential to protect autocrine activity from heat inactivation. The results of this preliminary experiment are presented in Table 3.2.4.5(i)a.

Table 3.2.4.5(i)a Heat stability of HEp-2 autocrine activity in crude CM in the presence and absence of heparin

TREATMENT		Lot C2/3 CM	Ham's F12
TEMPERATURE °C	TIME mins	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
		MINUS HEPARIN	
Untreated (n=39)	0	15.90 ± 1.87	4.44 ± 1.52
65°	10	12.15 ± 1.87	4.57 ± 0.03
	20	14.92 ± 3.15	4.35 ± 0.53
	30	14.88 ± 4.64	5.13 ± 0.75
	60	17.99 ± 0.28	5.69 ± 1.01
100°	5	11.51 ± 1.16	3.52 ± 0.31
		PLUS HEPARIN	
Untreated (n=6)	0	10.12 ± 1.85	3.26 ± 0.88
65°	10	14.54 ± 0.36	3.82 ± 0.34
	20	20.38 ± 1.41	5.33 ± 1.02
	30	18.68 ± 0.40	6.01 ± 0.39
	60	15.60 ± 1.81	0.00 ± 0.00
100°	5	7.06 ± 0.02	1.34 ± 0.63

n=3 for all detereminations unless otherwise stated.

These data indicate that both heating and added heparin have an effect on control medium irrespective of their effects on autocrine stability. Three trends emerge when the data in Table 3.2.4.5(i)a are analysed.

1. Added heparin reduces overall growth in CM and control medium by 30-40% (confirming observations in Sections 3.2.4.3 & 3.2.4.4).

2. Boiling control medium with added heparin results in a 70% reduction in growth in this sample compared to untreated control medium.

3. Heating control medium at 65°C increases overall growth after 10, 20, and 30 minute incubations.

The relative changes in control growth are mirrored by quantitatively similar changes in CM-stimulated growth, suggesting that the effects of heating and heparin together are additive. The *excess growth over control* supported in CM samples is thus best determined by subtracting the value for growth in the corresponding treatment control. The values for excess growth over control are then presented in Table 3.2.4.5(i)b as *percentage autocrine activity remaining* with respect to that in the untreated control.

The absence of growth in the control medium after 1 hour at 65°C cannot be explained. It was noted that some of the heat treatments (particularly those at 65°C) resulted in barely significant reductions in growth. Given the inherent variation in bioassay growth the trends observed in these cases may reflect varitaion about the respective means.

Table 3.2.4.5(i)b Percentage autocrine activity remaining in heat-treated HEp-2 CM with and without added heparin

SAMPLE TREATMENT	Unconcentrated CM Minus Heparin	% ACTIVITY REMAINING	Unconcentrated CM Plus Heparin	% ACTIVIITY REMAINING
None	11.46	100	6.86	100
65°C, 10 mins	7.58	66	10.72	156
65°C, 20 mins	10.57	92	15.05	219
65°C, 30 mins	9.75	85	12.67	185
65°C, 60 mins	12.30	107	N.D.	N.D.
100°C, 5 mins	7.99	70	5.72	83

N.D; Not determined.

This experiment indicated that 30% of the autocrine stimulatory activity was abolished after boiling for 5 minutes. When boiled for 5 minutes in the presence of heparin, an additional 10% of the stimulatory activity was stable. Heating at 65°C for 10 minutes also led to a 30% reduction in

autocrine activity. However, heating at 65°C for longer times brought the activity back to 100%. Added heparin augmented the autocrine activity when CM was heated to 65°C. A combination of heating at 65°C and added heparin for 30 minutes, or 1 hour, resulted in a two fold increase in the relative amount of autocrine stimulatory activity remaining.

The heat-treated samples of CM and control medium tested in the previous experiment were stored at 4°C for 4 days, and then assayed again for autocrine activity. The results are shown in Table 3.2.4.5(ii)a.

Table 3.2.4.5(ii)a Stability of heat-treated HEp-2 autocrine activity in crude CM after storage at 4°C

TREATMENT		Lot C2/3 CM	Ham's F12
TEMPERATURE °C	TIME mins	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
		MINUS HEPARIN	
Untreated (n=35)	0	24.49 ± 4.24	10.90 ± 1.54
65°	10	27.37 ± 1.86	11.44 ± 0.71
	20	26.30 ± 3.41	11.86 ± 1.44
	30	26.81 ± 2.99	11.76 ± 1.03
	60	24.39 ± 0.78	10.30 ± 1.19
100°	5	16.55 ± 0.92	10.12 ± 0.43
		PLUS HEPARIN	
Untreated (n=6)	0	19.80 ± 1.74	10.31 ± 1.03
65°	10	23.28 ± 1.76	9.64 ± 0.95
	20	23.19 ± 1.98	6.98 ± 0.94
	30	20.00 ± 2.36	4.33 ± 0.98
	60	20.89 ± 1.63	0.00 ± 0.00
100°	5	13.54 ± 1.90	5.51 ± 0.27

n=3 for all determinations unless otherwise stated.

This data was expressed as *growth in excess of the control value* and presented as *percentage autocrine activity remaining* in Table 3.2.4.5(ii)b.

Table 3.2.4.5(ii)b Percentage autocrine activity remaining in heat-treated crude HEp-2 CM after storage at 4°C

SAMPLE TREATMENT	Unconcentrated CM Minus Heparin	% ACTIVITY REMAINING	Unconcentrated CM Plus Heparin	% ACTIVIITY REMAINING
None	13.59	100	9.49	100
65°C, 10 mins	15.93	117	13.64	144
65°C, 20 mins	14.44	106	16.21	171
65°C, 30 mins	15.05	110	15.67	165
65°C, 60 mins	14.09	104	N.D.	N.D.
100°C, 5 mins	6.43	47	8.03	85

N.D; Not determined.

In this experiment an apparent 50% of the autocrine activity was lost in the CM boiled for 5 minutes. However, the level of activity remaining in the CM boiled with heparin was unchanged, suggesting a protective role for heparin under these conditions. No reduction in stimulatory activity was apparent in the CM treated at 65°C, in this case. On the contrary, treatment at this temperature increased the relative stimulatory activity as observed in the previous experiment {Table 3.2.4.5.(i)b}.

It was concluded that 30-50% of the autocrine activity in crude HEp-2 CM was labile after boiling for 5 minutes, and that heparin can protect a portion of this heat labile activity from inactivation. The results of the 65°C treatment were inconclusive.

3.2.4.6 A comparison of the heat stability of autocrine activities in crude CM with that in the R10-30 and R30 ultrafiltration fractions

The next experiment compared the heat stability of autocrine stimulatory activity in crude CM to that in two 'active' ultrafiltration fractions, R10-30 and R30. The more sensitive Lot C4 of crude HEp-2 CM was used here (Section 3.2.4.4).

Samples of CM and control Ham's F12 were heated for 1 hour at 65°C in the presence and absence of 10 μg/ml heparin. Separate aliquots were boiled for 5 minutes with and without added heparin. The activity of these samples was determined in the HEp-2 autocrine assay and the results are presented in Table 3.2.4.6a.

Table 3.2.4.6a Comparison of the heat stability profiles for autocrine activity in crude HEp-2 CM and the R10-30 and R30 ultrafiltration fractions

SAMPLE	Lot C4 CM	R10-30 (10x)	R30 (10x)	Control*
TREATMENT TEMPERATURE °C	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
UNTREATED	34.39 ± 1.69	20.11 ± 3.69	20.42 ± 2.02	13.08 ± 3.66
100°C + Heparin	13.53 ± 2.97	10.53 ± 0.87	12.77 ± 0.90	7.97 ± 1.30
100°C - Heparin	8.29 ± 1.39	15.10 ± 1.17	18.93 ± 1.12	13.30 ± 0.03
65°C + Heparin	N.D.	10.92 ± 1.83	N.D.	10.26 ± 1.89
65°C - Heparin	19.59 ± 2.26	7.89 ± 0.60	19.61 ± 2.67	13.00 ± 1.89

[a] Ham's F12. N.D; Not determined.

Negative treatment effects on control growth were observed when heating was performed with added heparin. The data in table 3.2.4.6a were therefore expressed as 'growth in excess of the respective treatment control value' to allow for this treatment effect. These values are presented in Table 3.2.4.6b as *percentage autocrine activity remaining*.

Table 3.2.4.6b Percentage autocrine activity remaining in heat-treated crude and ultrafiltrated HEp-2 CM

SAMPLE TREATMENT	CM*	% ACTIVITY REMAINING	R10-30 (10x)	% ACTIVITY REMAINING	R30 (10x)	% ACTIVITY REMAINING
None	21.31	100	7.03	100	7.34	100
65°C, 1 hour, + Heparin	5.56	70	2.56	36	4.80	65
65°C, 1 hour, - Heparin	-5.01[b]	0	1.80	26	5.63	77
100°C - H, 5 minutes	N.D.	N.D.	0.66	9	N.D.	N.D.
100°C + H, 5 minutes	6.59	31	-5.11[b]	0	6.1	90

[a] Lot C4. [b] Negative values indicate that growth in CM samples were less than those in the corresponding control. N.D; Not determined.

The autocrine stimulatory activity in Lot C4 crude CM, assayed for heat stability in this experiment, was more labile to heating than Lot C2/3 assayed in the preliminary heat stability experiment {Table 3.2.4.5(i)a}. Therefore, the trypsin resistant early collection CM is also more heat resistant. Boiling Lot C4 reduced all activity in the absence of added heparin. Heparin protected 70% of the autocrine stimulatory activity in this lot of crude CM from heat inactivation at 100°C. After 1 hour at 65°C, 69% of the autocrine stimulatory activity was lost in Lot C4 CM.

All the R10-30 stimulatory activity was abolished after 1 hour at 65°C, and added heparin protected only a further 10% from inactivation by this treatment. However, the degree of heparin protection is not significant, 10% being within the margin of error for mean determinations. Boiling abolished 74% of the R10-30 autocrine activity, and in the presence of heparin a further 10% was lost. This again was within the margin of error for mean determinations.

The R30 autocrine stimulatory activity was almost completely stable to heating at 65°C, but 30% of this activity was lost after boiling. Apparently more activity was lost in the presence of heparin, but this cannot be considered conclusive.

In conclusion, the 65°C treatment distinguishes the R10-30 and the R30 autocrine activities. R30 autocrine stimulatory activity is stable at 65°C for 1 hour, but R10-30 activity is completely labile.

3.2.4.7 Trypsin sensitivity of heat resistant autocrine activity in early collection crude CM

The stability of the heat and trypsin resistant autocrine stimulatory activity observed in the early collection lot of HEp-2 CM (Lot C2/3) was examined after exposure to extended boiling time, followed by trypsin treatment.

Briefly, a sample of Lot C2/3 CM was boiled for 10 minutes. Half of this boiled CM was then incubated with 10 μg/ml trypsin (purified Sigma grade III) at 37°C for three hours. Soyabean trypsin inhibitor was added (to a final concentration of 20 μg/ml) to an aliquot of 'boiled and trypsin treated' CM, as well as to a 'boiled only' sample (to act as a TI control).

The excess growth in the untreated CM over the untreated Ham's F12 control was used as a reference in calculating the reduction in CM-stimulated growth after treatment. The level of growth supported by the treated CM over the untreated control Ham's F12 value was expressed as a percentage of that obtained in the untreated sample. The results of are presented in Table 3.2.4.7.

Table 3.2.4.7 The effect of trypsin on heat resistant autocrine activity in early collection crude CM

SAMPLE	Lot C2/3 Conditioned Medium		Ham's F12 Control Medium	
TREATMENT	Colony ± S.E.M. Area mm^2 mm^2	%Reduction in the Absolute Growth over the Control	Colony ± S.E.M. Area mm^2 mm^2	%Reduction in Control Growth w.r.t UT Ctrl[c]
Untreated	15.97 ± 0.75	0	2.03 ± 0.33	0
Boiled, 10 mins	10.62 ± 0.47	38	2.90 ± 0.72	N.S.
Boiled, 10 mins, + Trypsin (p)[a] 3 hrs + TI[b] post 3 hrs	7.21 ± 0.63	63	2.67 ± 0.50	N.S.
Boiled, 10 mins + Trypsin (p) 3 hrs	7.90 ± 1.01	58	2.34 ± 0.06	N.S
Boiled, 10 mins + TI only	7.91 ± 0.49	50	1.06 ± 0.17	50

[a] Purified trypsin (Sigma). [b] Soyabean trypsin inhibitor. N.S:- Not significant. [c] With respect to untreated control.

The results indicated that approximately 40% of the autocrine stimulatory activity was labile to boiling for 10 minutes. This confirms the findings of previous experiments with this lot of HEp-2 CM where 30-50% autocrine stimulatory activity was lost after boiling for 5 minutes (Section 3.2.4.5).

After trypsin treatment of boiled CM, total autocrine stimulatory activity was reduced by another 20%. This suggested that 40% of the total autocrine stimulatory activity was resistant to both boiling and trypsin exposure. It also indicated that ⅓ (33%) of the heat resistant activity was also trypsin sensitive.

Soyabean trypsin inhibitor alone had a negative effect on growth in the boiled control medium, when present in the absence of trypsin. This phenomenon was independent of the autocrine effect because a proportionate reduction in growth was observed in the correspondingly treated CM.

3.2.5 THE EFFECT OF HEPARIN ON CELL GROWTH IN THE HEp-2 ASSAY

This experiment was performed to confirm the inhibitory effect of heparin on HEp-2 growth observed in earlier experiments (Section 3.2.4) and to determine whether or not this was dose dependent. To this end, a range of heparin concentrations (diluted from a stock solution in Ham's F12 at 1 mg/ml, Section 2.9.5) were tested on HEp-2 under autocrine assay conditions. The results of this experiment are presented in Table 3.2.5.

Table 3.2.5 The effect of heparin on HEp-2 growth

[HEPARIN] µg/ml	Colony ± S.E.M. Area mm^2 mm^2
20.0	5.56 ± 0.73
15.0	5.58 ± 0.54
10.0	6.12 ± 1.00
7.5	6.28 ± 1.32
5.0	4.93 ± 0.79
2.5	5.74 ± 1.17
0.5	5.90 ± 1.36
Ham's F12 control	9.77 ± 1.01

The results indicated that heparin was inhibitory for HEp-2 cell growth at all concentrations assayed. However, no dose response relationship was apparent in this experiment.

3.2.6 PURE GROWTH FACTORS IN THE HEp-2 ASSAY

Polypeptides from a number of different growth factor classes, with known biochemical properties and characterised biological activities, were purchased commercially (Section 2.13.1) and tested in the Hep-2 assay to determine their mitogenic potential in this system. Stock solutions of growth factors were prepared from stocks at 4°C or freshly thawed frozen stocks at -20 °C as described in Section 2.13.2. Working stocks were prepared by dilution in Ham's F12 plus 1 mg/ml BSA to 2x the desired final concentration immediately before their addition to the assay (resulting in a ½ dilution in concentration).

In the following set of experiments growth factors were tested on HEp-2 cells under autocrine assay conditions. A control for the effect of BSA was included because this fraction of serum albumin is not necessarily inert in biological assays {Melsert *et al.*, (1989)}. The choice of concentrations used for the respective growth factors in preliminary experiments were based on those quoted for other cell systems in the literature. Growth stimulation is expressed as the *fold stimulation over control* (=sample value ÷ control value).

3.2.6.1 Epidermal growth Factor and Transforming growth factor α

The ability of EGF and TGFα to stimulate HEp-2 cell growth above the control level (Ham's F12 + 0.5 mg/ml BSA) was examined in the following preliminary experiment. The results are presented in Table 3.2.6.1(i).

Table 3.2.6.1(i) Proliferation of Hep-2 cells in response to EGF and TGFα

GROWTH FACTOR	CONCENTRATION ng/ml	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]
EGF	4.0	16.24 ± 3.03	1.87
	2.0	24.30 ± 1.21	2.80
	1.0	27.95 ± 5.20	3.22
TGFα	50.0	2.43 ± 1.10	-
	5.0	11.98 ± 1.95	1.34
	0.5	30.37 ± 3.37	3.49
Ham's F12 MEDIUM CONTROLS			
+ 0.50 mg/ml BSA		8.69 ± 3.93	
+ 0.25 mg/ml BSA		12.96 ± 0.44	
No BSA		6.32 ± 2.12	

[a] The increase in growth induced by growth factors was calculated with respect to the level of growth in the control medium (Ham's F12) containing 0.5 mg/ml bovine serum albumin (Sigma, fraction V).

BSA alone caused stimulation of growth in the HEp-2 assay with respect to growth in control medium without BSA. This highlighted the need to use the correct BSA control when scoring stimulation due to growth factors in the assay.

EGF and TGFα were stimulatory for HEp-2 cells in a dose dependent manner. Fold stimulation was maximal at the lowest concentrations assayed (1.0 and 0.5 ng/ml for EGF and TGFα respectively). Higher concentrations of both polypeptides resulted in lower growth measured. The degree of reduction was in the same order for both growth factors at concentrations up to 4 ng/ml for EGF and 5 ng/ml for TGFα. 50 ng/ml TGFα was inhibitory.

Because of the potential autocrine role of TGFα in many carcinoma cell lines, the following experiments aimed to define the active stimulatory range of concentrations for TGFα, particularly its minimum active concentration, in the HEp-2 system. A range of TGFα concentrations from 1-500 pg/ml were tested. The experiment was performed in duplicate with three days between assays and using the same working dilutions of growth factor. The same stock of cells, pretreated identically, was used in both assays in an effort to minimise variation in assay conditions. On each occasion the effect of TGFα on HEp-2 growth was deteremined in the 24-well plate and 96-well plate system (i.e. in parallel using the same stock of indicator cells). The results of the replicate 24-well assays are presented in Table 3.2.6.1(ii).

Table 3.2.6.1(ii) The effect of TGFα on HEp-2 growth as measured in the 24-well assay system

TGFα pg/ml	EXPERIMENT 1		EXPERIMENT 2	
	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]
500	5.38 ± 1.00	1.58	15.13 ± 3.99	2.32
250	9.06 ± 1.35	2.66	14.41 ± 2.77	2.21
100	8.78 ± 0.43	2.58	13.16 ± 0.69	2.02
50	7.15 ± 0.95	2.10	11.47 ± 1.30	1.76
10	6.82 ± 0.58	2.01	9.92 ± 2.69	1.52
5	7.00 ± 1.05	2.06	8.23 ± 0.10	1.26
1	5.48 ± 1.47	1.61	8.11 ± 2.24	1.25
Ham's F12 MEDIUM CONTROLS				
	Experiment 1		Experiment 2	
0.5 mg/ml BSA	3.54 ± 0.62 mm^2		5.68 ± 1.43 mm^2	
No BSA	N.D.		2.69 ± 0.51 mm^2	

[a] The increase in growth induced by growth factors was calculated with respect to the level of growth in the control medium (Ham's F12) containing 0.5 mg/ml bovine serum albumin (Sigma, fraction V). N.D; Not determined.

The level of control growth was lower in the first experiment than in the repeat. This probably reflects the metabolic status and/or viability of the cells at the time of plating. This example highlights the degree of inter-assay variation and the need for appropriate internal controls.

The mitogenic activity of TGFα on HEp-2 was confirmed in both experiments. The minimum active concentration of TGFα was 5 pg/ml. Maximal activity was reached between 250 and 500 pg/ml, with a sharp reduction in fold stimulation above this value, in agreement with preliminary results. The response of HEp-2 to the same range of TGFα concentrations in replicate 96-well assays is shown in Table 3.2.6.1(iii).

Table 3.2.6.1(iii) The effect of TGFα on HEp-2 growth as measured in the 96-well assay system

TGFα pg/ml	EXPERIMENT 1		EXPERIMENT 2	
	ABS ± S.D.[a]	X FOLD[b]	ABS ± S.D.[a]	X FOLD[b]
500	0.121 ± 0.039	2.12	0.017 ± 0.032	N.S.
250	0.109 ± 0.022	1.91	0.063 ± 0.052	N.S.
100	0.084 ± 0.033	1.47	0.085 ± 0.007	1.54
50	0.081 ± 0.009	1.42	0.071 ± 0.005	1.29
10	0.063 ± 0.011	N.S.	0.057 ± 0.010	N.S.
5	0.060 ± 0.008	N.S.	0.054 ± 0.007	N.S.
1	0.054 ± 0.005	N.S.	0.042 ± 0.010	N.S.
Ham's F12 Medium Controls				
+ 0.5 mg/ml BSA	0.057 ± 0.009		0.055 ± 0.007	
No BSA	0.050 ± 0.006		0.044 ± 0.006	

n=8 for all determinations. [a] Absorbance ± the standard deviation measured at 405nm. [b] Fold stimulation over control. N.S. no significant stimulation over control.

The inter-assay variation between the level of growth in the controls was not significant in the 96-well system as that observed above in the 24-well plate assay. However, the lowest mitogenic concentration of TGFα was between 50 and 100 pg/ml. This implied that the acid phosphatase method of was less sensitive in detecting the mitogenic response to this growth factor. The lack of activity at 250 and 500 pg/ml in experiment 2 may be due to inhibition by TGFα at these concentrations. However, this was more likely to have arisen due to plating errors, or as a result of cell detachment during the washing step (Section 2.12.5), because there was good reproducibility at other concentrations between the replicate experiments. In experiment 1, maximal mitogenic activity with TGFα was at 500 pg/ml in agreement with the result of the 24-well assay (performed in parallel).

Taken together these results indicate that TGFα is mitogenic for HEp-2 with maximal mitogenic activity in the range 50-500 pg/ml and with a lower limit, possibly as low as 5 pg/ml.

3.2.6.2 Transforming growth factor β

The response of HEp-2 cells to TGFβ under autocrine assay conditions was examined using three concentrations of the polypeptide, spanning a broad concentration range. The experiment was part of the assay described in Section 3.2.6.1(i) above. The results are presented in Table 3.2.6.2.

Table 3.2.6.2 The effect of TGFβ on the proliferation of HEp-2 cells

GROWTH FACTOR	CONCENTRATION ng/ml	Colony ± S.E.M. Area mm^2 mm^2
TGFβ	2.00	10.34 ± 2.42
	0.20	13.76 ± 1.19
	0.02	13.34 ± 1.79
Ham's F12 MEDIUM CONTROLS		
+ 0.50 mg/ml BSA		8.69 ± 3.93
+ 0.25 mg/ml BSA		12.96 ± 0.44
No BSA		6.32 ± 2.12

The results indicate that TGFβ is not inhibitory for HEp-2 cells over the concentration range assayed. The mean values for growth in the presence of this polypeptide appeared higher than the control but this was not significant. No dose-response relationship was apparent in this experiment.

3.2.6.3 Insulin and Insulin-like growth factors I & II

The effect of Insulin and insulin-like growth factors I and II on HEp-2 proliferation was examined in parallel with the determination of TGFα and β in the experiment outlined in Sections 3.2.6.1 and 3.2.6.2 above. The results are presented in Table 3.2.6.3.

Table 3.2.6.3 The effect of Insulin and Insulin-like growth factors I & II on HEp-2 proliferation

GROWTH FACTOR	CONCENTRATION ng/ml	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]
INSULIN	500	0.00 ± 0.00	-
	50	17.94 ± 3.62	2.06
	5	16.62 ± 0.65	1.91
IGF-I	100	19.33 ± 0.50	2.22
	50	23.41 ± 2.40	2.69
	10	23.21 ± 2.32	2.67
	1	16.47 ± 1.27	1.90
IGF-II	100	15.55 ± 1.38	1.79
	50	16.22 ± 1.11	1.87
	10	13.45 ± 0.75	1.55
Ham's F12 MEDIUM CONTROLS			
+ 0.50 mg/ml BSA		8.69 ± 3.93	
+ 0.25 mg/ml BSA		12.96 ± 0.44	
No BSA		6.32 ± 2.12	

[a] The increase in growth induced by growth factors was calculated with respect to the level of growth in the control medium (Ham's F12) containing 0.5 mg/ml bovine serum albumin (Sigma, fraction V).

All three growth factors stimulated proliferation of HEp-2. The most potent member of this family in the HEp-2 assay was IGF-II. This protein was stimulatory at all concentrations tested but maximal stimulation was seen in the range 10-50 ng/ml.

Insulin was the second most potent stimulator in this concentration range with a two fold stimulation over control apparent at 5 and 50 ng/ml. The inhibition at 500 ng/ml probably reflects the acidity of the less diluted stock insulin (acidification was necessary to dissolve this protein in the preparation of the stock, see Section 2.13.2).

The peak level of stimulation for IGF-II was higher than IGF-I and maximal stimulation with IGF-I was achieved between 50-100 ng/ml with this growth factor.

3.2.6.4 Acidic and Basic Fibroblast Growth Factors

The mitogenic potential of acidic and basic FGFs in the HEp-2 assay were examined over a range of concentrations in the following set of of replicate experiments. The results are presented in Table 3.2.6.4.

Table 3.2.6.4 The effect of aFGF and bFGF on the proliferation of HEp-2

GROWTH FACTOR (Concentration)	EXPERIMENT 1		EXPERIMENT 2	
aFGF (ng/ml)	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]
25.00	4.81 ± 0.68	-	8.43 ± 0.34	1.48
5.00	6.21 ± 1.47	1.75	9.24 ± 0.63	1.63
2.50	5.15 ± 0.69	1.45	7.08 ± 0.73	1.25
1.50	3.34 ± 0.44	-	6.63 ± 0.56	1.17
0.50	3.83 ± 0.97	-	5.02 ± 0.82	-
0.25	3.82 ± 0.47	-	5.41 ± 1.45	-
0.10	0.00 ± 0.00	-	6.22 ± 0.99	-
bFGF (pg/ml)	**EXPERIMENT 1**		**EXPERIMENT 2**	
500	4.94 ± 0.28	1.40	8.30 ± 0.74	1.46
250	7.62 ± 1.14	2.15	11.54 ± 2.41	2.03
100	6.13 ± 0.53	1.73	9.69 ± 0.45	1.70
50	6.38 ± 0.38	1.80	8.69 ± 0.87	1.53
10	3.78 ± 1.00	-	7.55 ± 1.90	-
5	5.14 ± 0.72	1.45	9.36 ± 2.30	1.65
1	4.19 ± 0.48	-	6.10 ± 0.70	-
Ham's F12 MEDIUM CONTROLS				
+ 0.5 mg/ml BSA	3.54 ± 0.62 mm^2		5.68 ± 1.43 mm^2	
No BSA	N.D.		2.69 ± 0.51 mm^2	

n=3 unless otherwise stated. N.D:- Not determined. [a] The increase in growth induced by growth factors was calculated with respect to the level of growth in the control medium (Ham's F12) containing 0.5 mg/ml bovine serum albumin (Sigma, fraction V). N.D; Not determined.

Both growth factors were active mitogenically over a narrow concentration range. Acidic FGF was active maximally between 2.5 - 5.0 ng/ml with the greatest fold stimulation over control at 5.0 ng/ml. Basic FGF was maximally active at a 10-50 fold lower concentration between 50-250 pg/ml with the highest fold stimulation at 250 pg/ml. Mitogenic activity indicated at 5 pg/ml bFGF was only significant in the first experiment (with respect to standard errors).

3.2.6.5 Interleukins 1α and 1β

Interleukin 1α (IL-1α) and Interleukin 1β (IL 1β) were characterised initially as hematopoietic growth factors but have since been been shown to be active on epithelial cells. They were included in the replicate assays outlined in Section 3.2.6.1 above using the same working stocks of the growth factors. The results of these experiments are presented in Table 3.2.6.5.

Table 3.2.6.5 The response of HEp-2 to IL-1α and IL-1β

GROWTH FACTOR (Concentration)	EXPERIMENT 1		EXPERIMENT 2	
IL-1α (pg/ml)	Colony ± S.E.M. Area mm² mm²	Fold Stimulation w.r.t. Control[a]	Colony ± S.E.M. Area mm² mm²	Fold Stimulation w.r.t. Control[a]
500	0.23 ± 0.17	-	7.08 ± 2.31	-
250	4.92 ± 1.11	-	7.53 ± 1.76	-
100	3.62 ± 0.30	-	8.97 ± 1.50	1.58
50	3.82 ± 0.87	-	9.14 ± 1.17	1.61
10	4.50 ± 0.70	-	7.40 ± 0.52	-
5	5.55 ± 0.35	1.57	6.36 ± 1.09	-
1	3.99 ± 0.94	-	7.00 ± 0.50	-
IL-1β (pg/ml)	EXPERIMENT 1		EXPERIMENT 2	
500	4.48 ± 0.52	-	5.94 ± 0.99	-
250	7.00 ± 0.90	1.98	5.62 ± 1.73	-
100	5.01 ± 1.59	-	7.01 ± 0.11	-
50	4.55 ± 1.00	-	9.10 ± 0.84	1.60
10	5.44 ± 1.40	-	6.08 ± 0.37	-
5	5.36 ± 0.60	1.51	7.27 ± 0.40	-
1	5.86 ± 0.43	1.66	6.64 ± 1.57	-
Ham's F12 MEDIUM CONTROLS				
+ 0.5 mg/ml BSA	3.54 ± 0.62 mm²		5.68 ± 1.43 mm²	
No BSA	N.D.		2.69 ± 0.51 mm²	

n=3 unless otherwise stated. N.D, Not determined. [a] The increase in growth induced by growth factors was calculated with respect to the level of growth in the control medium (Ham's F12) containing 0.5 mg/ml bovine serum albumin (Sigma, fraction V). N.D; Not determined.

IL-1α and IL-1β both demonstrated mitogenic activity in the HEp-2 assay. However, the doses required to effect this stimulation varied between experiments. Fold stimulation was scored where a particular concentration of the polypeptide resulted in growth significantly above the control.

On this basis IL-1α was active at 5 pg/ml in the first experiment, but 50 - 100 pg/ml of this factor was required to produce the same fold stimultion in the repeat experiment. Similarly, IL-1β was mitogenically active at 1 - 5 pg/ml in the first determination but a 10 fold higher concentrataion was required to reproduce this level of stimulation in the repeat. An identical shift in sensitivity was also seen between assays with TGFα which was analysed in the same assay (Section 3.2.6.1). Evidence for a biphasic response to IL-1β was noted in experiment 1 because a larger fold stimulation was seen at 250 pg/ml with this growth factor. However, this was not reproduced in the second experiment, possibly due to the change in sensitivity to the cytokines.

3.2.6.6 Platelet-derived growth factor

The main target cells for platelet-derived growth factor (PDGF) are mesenchymal and glial cells. The diluent medium for this growth factor was Ham's F12 but the concentration of bovine serum albumin used was 0.5 mg% (= 0.005 mg/ml BSA). The respective BSA control contained 0.005 mg/ml BSA. The results of this experiment are shown in Table 3.2.6.6.

Table 3.2.6.6 The response of HEp-2 to PDGF in the autocrine assay

GROWTH FACTOR	CONCENTRATION Units/ml[a]	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[b]
PDGF	2.00	2.11 ± 0.49	-
	0.20	7.35 ± 0.66	1.41
	0.02	5.34 ± 1.12	-
Ham's F12 MEDIUM CONTROLS			
+ 0.50 mg% BSA		5.22 ± 0.17 mm^2	
No BSA		6.26 ± 0.54 mm^2	

[a] A unit of PDGF activity. [b] The increase in growth induced by growth factors was calculated with respect to the level of growth in the control medium (Ham's F12) containing 0.5 mg% bovine serum albumin (Sigma, fraction V).

In this case control growth was inhibited in the presence of BSA. Stimulation was scored with respect to the BSA control. Therefore, at 2 U/ml this protein was inhibitory, but at 0.2 U/ml there was a slight increase in growth over the BSA control (but not with respect to the experiment control) equivalent to a 1.4 fold increase. The lowest concentration assayed, 0.02 U/ml supported only a control (BSA) level of growth. It was concluded from this data that PDGF may be weakly stimulatory at concentrations around 0.2 U/ml.

3.2.6.7 Bombesin/Gastrin-Related Peptide (GRP)

The effect of the peptide gastrin-related peptide (GRP or Bombesin) in the HEp-2 system was examined in the following experiment. Stock solutions and dilutions were made in Ham's F12 plus 1 mg/ml BSA. The results of this investigation are shown in Table 3.2.6.7.

Table 3.2.6.7 The effect of GRP on the proliferation of HEp-2

GROWTH FACTOR	CONCENTRATION ng/ml	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[a]
Bombesin	100	16.28 ± 1.01	1.87
	10	16.03 ± 1.79	1.84
	1	16.51 ± 1.98	1.90
Ham's F12 MEDIUM CONTROLS			
+ 0.50 mg/ml BSA		8.69 ± 3.93 mm^2	
+ 0.25 mg/ml BSA		12.96 ± 0.44 mm^2	
No BSA		6.32 ± 2.12 mm^2	

[a] The increase in growth induced by growth factors was calculated with respect to the level of growth in the control medium (Ham's F12) containing 0.5 mg/ml bovine serum albumin (Sigma, fraction V).

This neuropeptide gave an almost two-fold increase in growth relative to the control (plus BSA) at all concentrations tested. However, the fold stimulation was the same over the entire concentration range assayed.

3.2.7 DETERMINATION OF THE TOXICITY OF CHAPS TO HEp-2 CELLS UNDER AUTOCRINE ASSAY CONDITIONS

The zwitterionic detergent, 3-[(3-Cholamidopropyl) dimethylamonio]-1-propane sulfonate or CHAPS, has been reported to stabilise purified fibroblast growth factors during frozen storage and in solution {Matuo *et al.*, (1988)} where it was found to be less toxic than a series of other detergents when tested on balb/c 3T3 cells *in vitro*. It has been employed in the purification of an FGF-like growth factor from medium conditioned by a clone of the Shionogi carcinoma cell line SC 115 (derived from a mouse mammary tumour) {Nonomura *et al.*, (1988) and Sato *et al.*, (1988)}.

The next experiment was designed to assess the toxicity of this detergent on HEp-2, grown under autocrine assay conditions, with a view to using this detergent as a stabilising agent in the purification of HEp-2 autocrine activity. HEp-2 were exposed to 0.1% (w/v) CHAPS and a series of dilutions thereof. The optimum concentration of CHAPS reported for stabilising the activity of fibroblast growth factors was 0.1% w/v. The results of this investigation are presented in Table 3.2.7.1.

Table 3.2.7.1 The growth of HEp-2 in the presence of CHAPS

[CHAPS] % w/v	Colony ± S.E.M Area mm^2 mm^2
0.001	6.85 ± 0.65
0.005	7.54 ± 2.50
0.010	6.50 ± 0.54
0.050	9.60 ± 0.73
0.100	7.24 ± 1.06
Ham's F12[a]	6.80 ± 0.65

[a] Control medium without added CHAPS.

CHAPS did not exhibit any toxic effects on HEp-2 under autocrine assay conditions. In the following experiment the same concentration range of CHAPS was assayed again, but in this case the effect of the detergent on the detection of the autocrine stimulatory effect in crude CM was also examined. The results are shown in Table 3.2.7.2.

Table 3.2.7.2 The effect of CHAPS on the measured HEp-2 autocrine activity

[CHAPS] % w/v	Ham's F12 Control	Lot 8 CM
	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
0.100	7.90 ± 1.16	27.87 ± 3.66
0.050	8.54 ± 0.76	39.56 ± 5.40
0.010	11.93 ± 1.22	42.59 ± 4.72
0.005	12.61 ± 0.75	38.05 ± 3.78
0.001	12.69 ± 0.80	40.45 ± 1.90
Ham's F12[a]	13.69 ± 1.56	37.44 ± 5.13

[a] Control medium without added CHAPS.

Toxicity on HEp-2 cells was detected in control medium at concentrations of CHAPS greater than 0.01% (w/v). Toxicity in the presence of crude HEp-2 CM was only detected at the highest concentration of detergent assayed, 0.1% (w/v). These results suggested that CHAPS might be included as a potential stabilisng agent in the chromatographic separation of the HEp-2 autocrine stimulatory activity.

3.3.1 SEPARATION OF CONCENTRATED HEp-2 CM ON A BIOGEL P30 COLUMN

A 58 × 1.9 cm Bio-Gel P30 column described in Section 2.11.1 was used to separate a 20x sample of HEp-2 CM {prepared by ultrafiltration using a 1000 kDa molecular weight cut-off membrane (R1)}. The column was run in 100 mM phosphate buffer containing 100 mM NaCl at 4°C and 110 × 1.5 ml fractions were collected at a flow rate of 8 mls/hour. The absorbance of each fraction was measured at 280 nm.

Fractions 1-3, 4-7, 7-9, etc. and every three consecutive fractions thereafter were pooled and dialysed against distilled H_2O overnight before they were lyophilised (Section 2.8.3) and reconstituted in 2.5 mls of serum-free Ham's F12. All samples were filter-sterilised by passage through 0.22 μm filters (Millex, low protein binding, Millipore). 0.5 mls of each fraction was assayed in the HEp-2 autocrine assay without prior dilution. However, it was not possible to determine activity in these samples due to contamination but the elution profile of CM illustrated that this column separated proteins in several size ranges (figure 3.1.1,A).

The same column was used to separate a second sample of Hep-2 concentrated CM. A 2 ml sample of a 60x CM concentrate (prepared by ultrafiltration with a 1000 MW cut-off membrane) was loaded onto the column in 25 mM phosphate running buffer at pH 7.4 (without NaCl). 60 × 2 ml fractions were collected and lyophilised. These were then reconstituted into 4 mls of Ham's F12, filter-sterilised and tested in the autocrine assay. In this experiment protein concentration was measured using the BioRad Protein Assay (see Section 2.11.6). The results of these determinations are presented in Table 3.3.1 and the elution profiles for protein and autocrine activity are shown in figure 3.3.1,B.

Table 3.3.1 Separation of 60x R1 HEp-2 CM on a BioGel P30 gel-filtration column

FRACTION NUMBER	[PROTEIN] µg/ml	Colony Area mm² ± S.E.M. mm²	FRACTION NUMBER	[PROTEIN] µg/ml	Colony Area mm² ± S.E.M. mm²
1	<0.02	13.64 ± 0.37	26	0.09	9.28 ± 1.14
2	0.04	11.60 ± 1.70	27	0.04	13.66 ± 0.94
3	0.03	13.12 ± 0.73	28	<0.02	18.04 ± 0.06
4	0.04	11.00 ± 0.22	29	0.02	18.21 ± 1.00
5	<0.02	11.37 ± 0.24	30	0.02	11.50 ± 1.47
6	<0.02	12.73 ± 0.94	31	0.08	13.26 ± 1.73
7	0.03	16.20 ± 0.62	32	0.05	16.27 ± 5.10
8	<0.02	10.89 ± 0.10	33	0.08	14.81 ± 1.01
9	0.02	10.58 ± 2.45	34	0.06	9.34 ± 0.02
10	<0.02	13.96 ± 0.76	35	0.02	6.60 ± 0.19
11	<0.02	13.53 ± 2.36	36	0.09	0.00 ± 0.00
12	1.78	10.92 ± 0.04	37	0.10	0.00 ± 0.00
13	>1.80	16.02 ± 1.19	38	0.11	0.72 ± 0.04
14	1.04	20.70 ± 1.42	39	0.10	5.39 ± 1.00
15	0.64	14.95 ± 0.14	40	0.09	5.31 ± 0.64
16	0.62	13.35 ± 1.42	41	0.09	2.98 ± 0.49
17	0.50	15.82 ± 0.02	42	0.03	8.42 ± 0.18
18	0.34	12.97 ± 0.64	43	0.09	6.64 ± 0.48
19	0.30	12.06 ± 1.76	44	0.05	7.28 ± 0.53
20	0.26	12.91 ± 1.02	45	0.09	7.03 ± 0.37
21	0.33	12.16 ± 2.75	46	0.09	5.89 ± 1.05
22	0.18	14.17 ± 2.43	47	0.02	8.29 ± 0.89
23	0.11	13.93 ± 2.52	48	0.07	8.59 ± 0.13
24	0.09	15.42 ± 2.89	49	0.04	9.43 ± 0.27
25	0.06	11.26 ± 2.48	50	0.02	11.98 ± 0.36
HEp-2 CM (1x)[a]		17.61 ± 1.14	**Ham's F12 Control**		10.70 ± 1.62

[a] This was diluted from a 60x concentrate of HEp-2 CM prepared with a 1000 MW cut-off membrane.

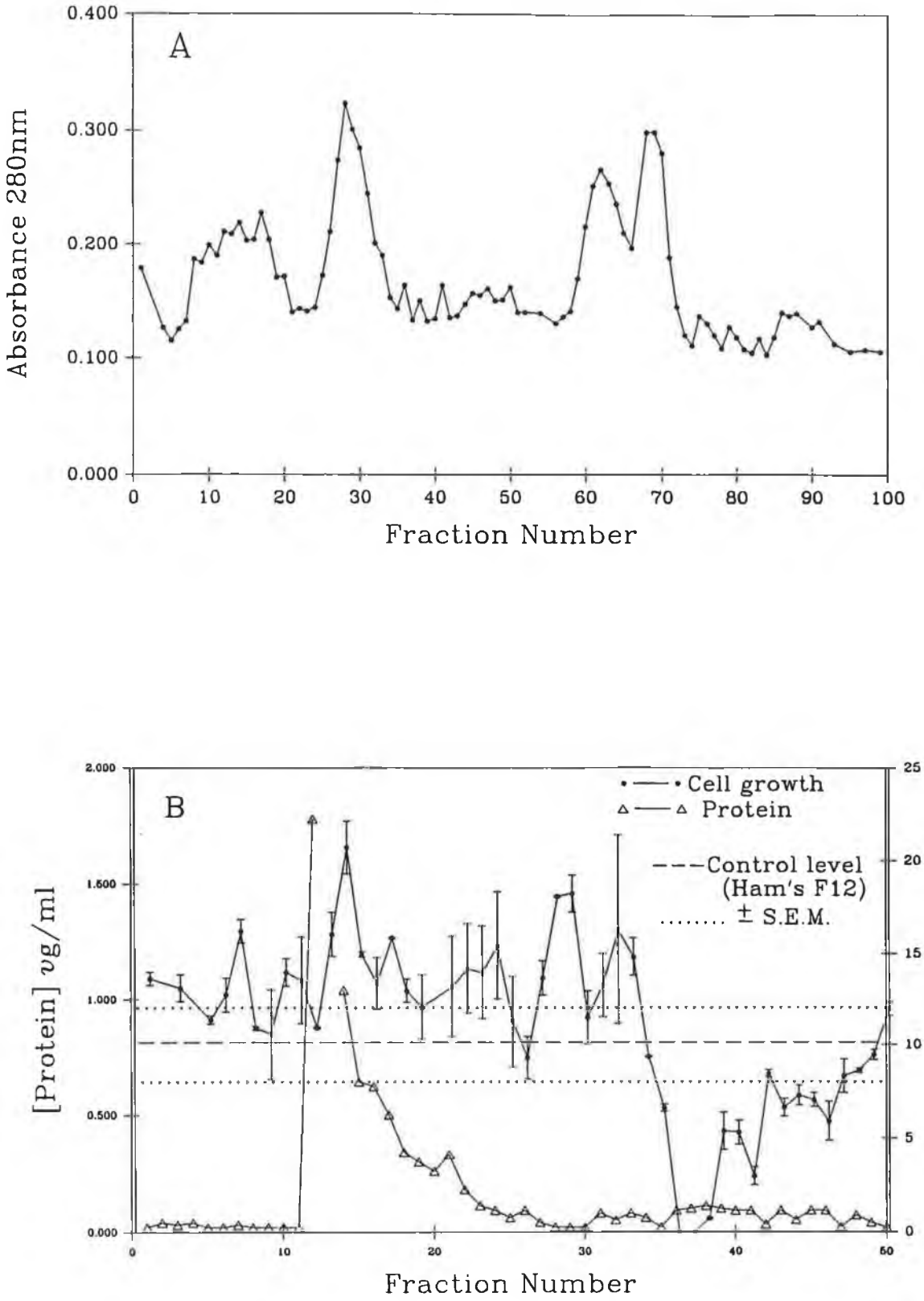


Figure 3.3.1 **Separation of HEp-2 CM, concentrated by ultrafiltration, on a BioGel P30 gel-filtration column. A; elution profile of a 20x (R1) CM concentrate. B; elution profile and growth stimulatory activity profile in a 63x (R1) CM concentrate.**

Autocrine activity eluted in a number of fractions containing different molecular weight species. Fractions 1 & 7 contained some stimulatory activity which eluted within the expected void volume. The principal stimulatory activity in the high molecular weight range was associated with fractions 13-15, eluting between 26-30 mls on the chromatogram, and this too is close to the expected exclusion limit for this gel (figure 3.3.1,B). Mitogenic activity in a single fraction (Nº 17) elutes at 34 mls. In the intermediate size range on this coulmn, three stimulatory activities elute. The first at 48 mls in fraction 24, and then two bands of activity in fractions 27-29 (54-58 mls) and 32 & 33 (64-66 mls). A broad band of inhibitory activity eluted from fraction 35 to 46 (70-92 mls).

Although molecular weight markers were not run on this column, the data confirm the predictions for the size of autocrine stimulatory and inhibitory activity. It would appear that at least one large (i.e > 30 kDa) and at least three intermediate molecular weight stimulators (i.e, 10-30 kDa) are present in HEp-2 CM, as well as a low MW inhibitory activity.

3.3.2 THE USE OF HEPARIN-SEPHAROSE AFFINITY CHROMATOGRAPHY IN THE PURIFICATION OF HEp-2 AUTOCRINE ACTIVITY

Several classes of growth factors have been shown to have an affinity for immobilised heparin. Among those with the greatest affinity are the so-called fibroblast growth factors (FGFs). Heparin affinity chromatographic systems has been successfully applied to the purification of FGFs because of their tight binding and differential elution from this matrix. The following experiment was performed to determine whether the autocrine activity in HEp-2 CM was heparin binding.

3.3.2.1 Preliminary evidence for heparin-binding autocrine activity in concentrated HEp-2 CM: Batch elution in NaCl from heparin-Sepharose

A 2 ml sample of the 60x concentrate of HEp-2 CM used in Section 3.3.1 was applied to a 5 ml heparin-Sepharose column and bound material was eluted by successive batches of running buffer containing NaCl with incremental increases in molarity from 0.1 - 5.0 M NaCl as described in Section 2.11.2.1. Sample dilutions were made in serum-free Ham's F12 such that the final concentration was equivalent to 1x with respect to the 60x sample loaded. Dialysed and filter-sterilised fractions from this separation were assayed for autocrine activity and the results are presented in Table 3.3.2.1.

Table 3.3.2.1 Elution of HEp-2 CM autocrine activity from a heparin-Sepharose column with a 0 - 5M NaCl step gradient.

Fraction [NaCl] Molar	Dilution Equivalent[a]	Colony ± S.E.M. Area mm^2 mm^2	Fold Stimulation w.r.t. Control[b]
Wash	1/20	0.52 ± 0.13	N.S.
0.1 M	1x	0.65 ± 0.21	N.S.
0.5 M	1x	0.66 ± 0.12	N.S.
1.0 M	1x	1.07 ± 0.43	3.24
1.5 M	1x	2.18 ± 1.18	6.61
2.0 M	1x	2.55 ± 0.87	7.73
5.0 M	1x	1.42 ± 0.48	4.30
Ham's F12 Control		0.33 ± 0.18	-

[a] Dilutions were made to ensure that all samples were at a concentration equivalent to the untreated CM (i.e. 1x). [b] Fold stimulation was calculated with respect to the level of growth in the control medium. N.S; No significant increase with respect to the control.

The control level of growth in this experiment was very low. However, significant stimulation above the control value was apparent in all eluates greater than 1.0 M NaCl. The largest fold stimulation over control eluted in the 1.5 and 2.0 M NaCl fractions respectively.

3.3.2.2 Separation of autocrine activity in HEp-2 CM by heparin-Sepharose chromatography using a 0 - 2.5 M continuous NaCl gradient

The aim of this experiment was to define the precise affinity of the heparin-binding autocrine mitogens. 300 mls of crude HEp-2 CM was pre-equilibrated in running buffer (100mM phosphate pH 7.4 using a Sephadex G-15 as described in Section 2.11.1.1) and then loaded onto a 10 ml heparin-Sepharose column. A continuous NaCl gradient of 0-2.5 M salt was applied as described in Section 2.11.2.2. The bulk of the protein in the CM washed through the column in the first 150 mls before the application of the gradient. 28 × 5 ml fractions were collected after 132 mls had run through. The elution profile from 82 mls to the end of the chromatogram is shown in figure 3.3.2.2.

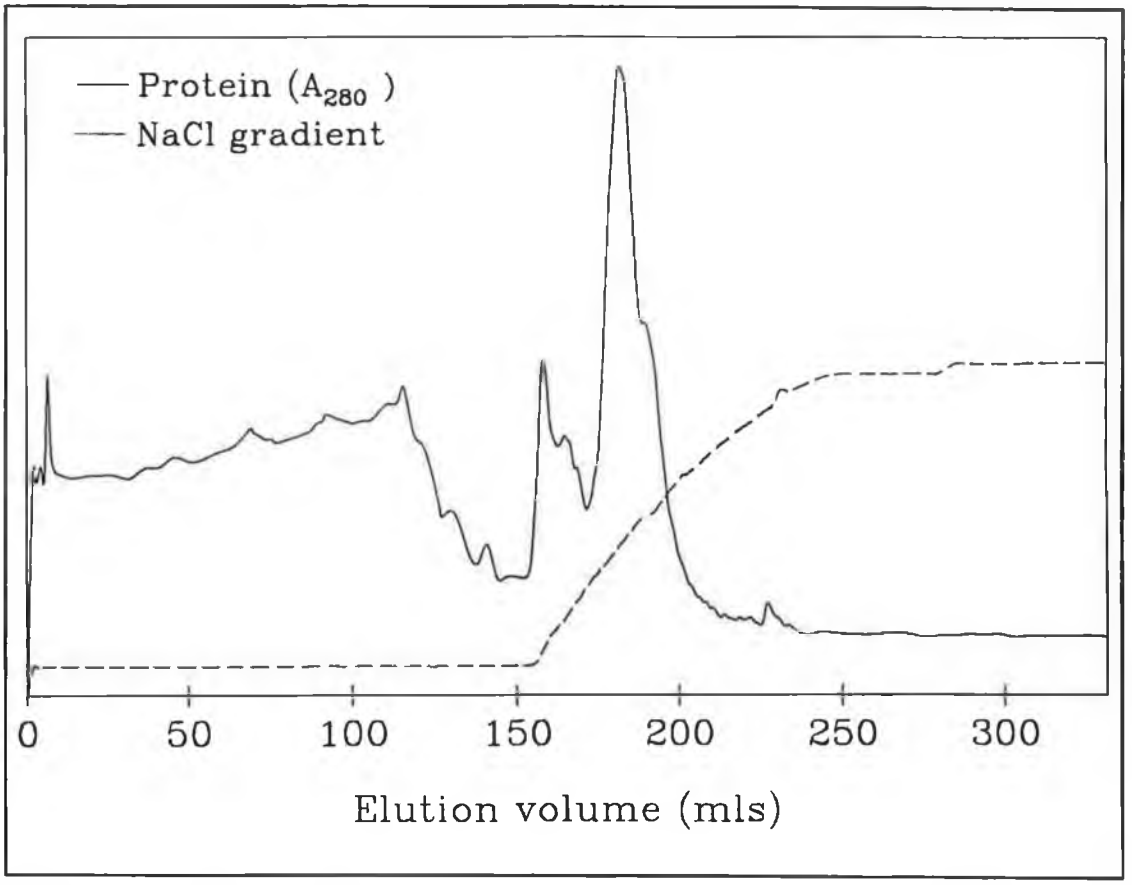


Figure 3.3.2.2 The separation of concentrated (63x) Hep-2 CM on a 10 ml heparin-Sepharose column using a 0-2.5 M NaCl gradient.

Two heparin-bound protein peaks eluted from the column on the NaCl gradient. The first from 0-0.7 M salt, the second from 0.7-1.8 M salt.

All fractions were dialysed and filter-sterilised before assay as described in Sections 2.8.2 and 2.1.3 respectively. The results of the experiment are presented in Table 3.3.2.2 parts a, b and c.

Table 3.3.2.2a Separation of HEp-2 autocrine activity using a continuous NaCl gradient on heparin-Sepharose

Fraction Dilutions		None	1/5
FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
1	0.00	0.00 ± 0.00	3.80 ± 1.09
2	0.00	0.00 ± 0.00	3.92 ± 0.73
3	0.00	0.00 ± 0.00	3.24 ± 0.27

Table 3.3.2.2b

Fraction Dilutions		1/2	1/4	1/10
FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
4	0.00	0.85 ± 0.34	2.62 ± 0.40	2.80 ± 1.16
5	0.00	2.79 ± 0.37	2.28 ± 0.80	3.14 ± 0.40
6	0.10	0.17 ± 0.10	1.84 ± 0.58	2.62 ± 0.35
7	0.33	0.59 ± 0.15	2.91 ± 0.06	2.90 ± 0.87
8	0.50	4.20 ± 0.43	4.95 ± 1.34	3.75 ± 0.24
9	0.70	0.00 ± 0.00	2.77 ± 0.23	5.69 ± 0.86
10	0.86	0.18 ± 0.06	3.02 ± 0.10	4.34 ± 0.39
11	1.00	1.02 ± 0.39	4.73 ± 0.59	6.08 ± 0.73
12	1.20	2.93 ± 0.92	6.54 ± 1.48	6.39 ± 1.32
13	1.30	6.79 ± 1.07	6.52 ± 1.94	5.67 ± 1.07
14	1.50	7.52 ± 1.63	6.68 ± 0.84	4.42 ± 0.57

Table 3.3.2.2c

Fraction Dilutions		None	1/5
FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
15	1.50	0.00 ± 0.00	7.86 ± 0.68
16	1.73	0.00 ± 0.00	8.20 ± 0.85
17	1.86	0.00 ± 0.00	0.00 ± 0.00
18	1.96	0.00 ± 0.00	4.06 ± 0.88
19	2.10	0.00 ± 0.00	7.41 ± 1.71
20	2.20	0.00 ± 0.00	2.43 ± 0.27
21	2.30	0.00 ± 0.00	3.09 ± 0.93
22	2.40	0.00 ± 0.00	3.84 ± 1.11
23	2.45	0.00 ± 0.00	5.11 ± 0.59
24	2.48	0.00 ± 0.00	6.17 ± 0.85
25	2.50	0.00 ± 0.00	6.08 ± 0.85
26	2.50	0.00 ± 0.00	6.90 ± 0.73
27	2.50	0.00 ± 0.00	2.01 ± 0.23
28	2.50	0.00 ± 0.00	4.03 ± 0.82
EXPERIMENT CONTROLS			
Dialysed Conditioned Medium			0.00 ± 0.00
Conditioned Medium - Column Wash Through			0.00 ± 0.00
Conditioned Medium - Post Gel filtration[a]			0.00 ± 0.00
Ham's F12 Control Medium			**3.84 ± 0.69**

n=3 for all determinations unless otherwise stated. [a] Sample of CM after desalting procedure on Sephadex G-15 column.

Inhibition was apparent in fractions eluted from the column which had been assayed without dilution. This was unlikely to be due to high osmolarity because of the extensive dialysis performed. The only practical alternative to desalting large numbers of fractions from a heparin-Sepharose column was to increase the magnitude of dilution used. Klagsbrun *et al.*, (1987) recommend that no more than 10 μl of a fraction containing 1.5 M NaCl should be added to 200 μl of medium on top of cells. In this experiment the complete inhibition of growth in CM dialysed as a control indicates that dialysis and not high salt concentration caused the reduction in growth. At dilutions of ¼ and greater, inhibition was eliminated. Nevertheless, in one fraction complete inhibition was still detected at a 1/5 dilution. This inhibitory activity eluted at a concentration of 1.86-1.96 M NaCl in fraction 17.

Autocrine stimulatory activity was detected as growth over control in two major regions of the gradient. The first eluted in fractions 11-16, between 1.0 and 1.9 M NaCl and the second activity eluted at 2.45-2.50 M NaCl in fractions 24-26. Two mitogens eluted in single fractions from the gradient at 0.70 M and 2.1 M NaCl in fractions 9 and 19 respectively.

3.3.3 HEPARIN-SEPHAROSE CHROMATOGRAPHY OF HEp-2 CELL EXTRACTS

In other cell systems where the heparin-binding acidic and basic fibroblast growth factors are produced, higher concentrations of these species have been measured intracellularly {Klagsbrun *et al.*, (1987)}. The evidence for autocrine stimulatory activities with high affinities for heparin prompted the next experiments with HEp-2 extracts.

Two HEp-2 cell extracts were prepared in buffers with high and low salt respectively. Both contained a cocktail of protease inhibitors and 1% w/v CHAPS detergent (to avoid protein degradation and denaturation). The pH was buffered with 20 mM HEPES to pH 7.5 as described in Section 2.10.1. Before heparin-Sepharose chromatography, extracts were equilibrated in running buffer using a Sephadex G-15 column as described in Section 2.11.1.1. Protein in high salt extract was retrieved in 10 mls of 100 mM phosphate (pH 7.4) and that in low salt extract in 13 mls.

3.3.3.1 The separation of High Salt HEp-2 Cell Extract on heparin-Sepharose

An 8 ml sample of high salt extract was applied to a 10 ml column and a 0 - 3 M NaCl gradient developed at a flow rate of 1 ml/minute as described in Section 2.11.2.2. A total of 64 × 3 ml fractions were collected from the start of the wash through to 192 mls eluted in total. The elution profile is shown in figure 3.3.3.1.

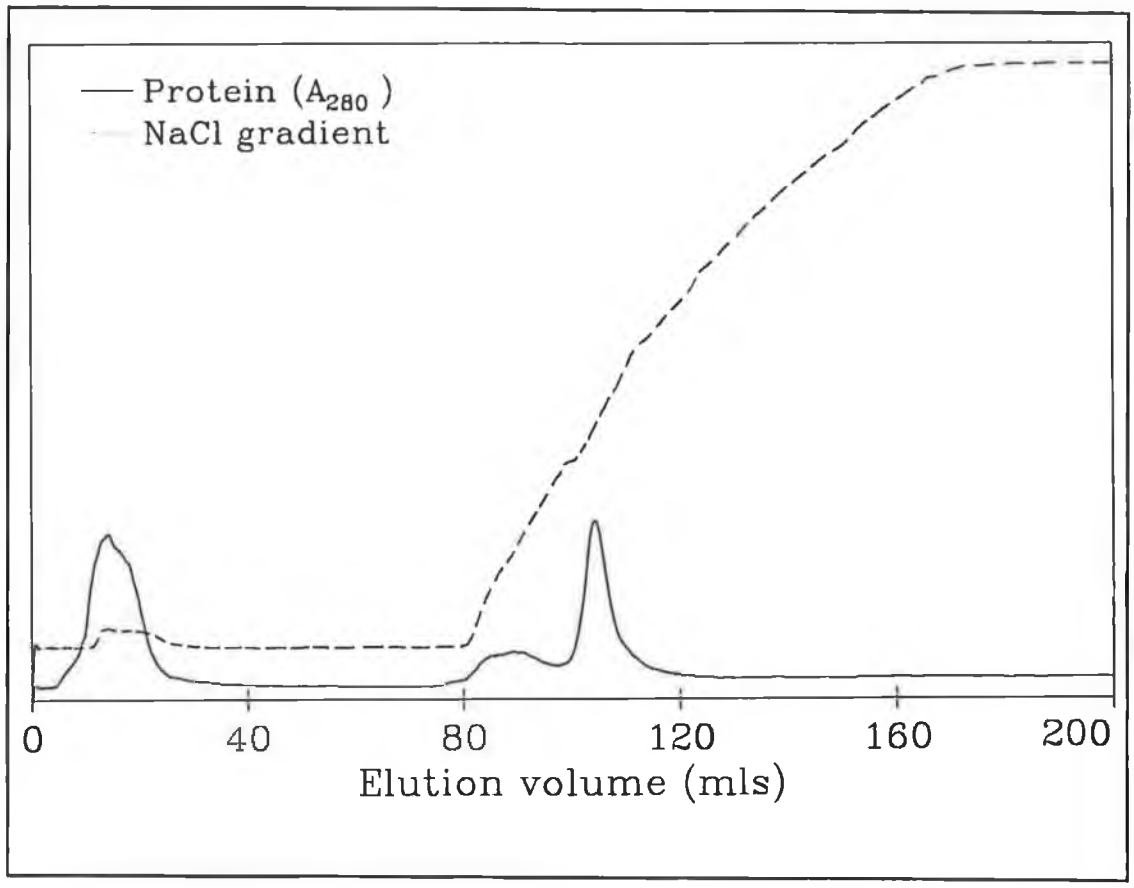


Figure 3.3.3.1 The separation of HEp-2 cell extract (in 1.5 M NaCl) using a 0-3 M NaCl gradient on a 10 ml heparin-Sepharose column.

Three protein peaks separated on the heparin column. The first, eluting after 40 mls had no affinity for heparin. A broad band of protein eluted from the start of the gradient up to 1.0 M NaCl. A second sharper band with higher affinity eluted between 1.0 and 1.65 M NaCl. The latter peak was centered at 1.4 M NaCl on the gradient.

The following fractions were dialysed (see Section 2.8.2 for details) and assayed after filter-sterilisation in the HEp-2 autocrine assay. Fractions 1-19, (0-57 mls, inclusive of the non-bound protein peak); fractions 23-39 (66-117 mls, inclusive of the two heparin-binding protein peaks); fraction 42, and every third fraction thereafter. 1 ml of cell extract (from the Sephadex G-15 column) was dialysed as a control. Samples were serially diluted in serum-free Ham's F12 to a final volume of 0.5 ml before being plated in the assay, the results of which are presented in Table 3.3.3.1a.

Table 3.3.3.1a Elution of autocrine activity in HEp-2 high salt extract from heparin-Sepharose

Fraction Dilutions		1/5	1/25	1/50
FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
1	0.00	2.10 ± 0.71	2.92 ± 0.97	2.11 ± 0.62
2	0.00	1.43 ± 0.28	2.86 ± 0.38	2.91 ± 0.66
3	0.00	2.79 ± 0.68	2.39 ± 0.63	3.12 ± 0.71
4	0.00	4.20 ± 0.88	4.53 ± 0.46	4.30 ± 0.45
5	0.00	2.79 ± 0.52	5.56 ± 1.26	0.00 ± 0.00
6	0.00	2.44 ± 0.56	4.93 ± 0.91	5.38 ± 1.01
7	0.00	4.00 ± 0.72	4.83 ± 0.92	4.74 ± 1.02
8	0.00	4.04 ± 0.65	4.61 ± 0.78	5.39 ± 0.99
9	0.00	5.70 ± 0.91	4.63 ± 0.44	3.35 ± 0.81
10	0.00	3.06 ± 0.63	2.15 ± 0.41	5.40 ± 0.36
11	0.00	4.91 ± 0.50	4.67 ± 0.83	3.80 ± 0.84
12	0.00	4.17 ± 0.24	4.30 ± 1.17	3.28 ± 0.53
13	0.00	3.18 ± 0.43	4.77 ± 0.63	4.14 ± 0.97
14	0.00	2.57 ± 0.47	4.90 ± 0.92	5.20 ± 0.85
15	0.00	4.03 ± 0.46	4.32 ± 0.76	5.47 ± 0.69
16	0.00	4.23 ± 0.85	4.90 ± 0.50	5.76 ± 0.93
17	0.00	3.22 ± 0.48	4.37 ± 0.61	6.63 ± 0.57
18	0.00	1.41 ± 0.25	3.98 ± 0.39	3.65 ± 0.71
19	0.00	4.20 ± 0.69	7.30 ± 0.90	4.77 ± 0.90

Fraction Dilutions		1/5	1/25	1/50
FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
22	0.00	5.67 ± 0.73	8.20 ± 2.23	5.97 ± 1.18
23	0.00	4.37 ± 1.27	5.66 ± 0.74	6.79 ± 1.70
24	0.00	5.45 ± 1.49	4.97 ± 1.16	3.96 ± 1.69
25	0.10	N.D.	4.25 ± 1.10	6.30 ± 1.67
26	0.25	4.24 ± 0.77	6.38 ± 0.81	7.49 ± 2.24
27	0.40	2.96 ± 0.89	6.85 ± 2.46	5.08 ± 0.97
28	0.62	0.32 ± 0.21	3.59 ± 1.80	4.00 ± 0.94
29	0.70	4.04 ± 1.02	8.69 ± 3.18	7.07 ± 2.17
30	0.90	2.08 ± 0.75	4.00 ± 0.57	3.92 ± 1.23
31	1.00	0.00 ± 0.00	0.18 ± 0.13	3.28 ± 1.79
32	1.16	4.72 ± 1.62	2.27 ± 0.42	2.52 ± 0.59
33	1.36	3.60 ± 1.02	2.39 ± 0.75	1.26 ± 0.45
34	1.50	3.64 ± 1.49	2.58 ± 1.19	2.26 ± 0.32
35	1.63	4.16 ± 0.62	3.90 ± 0.41	1.73 ± 0.61
36	1.70	3.71 ± 0.72	3.96 ± 0.88	3.71 ± 0.79
37	1.78	2.15 ± 0.20	3.42 ± 0.89	4.04 ± 0.30
38	1.89	3.62 ± 0.99	2.94 ± 0.79	4.18 ± 0.57
39	2.00	4.82 ± 0.55	0.49 ± 0.11	0.72 ± 0.19
42	2.26	1.33 ± 0.09	0.32 ± 0.10	0.91 ± 0.46
45	2.50	1.79 ± 0.68	0.71 ± 0.38	1.18 ± 0.36
48	2.72	2.84 ± 0.71	1.92 ± 0.69	1.08 ± 0.50
51	2.90	1.45 ± 0.69	1.99 ± 0.94	1.62 ± 0.94
54	3.00	3.43 ± 0.40	3.93 ± 0.76	3.93 ± 0.69
57	3.00	3.06 ± 0.42	3.34 ± 1.07	3.83 ± 1.56
60	3.00	4.15 ± 1.01	3.73 ± 0.65	4.88 ± 0.95
CELL EXTRACT[a]		3.77 ± 1.97	6.46 ± 1.64	5.89 ± 1.38
Ham's F12 Experiment Control Mean = 2.35 ± 1.34 mm^2				

[a] Desalted by passage through a Sephadex G-15 column. N.D; not determined.

The significant inhibiton detected in 1/25 and 1/50 dilutions of fractions 39 and 42 (eluting at 2.0 and 2.26 M NaCl respectively) is most likely explained by poor growth on the particular plate on which these samples were analysed. The plate control in this case at 0.01 mm^2 was barely detectable (compared to the experiment control). For technical reasons mentioned in Section 3.2.3.2, interplate variations in plating densities are more likely to occur in large experiments where many plates must be seeded.

Growth in the assayed fractions was only considered significantly greater than control if the sample mean value *minus the standard error on that determination*, was greater than the value for the experiment control mean value *plus the standard error on that determination*. The levels of growth supported by the fractions eluted in this separation were at best only slightly in excess of the control. In order to analyse these results with greater clarity the original data in Table 3.3.3.1a are presented in Table 3.3.3.1b as *absolute growth in excess of the control mean* {sample mean - control mean}. Only fractions which demonstrated significant stimulation as outlined above were treated in this way.

Table 3.3.3.1b Absolute growth over control in High Salt HEp-2 extract separated on heparin-Sepharose

FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	FOLD STIMULATION IN SAMPLES RELATIVE TO THE Ham's F12 MEDIUM CONTROL		
SAMPLE DILUTIONS		1/5	1/25	1/50
4	0.00	-	2.18	1.95
5	0.00	-	3.21	-
6	0.00	-	2.58	3.03
7	0.00	-	2.48	2.39
8	0.00	-	2.26	3.04
9	0.00	3.35	2.28	-
10	0.00	-	-	3.05
11	0.00	2.56	2.32	-
12	0.00	1.82	-	-
13	0.00	-	2.42	-
14	0.00	-	2.55	2.85
15	0.00	-	-	3.12
16	0.00	-	2.55	3.41
17	0.00	-	2.02	4.28
19	0.00	-	4.95	2.42
22	0.00	3.32	5.85	3.62
23	0.00	-	3.31	4.44

FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	FOLD STIMULATION IN SAMPLES RELATIVE TO THE Ham's F12 MEDIUM CONTROL		
SAMPLE DILUTIONS		1/5	1/25	1/50
24	0.00	3.10	2.62	-
25	0.10	-	-	3.95
26	0.25	-	4.03	5.14
27	0.40	-	4.50	2.73
29	0.70	-	6.34	4.72
37	1.78	-	-	1.69
39	2.00	2.47	-	-
60	3.00	-	-	2.53
Cell Extract	1.50	-	4.11	3.54

On the basis of the data in Table 3.3.3.1b there was some evidence for the presence of high affinity heparin-binding stimulatory activity in the HEp-2 (high salt) extract, although it should be noted that fractions were stored at 4°C for 7 days before being assayed which may have reduced the activity of the heparin-binding species particularly. Stimulatory activity was also recovered from this separation in the non-heparin binding eluate.

Three heparin-binding stimulatory activities appeared to elute on the gradient. The first was associated with the weak to moderate affinity heparin-binding protein peak. Autocrine stimulatory activity with weak affinity for heparin eluting between 0.10-0.40 M NaCl (fractions 25-27, after 72 mls), and activity with moderate affinity eluting between 0.70-0.85 M NaCl (fraction 29, after 84 mls), account for the first species. The second activity eluted at the trailing end of the high affinity protein peak at 1.78 and 2.0 M NaCl (fractions 37 & 39, between 108 and 114 mls). The third activity with very high affinity, eluted at 3.0 M NaCl (fraction 60, after 180 mls).

Mitogenic activities eluting from the gradient at 0.7 and 2.1M NaCl were also detected in HEp-2 CM separated on a 10 ml heparin-Sepharose column developed with a 0-2.5 M NaCl continuous gradient (Section 3.3.2.2). However, a low affinity heparin-binding mitogen similar to the one detected in the cell extract was not isolated from the CM sample.

In the case of the crude 'desalted' cell extract control, evidence for the presence of stimulatory activity was only clear at a 1/25 dilution of this sample. Further dilution to 1/50 did not appear to significantly reduce this level of growth.

3.3.3.2 The separation of Low Salt HEp-2 Cell Extract on heparin-Sepharose

A 10 ml sample of low salt HEp-2 cell extract was loaded onto the same 10 ml heparin-Sepharose column described in the separation of high salt extract (after suitable washing in high salt buffer, see Section 2.11.2.2). The column was run under the same conditions on the FPLC system and sixty 3 ml fractions were collected. The elution profile is shown in figure 3.3.3.2.

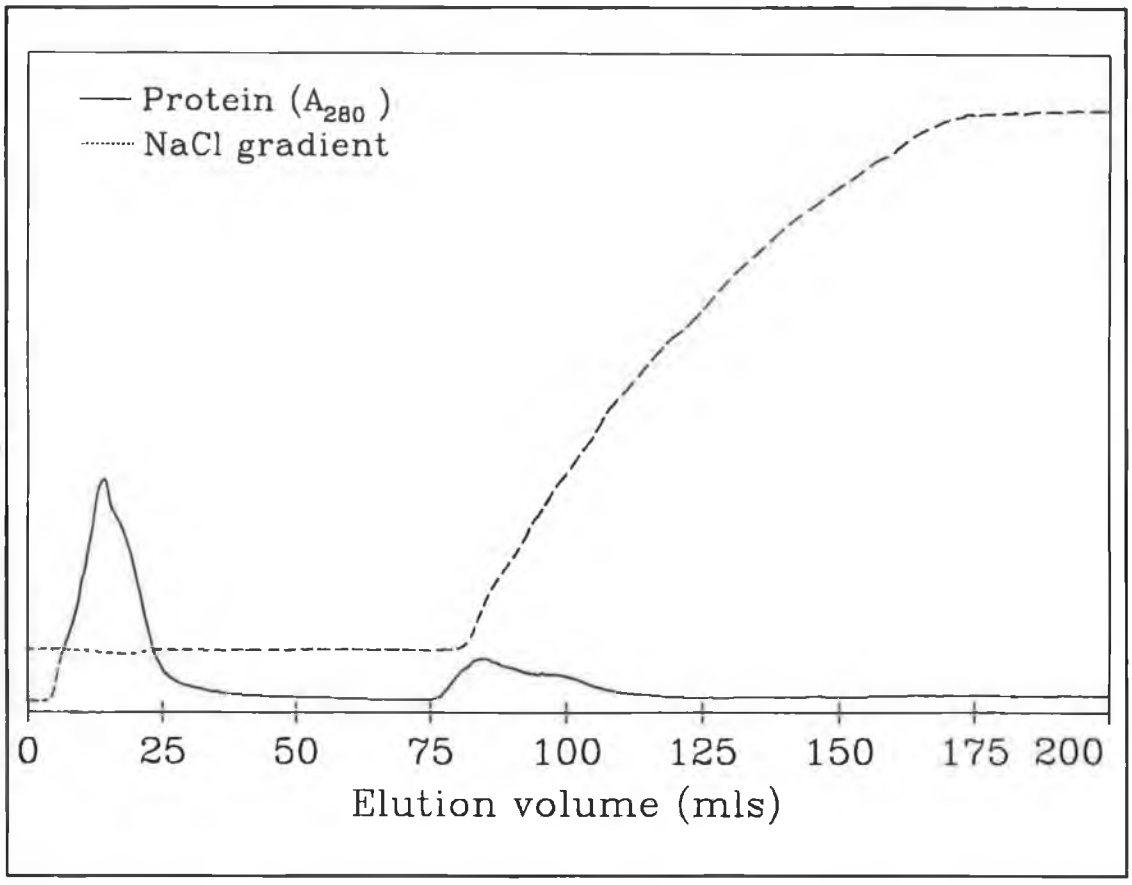


Figure 3.3.3.2 The separation of HEp-2 cell extract (in Ham's F12) using a 0-3 M NaCl gradient on a 10 ml heparin-Sepharose column.

Unbound protein eluted in the first 40 mls as with the high salt extract. In this case however, only a weak affinity heparin-binding peak eluted from the gradient between 0-1.0 M NaCl, and centered at 0.5 M NaCl. The high affinity binding mitogenic species present in the high salt extract was absent from this separation.

Several representative fractions were selected from the non-bound protein (N^{o}s 2,6,9 and 12) and heparin-bound protein (N^{o}s 24,26,29,30,32,34,36,38,and 40) eluting from the column. These were dialysed as described in Section 2.8.2 and assayed in the same assay as the high salt fractions in Section 3.3.3.1. The original data of this experiment are shown in Table 3.3.3.2(i)a.

Table 3.3.3.2(i)a Autocrine activity in Low Salt HEp-2 Cell Extract separated on heparin-Sepharose

Fraction Dilutions		1/5	1/25	1/50
FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
2	0.00	5.84 ± 0.04	3.56 ± 0.31	5.36 ± 0.36
6	0.00	4.75 ± 0.34	3.46 ± 0.06	2.62 ± 0.34
9	0.00	4.22 ± 0.01	3.76 ± 0.08	0.14 ± 0.06
12	0.00	1.52 ± 0.02	3.84 ± 0.43	5.24 ± 0.55
24	0.00	6.95 ± 0.94	4.67 ± 0.61	3.13 ± 0.59
26	0.25	5.42 ± 0.18	6.17 ± 1.66	0.50 ± 0.19
29	0.79	1.32 ± 0.47	1.08 ± 0.47	0.44 ± 0.05
30	0.94	1.01 ± 0.06	1.08 ± 0.15	1.06 ± 0.16
32	1.23	1.48 ± 0.70	1.52 ± 0.27	2.01 ± 0.59
34	1.51	2.02 ± 0.79	1.31 ± 0.47	1.63 ± 0.54
36	1.70	2.49 ± 0.43	3.56 ± 0.98	3.32 ± 0.66
38	1.86	5.20 ± 0.32	4.05 ± 0.26	2.24 ± 0.74
40	2.11	4.65 ± 1.48	4.27 ± 1.32	4.68 ± 1.12
Ham's F12 Experiment Control Medium = 2.35 ± 1.34 mm^2				

Table 3.3.3.2(i)b presents these results as absolute growth over the control (scored on the same basis as in Section 3.3.3.1).

Table 3.3.3.2(i)b Absolute autocrine stimulation over control in Low Salt HEp-2 extract separated on heparin-Sepharose

FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	FOLD STIMULATION IN SAMPLES RELATIVE TO THE Ham's F12 MEDIUM CONTROL		
SAMPLE DILUTIONS		1/5	1/25	1/50
2	0.00	3.49	-	3.01
6	0.00	2.40	-	-
9	0.00	1.87	-	-
12	0.00	-	-	2.89
24	0.00	4.60	2.32	-
26	0.25	3.07	3.82	-
38	1.86	2.85	1.70	-

Two heparin-binding autocrine stimulatory activities eluted from the gradient in this case. The first was associated with the weakly heparin-bound peak in fractions 24 & 26 (from 0-0.5 M NaCl). A second tightly bound activity eluted at 1.95 M NaCl in fraction 38. Autocrine stimulatory activity eluted from the column at the same NaCl concentrations during the separation of high salt extract.

Non-bound autocrine activity was also detected in the eluate of this extract (fractions 2,6,9, and 12) consistent with the result of the high salt extract separation.

In the following assay the low salt extract fractions were assayed again. All fractions up to and including fraction 44 were included and every second fraction thereafter. To avoid the dialysis step, fractions not already dialysed were diluted directly into the assay. For this reason, higher dilutions were used in this experiment. Appropriate dilution controls included the equivalent volume for dilution of phosphate buffer with and without 3 M NaCl.

The original data for this experiment are presented in Table 3.3.3.2(ii)a, & b and fractions dialysed before the assay are denoted by shading in the tables.

Table 3.3.3.2(ii)a Autocrine activity in Low Salt HEp-2 Extract after separation on a heparin-Sepharose column

Fraction Dilutions		1/50	1/100
FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
1	0.00	18.38 ± 2.43	20.30 ± 1.36
2	0.00	18.50 ± 0.31	16.25 ± 1.76
3	0.00	22.66 ± 1.83	19.70 ± 2.54
4	0.00	22.26 ± 2.00	20.08 ± 0.97
5	0.00	22.96 ± 1.44	23.01 ± 0.58
6	0.00	20.27 ± 3.75	16.04 ± 3.16
7	0.00	21.06 ± 3.86	25.18 ± 2.00
8	0.00	21.70 ± 4.04	26.28 ± 1.57
9	0.00	25.28 ± 4.91	18.86 ± 2.19
10	0.00	22.31 ± 2.11	24.48 ± 0.73
11	0.00	23.91 ± 2.98	21.55 ± 0.45
12	0.00	23.63 ± 1.94	23.56 ± 2.80
13	0.00	20.41 ± 0.05	24.51 ± 1.88
14	0.00	25.46 ± 4.37	23.23 ± 2.21
15	0.00	15.93 ± 3.23	21.15 ± 0.86
16	0.00	18.11 ± 3.34	18.00 ± 2.88
17	0.00	15.71 ± 2.55	17.73 ± 2.53
18	0.00	18.65 ± 1.82	19.94 ± 3.24
19	0.00	21.90 ± 1.45	20.65 ± 2.77
20	0.00	17.61 ± 1.53	18.31 ± 5.60
21	0.00	20.27 ± 0.39	20.20 ± 2.00
22	0.00	16.72 ± 1.99	24.70 ± 2.26
23	0.00	19.69 ± 1.46	15.18 ± 0.83
24	0.00	17.97 ± 2.92	21.24 ± 3.13
25	0.08	19.57 ± 2.40	15.78 ± 4.61
26	0.25	25.29 ± 4.24	23.68 ± 3.39
27	0.47	22.81 ± 2.35	23.87 ± 2.93
28	0.64	20.12 ± 1.02	25.08 ± 3.56
29	0.79	12.84 ± 2.77	17.14 ± 1.05

Fraction Dilutions		1/50	1/100
FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
30	0.94	18.88 ± 2.95	16.07 ± 2.48
31	1.06	5.21 ± 0.88	16.27 ± 0.39
32	1.23	22.54 ± 3.54	11.39 ± 2.55
33	1.36	9.03 ± 1.37	16.20 ± 3.00
34	1.51	22.39 ± 1.83	14.67 ± 0.18
35	1.59	6.54 ± 1.39	13.66 ± 2.13
36	1.70	13.59 ± 4.48	18.42 ± 1.09
37	1.78	6.86 ± 1.87	11.79 ± 3.06
38	1.86	8.21 ± 4.71	15.48 n=1
39	1.97	0.00 ± 0.00	7.19 ± 2.10
40	2.11	20.60 ± 1.73	18.85 ± 1.69
41	2.16	2.31 ± 0.14	6.27 ± 4.22
42	2.24	0.09 ± 0.05	11.43 ± 5.31
43	2.32	0.58 ± 0.24	10.95 ± 4.00
44	2.40	1.36 ± 0.82	6.95 ± 3.40
46	2.55	0.12 ± 0.04	4.97 ± 2.54
48	2.70	0.81 ± 0.58	4.66 ± 1.18
50	2.85	0.52 ± 0.14	6.59 ± 1.03
52	2.95	0.20 ± 0.01	2.34 ± 1.70
54	2.99	0.23 ± 0.15	3.10 ± 1.45
56	3.00	0.02 ± 0.02	1.00 ± 0.80
58	3.00	0.00 ± 0.00	0.10 ± 0.02
60	3.00	0.39 ± 0.36	5.67 ± 2.92
EXPERIMENT CONTROLS			
CELL EXTRACT		5.10 ± 0.44	16.48 ± 2.09
Gel-filtered CELL EXTRACT		6.48 ± 2.65	27.59 ± 0.56
Phosphate Buffer - NaCl		21.54 ± 3.57	21.29 ± 4.53
Phosphate Buffer + 3M NaCl		0.17 ± 0.11	2.54 ± 1.16
MEDIUM (DMEM/Ham'F12)		16.87 ± 4.94	16.58 ± 5.01

Table 3.3.3.2(ii)b

Fraction Dilutions		1/200	Absolute Growth in Excess of the Control level
FRACTION NUMBER	Corresponding [NaCl] at gradient elution MOLAR	Colony ± S.E.M. Area mm^2 mm^2	
37	1.78	30.48 ± 8.05	12.81
38	1.86	34.52 ± 3.15	16.85
39	1.97	27.15 ± 3.49	9.48
40	2.11	30.59 ± 1.16	12.92
41	2.16	28.99 ± 1.70	11.32
42	2.24	32.29 ± 1.98	14.62
43	2.32	31.38 ± 1.21	13.71
44	2.40	17.00 ± 2.57	-
46	2.55	19.48 ± 1.73	-
48	2.70	20.31 ± 1.14	-
50	2.85	15.82 ± 2.24	-
Phosphate Buffer + 3 M NaCl		**17.67 ± 2.74**	-

Overall growth was higher in this experiment than the first experiment with heparin-purified extracts (3.3.3.1a). The controls with phosphate buffer plus 3 M NaCl were significantly reduced with respect to the medium control at 1/50 and 1/100 dilutions but at 1/200 the growth was in the same order as the medium control. Phosphate buffer without NaCl did not alter the level of control growth significantly with the volumes used to dilute undialysed samples. When the same criteria are used to score autocrine stimulation as outlined in Section 3.3.3.1, the following pattern emerges.

No autocrine stimulation was detected in fractions 1-30 (i.e. up to 1.1 M NaCl) at either dilution tested with respect to the phosphate controls. In fractions 31-40 inhibition (perhaps due to high osmolarity) becomes apparent at a 1/50 dilution but not in the dialysed fractions. No evidence for autocrine stimulation however was evident in the dialysed fractions. All fractions after Nº 40 show inhibition of growth which increases along with increasing NaCl concentration in later fractions from the gradient. It is impossible to draw any conclusions about stimulatory acitivity in fractions from this part of the separation (in the absence of proper controls for the salt inhibition).

No inhibition was apparent in fractions diluted 1/200. Significant stimulation over the phosphate control (with 3M NaCl) was observed in fractions 37-43 eluting between 1.8 and 2.4 M NaCl. This was consistent with the previous analysis of these fractions {Table 3.3.3.2(i)b} In Table 3.3.3.2(ii)b the excess growth over control is also presented.

In summary these experiments indicate that HEp-2 autocrine stimulatory activity may be recovered from the intracellular compartment of HEp-2 cells. Part of this activity has affinity for heparin. The heparin-binding autocrine activity can be divided into three classes. The first has a relatively low affinity for heparin, eluting from heparin-Sepahrose between 0-0.55 M NaCl. The second with an intermediate affinity for heparin and was recovered between 0.75-0.85 M NaCl. A third autocrine mitogenic species with high affinity eluted between 1.8 and 2.4 M NaCl. The intermediate affinity species was only recovered in the high salt HEp-2 extract.

3.3.4 CATION EXCHANGE CHROMATOGRAPHY

A two-step purification procedure using cation exchange followed by heparin-Sepharose chromatography has been successfully employed in the purification of several growth factors including a chondrosarcoma-derived basic FGF which was purified to apparent homogeneity using just these two steps {Shing *et al.*, (1983); Shing *et al.*, (1984)}. Given that at least part of the autocrine stimulatory activity in HEp-2 CM was heparin-binding, the use of this technique as a purification step in the separation of the HEp-2 autocrine mitogenic activity was investigated.

3.3.4.1 The use of CM-Sepharose fastflow in the purification of autocrine activity in HEp-2 conditioned medium

The conductivity of a 200 ml sample of HEp-2 conditioned medium was adjusted to 6 mS and the pH adjusted to 6.0 before it was applied to a 10 ml column of CM-Sepharose fastflow (Pharmacia). The column was developed as described in Section 2.11.3 and bound material eluted with 40 ml batches of buffer containing KCl with concentrations increasing incrementally. Because of its ability to stabilise heparin-binding growth factors and other unstable proteins, the detergent CHAPS was included in the running buffer (50 mM potassium phosphate) at a final concentration of 0.1% w/v. The elution profile for this separation is shown in figure 3.3.4.1.

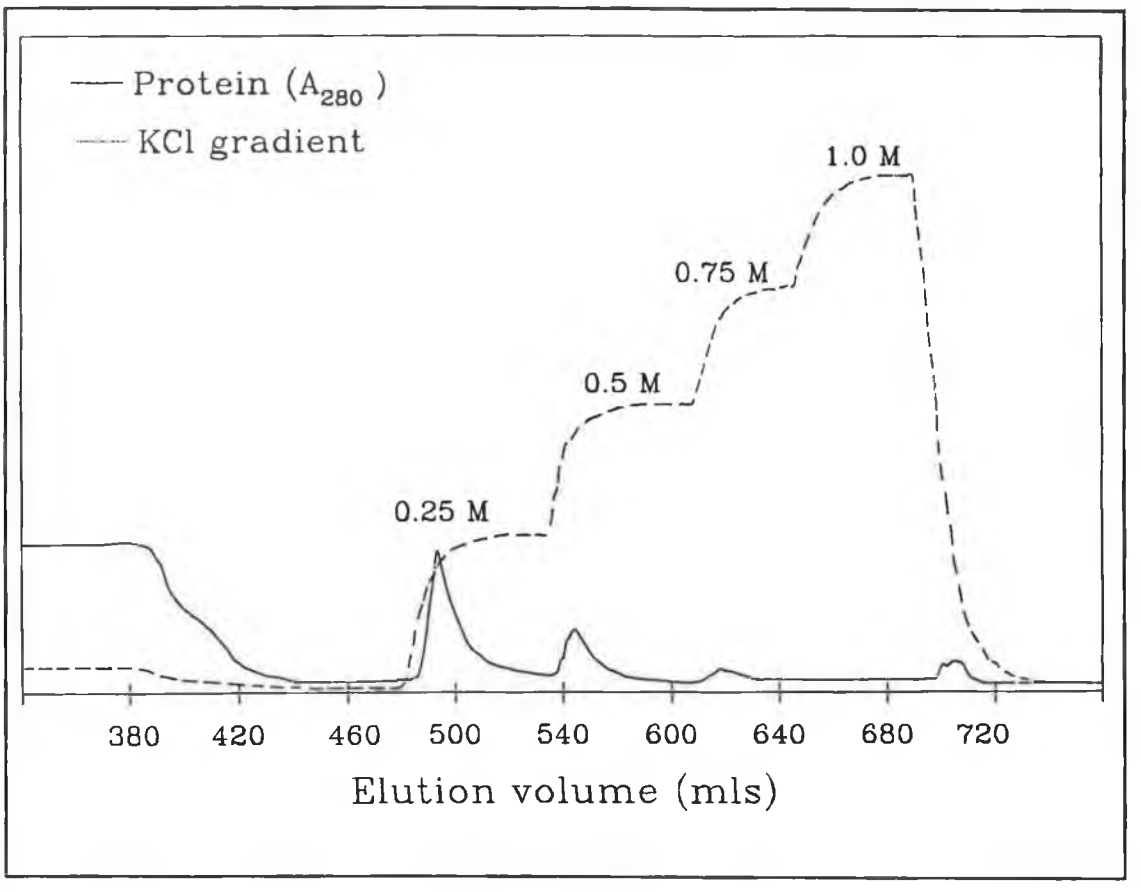


Figure 3.3.4.1 The separation of crude HEp-2 CM on a cation exchange column (CM-Sepharose Fastflow)

The elution profile showed that the bulk of the protein in HEp-2 CM did not bind to the column. Batch elution with KCl resulted in the separation of three protein peaks eluting at 0.25 M, 0.5 M, and 0.75 M KCl respectively.

Fractions of 20 mls were collected from the column after the start of the gradient. 5 mls of each fraction was dialysed as described in Section 2.8.2 and filter-sterilised before assay. As a control for the combined effects of dilution and dialysis, separate 5 ml aliquots of the same CM lot were (1) diluted in potassium phosphate, and (2) diluted in potassium phosphate and also dialysed with fractions from the column separation. The results of this experiment are presented in Table 3.3.4.1.

Table 3.3.4.1 The separation of HEp-2 autocrine activity using cation exchange chromatography

DILUTIONS	1/5	1/10	1/20
Fraction Number	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
1	3.21 ± 0.24	3.07 ± 0.50	2.61 ± 0.75
2	5.13 ± 0.42	4.94 ± 0.67	3.80 ± 0.52
3	2.68 ± 0.36	2.54 ± 0.23	2.34 ± 0.14
4	2.25 ± 0.21	2.92 ± 0.50	2.18 ± 0.09
5	2.77 ± 0.37	2.33 ± 0.30	1.80 ± 0.34
6	1.80 ± 0.12	1.59 ± 0.19	1.28 ± 0.14
7	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
8	1.97 ± 0.31	1.81 ± 0.30	2.22 ± 0.75
9	1.63 ± 0.14	1.68 ± 0.27	1.71 ± 0.17
10	0.85 ± 0.21	1.28 ± 0.34	1.46 ± 0.16
EXPERIMENT CONTROLS			
Ham's F12 +0.02% CHAPS	1.57 ± 0.25	1.84 ± 0.34	2.15 ± 0.17
Unbound Protein	N.D.	1.47 n=1	1.31 n=1
Column Wash through	N.D.	2.83 n=1	2.63 n=1
Dialysed CM	9.10 ± 0.94	5.46 ± 1.06	4.01 ± 0.96
Diluted[a] & Dialysed CM	N.D.	4.83 ± 0.28	4.52 ± 0.90
Diluted & Dialysed CM at a dilution equivalent to 1/40			2.43 ± 0.97
Unconcentrated Lot 8 CM (n=37)			30.00 ± 2.35
Ham's F12 Experiment Control Mean (n=32)			**1.33 ± 0.25**

n=3 unless otherwise stated. [a] Dilutions of this CM sample take into account that it had already been diluted ½ prior to the final ½ dilution in the assay. The actual dilution of this sample was half of that of the 'Dialysed CM' sample above it. N.D; not determined.

Autocrine stimulatory activity was recovered from the column in the first five fractions. Most of the stimulatory activity was present in fraction 2, which contains the bulk of the protein eluting at 0.25 M KCl. Significant stimulatory activity was also detected either side of this in fractions 1 & 3, but the activity in these fractions was lower than in fraction 2. A second stimulatory autocrine activity eluted in fractions 4 & 5 associated with the second band of protein to elute from the gradient in the 0.5 M KCl batch. Complete inhibition was associated with fraction 7 at all dilutions assayed. This corresponds to the third protein band which eluted from the column at 0.75 M KCl. There was evidence that stimulatory activity came through the column in the wash, however because of contamination in the assay only one determination was readable. No evidence for stimulatory activity was apparent in the bulk of unbound protein, but the same problem with contamination in replicate wells prevented the significance of this result being evaluated.

Dilution of the CM before dialysis did not significantly alter the autocrine stimulatory activity with respect to the 'dialysed only' CM at an equivalent dilution.

3.3.4.2 Reconstitution of HEp-2 CM fractions separated by cation exchange chromatography

Separation of two autocrine stimulatory and one inhibitory activity using cation exchange chromatography suggested that the overall autocrine effect might derive from the interaction of synergistic as well as antagonistic species in HEp-2 CM. To investigate this possibility the following experiment was performed.

In this pilot experiment every fraction from the separation in Section 3.3.4.1 was mixed 1:1 with the most active stimulatory fraction, fraction 2. All combinations were assayed at dilutions of 1/5 and 1/10 in the HEp-2 assay. CM controls included 'dialysed only', 'diluted ½ and dialysed', 'untreated CM'. The results of this experiment are presented in Table 3.3.4.2.

Table 3.3.4.2 The activity of reconstituted HEp-2 CM fractions separated by cation exchange chromatography

DILUTIONS	1/5	1/10
SAMPLE	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
Fractions 2 + 1	9.99 ± 1.10	11.08 ± 0.77
Fractions 2 + 3	9.54 ± 0.56	10.20 ± 0.15
Fractions 3 + 4	11.50 ± 0.81	9.49 ± 1.00
Fractions 2 + 5	7.70 ± 1.34	12.43 ± 0.45
Fractions 2 + 6	11.29 ± 0.28	9.94 ± 0.25
Fractions 2 + 7	11.18 ± 0.08	11.76 ± 1.15
Fractions 2 + 8	11.79 ± 1.82	12.98 ± 0.93
Fractions 2 + 9	11.52 ± 0.89	11.66 ± 0.20
Fractions 2 + 10	11.79 ± 0.81	11.39 ± 1.79
Fractions 2 + Unbound	8.13 ± 0.25	9.33 ± 0.46
Fraction 2 + Wash	9.08 ± 0.89	9.33 ± 0.05
EXPERIMENT CONTROLS		
Dialysed CM	4.76 ± 1.24	8.91 ± 1.66
Diluted & Dialysed CM	0.05 ± 0.03	9.18 ± 1.72
Untreated CM	11.37 ± 0.79	10.70 ± 1.75
Untreated CM, diluted ½		11.48 ± 1.13
Untreated & Undiluted CM		17.62 ± 2.89
Ham's F12 Experiment Control Mean = 6.62 ± 0.86 mm^2		

n=3 for all determinations.

Dialysis of CM caused the growth stimulatory response in this sample to be reduced. The inhibition was apparent at a dilution of 1/5 where growth in the dialysed CM was reduced significantly with respect to the untreated CM at an equivalent dilution. This 'dialysis effect' resulted in almost complete inhibition where the CM had been diluted prior to dialysis. Despite the effect of dialysis, stimulation over the control was detected in all of the fraction combinations. A 1/5 dilution of the fraction 5/fraction 2 combination supported only a control level of growth. This may suggest that the respective autocrine stimulatory species in these fractions are mutually antagonistic at this particular concentration. Alternatively, the dialysis effect noted above may account for the loss of stimulatory activity, because the same combination was mitogenic at a 1/10 dilution. The inhibitory species in fraction 7 (see previous Section 3.3.4.1) was masked by the addition of fraction 2.

3.3.5 A TWO STEP PURIFICATION OF THE HEp-2 AUTOCRINE ACTIVITY

The following experiment was performed to establish whether the autocrine stimulatory activity separated on the CM-Sepharose fastflow column at 0.25 M KCl (fraction №2, Section 3.3.4.1) could be further purified by heparin-Sepharose chromatography.

Briefly, a 10 ml sample of the 0.25 M KCl eluate from the CM-Sepharose separation above (Section 3.3.4.1) was diluted ½ with 50 mM potassium phosphate buffer, pH 7.4. The diluted CM was applied to a 10 ml heparin-Sepharose column and a 0 - 3 M linear NaCl gradient was developed in the running buffer (100 mM phosphate, pH 7.4). Sixty 3 ml fractions were collected at a flow rate of 1 ml/minute. The elution profile is shown in figure 3.3.3.5.

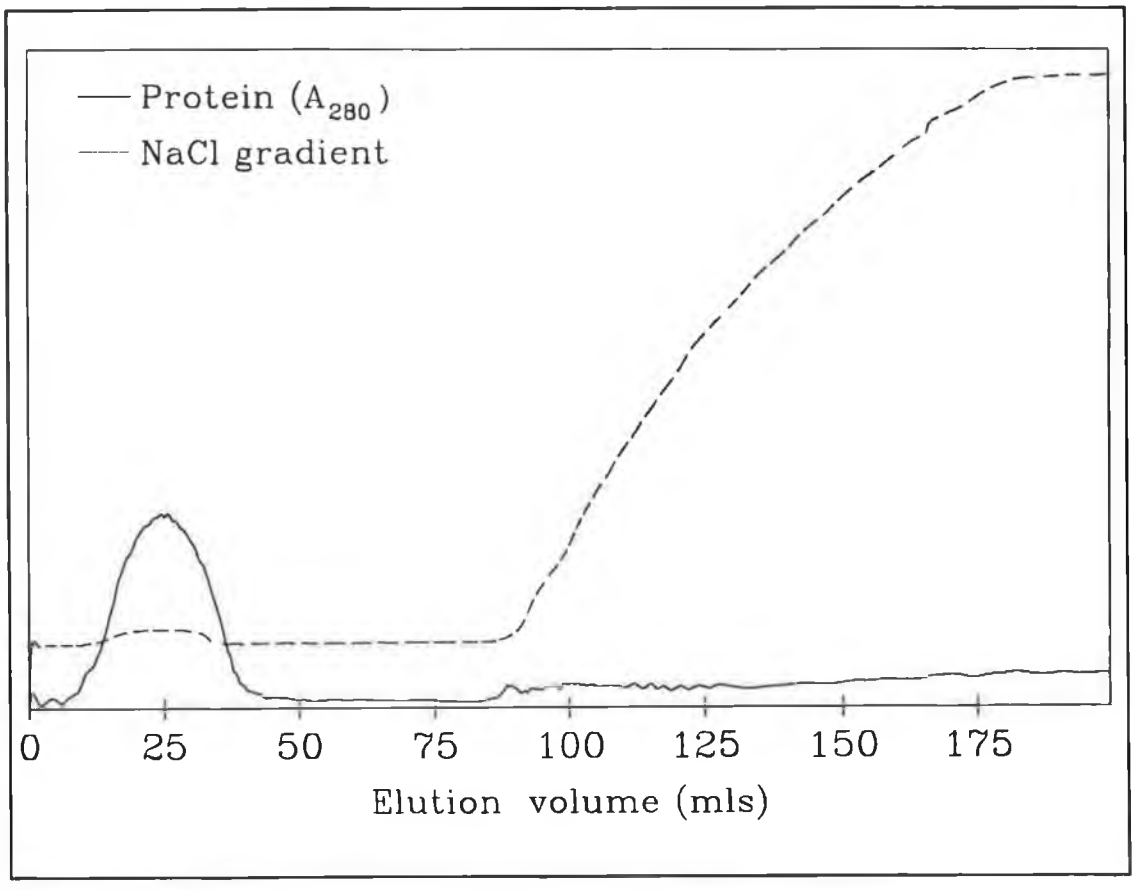


Figure 3.3.3.5 The separation of a partially purified fraction of HEp-2 CM (eluted from CM-Sepharose fastflow column at 0.25 M KCl) using a 0-3 M NaCl gradient on a 10 ml heparin-Sepharose column

The bulk of the protein in this sample did not bind to heparin, but eluted in the first 50 mls of the separation, and before the gradient. No discernable protein peaks eluted with the gradient.

Fractions (stored at -20°C before assay) were diluted 1/100 in serum-free Ham's F12 before the final ½ dilution in the assay. The 0.25 M KCl eluate was included as a control in this experiment. The results are presented in Table 3.3.5.

Table 3.3.5 Autocrine activity from the 0.25 M KCl cation exchange eluate separated on heparin-Sepharose

Dilution	1/100	Dilution	1/100
Fraction N° {[NaCl]}	Colony ± S.E.M. Area mm^2 mm^2	Fraction N° {[NaCl]}	Colony ± S.E.M. Area mm^2 mm^2
1 {0.00}	7.01 ± 0.64	24 {0.00}	17.54 ± 1.83
2 {0.00}	14.37 ± 0.21	25 {0.00}	0.08 ± 0.05
3 {0.00}	16.28 ± 1.55	26 {0.00}	16.64 ± 1.24
4 {0.00}	16.31 ± 2.08	27 {0.00-0.07}	0.00 ± 0.00
5 {0.00}	10.62 ± 0.85	28 {0.07-0.26}	18.97 ± 1.65
6 {0.00}	13.87 ± 1.26	29 {0.26-0.38}	14.38 ± 1.03
7 {0.00}	15.05 ± 1.98	30 {0.38-0.58}	15.76 ± 1.78
8 {0.00}	12.43 ± 0.30	31 {0.58-0.74}	0.00 ± 0.00
9 {0.00}	13.97 ± 0.62	32 {0.74-0.90}	15.20 ± 1.58
10 {0.00}	14.65 ± 0.88	33 {0.90-1.05}	16.14 ± 1.73
11 {0.00}	13.71 ± 2.13	34 {1.05-1.16}	17.13 ± 0.45
12 {0.00}	12.39 ± 1.04	35 {1.16-1.28}	17.71 ± 1.53
13 {0.00}	14.40 ± 2.30	36 {1.28-1.46}	12.91 ± 3.34
14 {0.00}	17.68 ± 1.49	37 {1.46-1.57}	16.92 ± 0.16
15 {0.00}	11.47 ± 0.85	38 {1.57-1.66}	16.92 ± 1.15
16 {0.00}	15.19 ± 1.56	39 {1.66-1.77}	15.44 ± 2.24
17 {0.00}	16.86 ± 1.33	40 {1.77-1.89}	14.78 ± 1.16
18 {0.00}	16.33 ± 1.18	41 {1.89-1.97}	17.81 ± 2.49
19 {0.00}	13.67 ± 1.24	42 {1.97-2.07}	18.05 ± 2.17
20 {0.00}	15.50 ± 0.86	43 {2.07-2.17}	14.26 ± 0.71
21 {0.00}	17.21 ± 1.08	44 {2.17-2.21}	15.69 ± 1.81
22 {0.00}	0.00 ± 0.00	45 {2.21-2.35}	10.87 ± 0.22
23 {0.00}	16.06 ± 1.69	46 {2.35-2.42}	13.31 ± 1.00

Dilution	1/100	Dilution	1/100
Fraction № {[NaCl]}	Colony ± S.E.M. Area mm^2 mm^2	Fraction № {[NaCl]}	Colony ± S.E.M. Area mm^2 mm^2
47 {2.42-2.46}	9.14 ± 0.90	54 {2.88-2.96}	12.29 ± 0.07
48 {2.46-2.53}	7.91 ± 0.78	55 {2.96-3.00}	13.18 ± 1.53
49 {2.53-2.64}	16.60 ± 2.74	56 {3.00}	4.28 ± 0.17
50 {2.64-2.68}	14.26 ± 1.54	57 {3.00}	8.85 ± 0.18
51 {2.68-2.75}	15.56 ± 1.05	58 {3.00}	0.86 ± 0.42
52 {2.75-2.80}	9.43 ± 0.56	59 {3.00}	5.23 ± 0.76
53 {2.80-2.88}	12.42 ± 0.59	60 {3.00}	0.63 ± 0.25
EXPERIMENT CONTROLS			
Fraction 2 (0.25 M KCl Eluate) **Diluted 1/5**			**29.70 ± 1.65**
Fraction 2 (0.25 M KCl Eluate) **Diluted 1/100**			**21.46 ± 1.05**
Ham's F12 Experiment Control Mean (n=8) 12.61 ± 1.72 mm^2			

n=3 unless otherwise stated.

Significant growth over the control was scored as autocrine stimulation using the same criteria as outlined in Section 3.3.3.1. On this basis, stimulatory activity was recovered in the bound and unbound eluate. Activity was also detected in the 0.25 M KCl fraction down to a dilution of 1/100.

Non-bound stimulatory activity was found in fractions 3 & 14, at the start, and the trailing end of the major non-bound protein peak (figure 3.3.3.5). Stimulatory activity eluted in several other places before the start of the gradient in fractions 17 & 18, 21, 24, and 26.

Loosely bound stimulatory activity came off the column in fraction 28 (0.25 M NaCl).

Tightly bound autocrine stimulatory activity eluted from the column on the gradient in fractions 34 & 35 (1.05 - 1.28 M NaCl), 37 & 38 (1.46 - 1.66 M NaCl), and in fractions 41 & 42 (1.89 - 2.07 M NaCl). There was also an indication of autocrine stimulatory activity eluting at 2.7 M NaCl (fraction 51) but the level of growth over control is barely significant in this case. The apparent inhibition seen in the last five fractions off the column may be due incomplete dialysis of these high salt containing fractions.

3.3.6 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

A 2 ml sample of an R10-30 (10x) HEp-2 CM concentrate was applied to a 1 ml phenyl-Sepharose column as described in Section 2.11.4. Fractions of 2 ml were collected as the column was washed through sequentially with 1M, and 0.5M $(NH_4)_2SO_4$ through to zero salt. The column was then developed with increasing concentrations of glycerol. After dialysis (Section 2.8.2) and filter-sterilisation the fractions were diluted ½ and ¼ in Ham's F12 and tested in the 24-well HEp-2 autocrine assay. The resulting activity in these fractions together with the corresponding buffer composition at this elution volume are tabulated in Table 3.3.6. In this experiment the same R10-30 sample was included as a control together with a sample of the crude CM used to make the R10-30 concentrate. An R30 (10x) prepared from the same CM lot was also included in this experiment.

Table 3.3.6 Autocrine activity in the 10-30 kDa fraction of HEp-2 CM eluted from a phenyl-Sepharose column

DILUTIONS		½	¼
FRACTION NUMBER	FRACTION ELUATE	Colony ± S.E.M. Area mm² mm²	Colony ± S.E.M. Area mm² mm²
1	Running Buffer	2.61 .1 0.86	4.14 ± 0.54
2		4.08 ± 0.57	2.36 ± 0.05
3	1.0 M $(NH_4)_2SO_4$	2.79 ± 1.43	2.57 ± 0.90
4		0.71 ± 0.46	3.68 ± 1.50
5		3.84 ± 0.58	1.82 ± 0.52
6	0.5 M $(NH_4)_2SO_4$	N.D.	3.61 ± 0.32
7		0.76 ± 0.49	2.72 ± 1.60
8	0 SALT	2.18 ± 0.20	4.20 ± 1.31
9		3.34 ± 0.43	2.38 ± 0.26
10		0.00 ± 0.00	0.00 ± 0.00
11	10% GLYCEROL	2.31 ± 0.67	4.58 ± 1.29
12		0.00 ± 0.00	0.00 ± 0.00
13		0.00 ± 0.00	0.00 ± 0.00
14	20% GLYCEROL	0.00 ± 0.00	0.00 ± 0.00
15		0.00 ± 0.00	0.00 ± 0.00
16		0.00 ± 0.00	0.00 ± 0.00
17	30% GLYCEROL	0.80 ± 0.29	2.64 ± 0.66
18		2.33 ± 1.21	1.30 ± 0.37
19		1.17 ± 0.66	2.33 ± 1.41
20		0.00 ± 0.00	0.86 ± 0.65
EXPERIMENT CONTROLS			
Lot 3 R10-30 (10x)			4.33 ± 1.34
Lot 3 R30 (10x)			0.18 ± 0.04
Unconcentrated Lot 3 CM			8.60 ± 1.61
Ham's F12 Experiment Control Mean			**1.57 ± 0.49**

n=3 for all determinations. N.D; Not determined.

The amount of stimulation in the R10-30 fraction was not greater than that in the crude lot of CM from which it had been prepared. The 10x concentrate of the R30 fraction was inhibitory, consistent with earlier observations (Section 3.2.3.2). Four autocrine stimulatory activities appeared in the eluate from this column. The first washed through in fractions 1 & 2, a second activity came off in 0.5 M $(NH_4)_2SO_2$ {fraction 5}, and a third in fractions 8 & 9 at zero salt. The fourth eluted in more hydrophobic conditions at 10% glycerol {fraction 11}. Complete inhibition was detected in fractions 12-16 (in both dilutions of these samples) eluted at 20% glycerol. This was unlikely to be due to incomplete dialysis becasue control growth was restored in subsequent fractions eluted by 30% glycerol. These results indicated that at least one of the autocrine stimulatory species in the R10-30 HEp-2 CM fraction is hydrophobic, but that the remainder are hydrophillic in nature. There was also evidence for a hydrophobic inhibitory species eluting in 20% glycerol.

3.4.1 ANTI-FIBROBLAST GROWTH FACTOR NEUTRALISING ANTIBODIES

Studies with purified growth factors (Section 3.2.6) provided indirect evidence for the presence of FGF receptors on HEp-2 cells and partial purification of HEp-2 CM separated two heparin-binding autocrine stimulatory activities with affinites for immobilised heparin that were coincidental with those reported for aFGF and bFGF respectively (Section 3.3.2). Taken together, these findings suggested the possibility of an FGF autocrine loop in HEp-2 cells. To investigate this hypothesis, polyclonal anti-FGF neutralising antibodies incubated with three stimulatory fractions of HEp-2 CM in an attempt to assess their potential to block their endogenous mitogenic activities. Concentrations of the respective neutralising antibodies used were based the efficacies quoted by the manufacturers as effective in the NR6-3T3 fibroblast cell line system (see Section 2.14.1).

3.4.1.1 Preliminary evidence for FGF-neutralisable autocrine activity in HEp-2 CM

Initially crude HEp-2 CM and an R10-30 ultrafiltration fraction were incubated with anti-acidic or anti-basic FGF antibodies (see Section 2.14.3 for details) Mitogenic concentrations of the relevant growth factors were used as controls for antibody function. Because FGFs are mitogenic over a narrow concentration range (Section 3.2.6), three concentrations were tested with and without antibody to ensure that activity was measured in the control. To check for possible growth regulatory activity in the antibody preparation, a separate control with antibody only in control medium was included.

Anti-acidic FGF antibody in crude HEp-2 CM

The effect of anti-acidic FGF on the autocrine stimulatory activity in crude CM (Lot C4) was determined in the first part of this experiment. Due to the limited quantity of this antibody available, only 25 μg/ml could be used for the controls for antibody function. However, this was still within the quoted efficacy range (Section 2.14.1). The results with this antibody are shown in Table 3.4.1.1a.

Table 3.4.1.1a The effect of Anti-acidic FGF neutralising antibody on the autocrine stimulatory activity in crude HEp-2 CM

SAMPLE	NO ANTIBODY	+ 25 μg/ml ANTIBODY
Acidic FGF ng/ml	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
5	6.64 ± 0.83	6.73 ± 0.62
4	6.57 ± 0.61	6.93 ± 1.61
3	N.D.	6.68 ± 1.34
CONDITIONED MEDIUM AND CONTROL		
SAMPLE	NO ANTIBODY	+ 50 μg/ml ANTIBODY
Lot C4 CM	11.14 ± 1.54	7.61 ± 0.42
Ham's F12[a]	5.92 ± 0.94	8.28 ± 0.90

n=3 unless otherwise stated. [a] n=9. N.D; Not determined.

Anti-aFGF antibody alone increased the overall control growth by 40%. In addition, the purified aFGF used was inactive mitogenically at all concentrations tested, making a definitive conclusion about the inhibitable mitogenic activity in crude CM impossible. Nevertheless, at 50 μg/ml anti-acidic FGF antibody significantly neutralised the autocrine stimulatory activity in crude HEp-2 CM.

Anti-basic FGF antibody in crude HEp-2 CM

The second part of the experiment looked at the effect of anti-basic FGF neutralising antibody on the autocrine stimulatory activity in Lot C4 HEp-2 CM under the same assay conditions. In this case the same concentration of antibody was used for control and CM samples. The results are presented in Table 3.4.1.1b.

Table 3.4.1.1b The effect of Anti-basic FGF neutralising antibody on the autocrine stimulatory activity in crude HEp-2 CM

SAMPLE	NO ANTIBODY	+ 50 μg/ml ANTIBODY
Basic FGF pg/ml	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
250	11.92 ± 1.46	7.17 ± 1.36
200	7.68 ± 1.06	4.60 ± 0.85
150	6.75 ± 0.78	3.53 ± 0.50
CONDITIONED MEDIUM AND CONTROL		
SAMPLE	NO ANTIBODY	+ 50 μg/ml ANTIBODY
Lot C4 CM	11.14 ± 1.54	8.95 ± 0.67
Ham's F12[a]	5.92 ± 0.94	8.79 ± 1.67

n=3 unless otherwise stated. [a] n=9.

Purified bFGF was active mitogenically above 200 pg/ml with maximal activity at 250 pg/ml. This was in good agreement with previous results with this growth factor (Section 3.2.6). The antibody alone was mitogenic for HEp-2 when added to the control medium; at 50 μg/ml anti-bFGF antibody control growth was increased by 48%.

The data in Tables 3.4.1.1a & 3.4.1.1b above were expressed *as sample growth in excess of control growth* and the relative amount of inhibition with respect to the untreated sample was calculated. These are shown in Table 3.4.1.1c.

Table 3.4.1.1c The percentage mitogenic activity remaining in HEp-2 CM and growth factor controls after addition of anti-FGF antibodies

TREATMENT	NO ANTIBODY	ANTIBODY (μg/ml)	% Autocrine Activity Remaining
SAMPLE	ABSOLUTE GROWTH OVER CONTROL[a]		
Basic FGF		**50 μg/ml Anti-bFGF**	
250 pg/ml	6.00	1.25	21
200 pg/ml	1.76	-1.32[b]	0
150 pg/ml	0.83	-2.39[b]	0
Crude HEp-2 CM	5.62	3.03	54
Acidic FGF		**25 μg/ml Anti-aFGF**	
5 ng/ml	0.72	0.81	113
4 ng/ml	0.65	1.01	155
3 ng/ml	-	0.76	-
Crude HEp-2 CM	5.22	1.69[c]	32

[a] This figure was calculated by subtracting the value for the control from that of the treated sample value.
[b] The growth values for these treatments were less then the control. [c] 50 μg/ml of anti-aFGF was used in the CM sample.

These results indicate that 50 μg/ml of the neutralising anti-acidic FGF antibody inhibited 68% of the total autocrine activity in crude CM (calculated with respect to Ham's F12 without antibody). However, this observation was inconclusive due the absence of a positive control for antibody function. The stimulation indicated in the anti-acidic FGF antibody controls suggests that a contaminating mitogen in this antibody preparation is active at the relatively high concentration of antibody used. The same was true for the anti-basic FGF antibody.

Anti-basic FGF neutralising antibody inhibited 80% of the maximal stimulation with the bFGF control (i.e 250 pg/ml bFGF). At lower bFGF concentrations, inhibition with 50 μg/ml anti-basic FGF antibody was greater. 100% inhibition of mitogenic respose to 200 pg/ml bFGF was seen, and at 150 pg/ml bFGF the level of growth in the presence of antibody was less than the control value. The apparent reduction in autocrine stimulation in CM under these conditions was 46% (with respect to Ham's F12 control).

Control growth was increased by 40% with anti-acidic FGF and by 48% in the presence of anti-basic FGF. These growth factors were purified from rabbit serum and may therefore contain mitogens for HEp-2. No mitogenic activity was reported in these antibody preparations by the suppliers, but they had used a different indicator cell line.

3.4.1.2 Neutralisation of R10-30 autocrine activity with anti-FGF antibodies

In the third part of this preliminary experiment the effect of the anti-FGFs on the autocrine stimulatory activity in a 10x R10-30 ultrafiltration fraction of HEp-2 CM was examined. A range of antibody concentrations from 5-50 μg/ml was incubated with the CM fraction before assay (see Section 2.14.3 for details). The results are presented in Table 3.4.1.2a. These results were then expressed as *sample growth in excess of control* and the residual activity was presented as a percentage of that in untreated R10-30 fraction (minus antibody) in Table 3.4.1.2b.

Table 3.4.1.2a Inhibition of autocrine activity in the R10-30 fraction of HEp-2 CM using anti-FGF antibodies

SAMPLE	ANTIBODY	
R10-30 (10x)	Anti-Acidic FGF	Anti-Basic FGF
[ANTIBODY] µg/ml	Colony ± S.E.M. Area mm^2 mm^2	Colony ± S.E.M. Area mm^2 mm^2
5	8.01 ± 0.19	5.97 ± 0.88
10	9.56 ± 1.05	8.57 ± 0.09
25	9.02 ± 0.75	8.82 ± 0.45
50	7.72 ± 0.96	7.76 ± 1.39
EXPERIMENT CONTROLS		
R10-30 (10x) - No Antibody		11.28 ± 1.78
Crude HEp-2 CM (Lot C4)		11.12 ± 1.54
Ham's F12 (n=9)		5.92 ± 0.94

n=3 unless otherwise stated.

Table 3.4.1.2b Percentage autocrine activity remaining in the R10-30 fraction of HEp-2 CM after addition of anti-FGF antibodies

[ANTIBODY] µg/ml	Absolute Growth in Excess of Control[a]	% Autocrine Activity Remaining
No Antibody	5.36	100
Anti-Acidic FGF		
5	2.09	39
10	3.64	68
25	3.10	58
50	1.80	34
Anti-Basic FGF		
5	0.05	1
10	2.65	49
25	2.90	54
50	1.84	34

[a] This figure was calculated by subtracting the value for the control from that of the treated sample value.

This data indicated that 99% of the autocrine stimulatory activity in the R10-30 fraction of HEp-2 CM could be inhibited by 5 μg/ml anti-basic FGF antibody. At higher antibody concentrations the percentage reduction in activity was less. This may reflect the effect of contaminating mitogenic activity in the antibody preparation or non-specific interactions at higher concentrations.

The inhibition of 61% of the R10-30 autocrine stimulatory activity by 5 μg/ml anti-acidic FGF appeared indicated cross-reactivity with one or both of the antibodies under the conditions used in this experiment. If only anti-basic FGF were cross-reactive, this would imply that an aFGF-like and a bFGF-like activity were present in the R10-30 fraction. However, if cross-reactivity were confined to the anti-acidic FGF antibody, then this would imply that at least 99% of the autocrine activity in this fraction could be attributed to a bFGF-like factor. This question of cross-reactivity was addressed in Section 3.4.1.4.

3.4.1.3 Determination of the ND_{50} values for two commercially available Anti-FGF neutralising antibodies in the HEp-2 assay system

Preliminary results suggested that the efficacies and cross-reactivity patterns of the commercial neutralising anti-FGF antibodies should be determined in the HEp-2 assay system. Antibody efficacy was addressed in the following experiment with a second lot of antibodies purchased from the same supplier. The suppliers expressed the efficacy of the second lot of antibodies in terms of their 'ND_{50}'values (see Section 2.14.1 for details).

Concentrations of purified aFGF and bFGF required to give maximal stimulation in this assay (2.5 ng/ml and 250 pg/ml respectively) were titrated against a range of both antibody concentrations from 0.1-10.0 μg/ml. BSA carrier protein was omitted from the incubations with growth factors (see Section 2.14.3 for details) because of its stimulatory effect on HEp-2 growth reported in Section 3.2.6 above.

Controls

The question of FGF stability under these incubation conditions (without BSA) was examined. To this end, two concentrations of FGFs without BSA and antibody were assayed. The same concentrations of FGFs incubated with BSA without antibody were also tested. The activity of antibody preparation alone was investigated by the addition of 10 μg/ml of the respective lots to control medium. A sample of Lot C4 HEp-2 CM (used in the stability studies, Section 3.2.4 above) was included to check for assay function. The results of this experiment are presented in Table 3.4.1.3.

Table 3.4.1.3 Determination of the ND_{50} for two commercially available anti-FGF neutralising antibodies in the HEp-2 assay system

2.5 ng/ml Acidic FGF		250 pg/ml Basic FGF	
+ Anti-Acidic FGF ANTIBODY µg/ml	Colony ± S.E.M. Area mm² mm²	+ Anti Basic FGF ANTIBODY µg/ml	Colony ± S.E.M. Area mm² mm²
0.1	6.57 ± 0.96	0.1	6.57 ± 0.89
0.5	5.71 ± 0.57	0.5	6.25 ± 0.43
1.0	6.10 ± 0.41	1.0	6.93 ± 0.36
5.0	5.42 ± 0.32	5.0	3.55 ± 0.27
10.0	5.82 ± 0.33	10.0	3.42 ± 0.43
No Acidic FGF		No Basic FGF	
10.0	4.35 ± 0.43	10.0	3.21 ± 1.18
GROWTH FACTOR CONTROLS MINUS BSA			
5.0 ng/ml aFGF	7.55 ± 0.21	250 pg/ml bFGF	7.12 ± 0.69
2.5 ng/ml aFGF	6.96 ± 0.93	125 pg/ml bFGF	4.94 ± 0.44
Ham's F12 Control - BSA (n=25) = 4.81 ± 1.13 mm²			
GROWTH FACTOR CONTROLS PLUS 0.25 mg/ml BSA			
5.0 ng/ml aFGF	0.00 ± 0.00	250 pg/ml bFGF	11.22 ± 2.05
2.5 ng/ml aFGF	0.00 ± 0.00	125 pg/ml bFGF	11.02 ± 0.63
Ham's F12 Control + 0.25 mg/ml BSA (n=8) = 9.47 ± 1.07 mm²			
Crude HEp-2 Conditioned Medium Lot C4 (n=6) = 15.55 ± 1.02 mm²			

n=3 unless otherwise stated.

The overall level of growth in the assay was greater in the growth factor samples when BSA was present. However, the relative increase over the respective control measured with growth factors was greater in the absence of BSA. This suggested that the absence of BSA did not adversely affect the stability of the growth factors during the short preparation period in low protein conditions (i.e. dilution from stocks). The complete lack of growth in the aFGF controls plus BSA cannot easily be explained. Neither antibody alone was mitogenic for HEp-2 cells at 10 µg/ml. Purified aFGF and bFGF gave significant stimulation over the control at 2.5 ng/ml and 250 pg/ml in the absence of BSA, respectively. The bFGF-stimulated growth was completely inhibited by 5 µg/ml of anti-basic FGF antibody, but no reduction was achieved with 1 µg/ml of the same antibody. The ND_{50} value for the anti-basic FGF antibody therefore was determined to be between 1 and 5 µg/ml in the HEp-2 system. This was in good agreement with the value of 2 µg/ml quoted by the supplier's in the NR6 system (Section 2.14.1). At 5 µg/ml anti-aFGF antibody (close to the 2 µg/ml ND_{50} specified for this antibody by the supplier's) partial inhibition of the response to 2.5 ng/ml aFGF was detected. The level of inhibition observed was not significant however, and no titratible inhibition was detected with the anti-aFGF antibody at other concentrations tested.

3.4.1.4 Neutralisation of autocrine activity in HEp-2 CM with anti-FGF antibodies of known efficacy in the HEp-2 system

The neutralising anti-FGF antibodies tested for their efficacy in the HEp-2 system (Section 3.4.1.3) above were used in to assess their ability to neutralise the autocrine stimulatory activity in crude HEp-2 CM (lot C4). This experiment was designed to address the question of cross-reactivity of both antibodies. The possibility of non-specific inhibition of growth stimulation was also investigated. To this end, TGFα (a known stimulator of HEp-2, Section 3.2.6) at 1 ng/ml was preincubated with both antibodies independently (see Section 2.14.3) and then tested for activity in the HEp-2 assay. The results of this experiment are shown in Table 3.4.1.4.

Table 3.4.1.4a The effect of anti-FGF antibodies on the autocrine activity in crude HEp-2 CM

SAMPLE	Lot C4 CM	Ham's F12[a]	2.5 ng/ml aFGF	250 pg/ml bFGF
[ANTIBODY] μg/ml	Colony ± S.E.M. Area mm² mm²	Colony ± S.E.M. Area mm² mm²	Colony ± S.E.M. Area mm² mm²	Colony ± S.E.M. Area mm² mm²
ANTI-ACIDIC FGF NEUTRALISING ANTIBODY				
5	11.63 ± 3.01	3.21 .1 0.71	3.81 ± 0.38	3.01 ± 0.78
10	13.00 ± 2.72	3.15 ± 0.11	N.D.	N.D.
ANTI-BASIC FGF NEUTRALISING ANTIBODY				
5	8.88 ± 0.19	3.63 .1 0.56	5.30 ± 0.83	2.94 ± 0.73
10	9.74 ± 0.98	3.39 ± 0.36	N.D.	N.D.
ANTI-ACIDIC & ANTI-BASIC FGF NEUTRALISING ANTIBODIES COMBINED[b]				
5	9.08 ± 0.41	N.D.	N.D.	N.D.
10	9.63 ± 0.52	N.D.	N.D.	N.D.
NO ANTIBODY				
0	11.47 ± 1.57[c]	2.53 ± 0.61[d]	4.72 ± 0.39[e]	3.82 ± 0.44[e]
Transforming growth factor α (1 ng/ml) Control				
No Antibody				8.30 ± 2.96
10 μg/ml Anti-Acidic FGF				9.10 ± 3.08
10 μg/ml Anti-Basic FGF				12.46 ± 0.41

n=3 unless otherwise stated. [a] Control medium. [b] 5 μg/ml and 10 μg/ml of each amtibody were used in combination. [c] n=15. [d] n=12. [e] n=6. N.D; Not determined.

The level of stimulation in response to acidic and basic FGF was low with respect to the control in this experiment. It was nevertheless significant (allowing for standard errors). Neither antibody alone significantly altered the control level of growth (Ham's F12) up to concentrations of 10 μg/ml (consistent with the result in Section 3.4.1.3). The amount of growth in all samples was expressed as *sample growth in excess of control* (Ham's F12) and in Table 3.4.1.4b below the effects of the respective antibodies can be judged by comparing the percentage activity

remaining in CM and growth factor samples after addition of antibody. The excess growth in samples without antibody were taken as 100% activity.

Table 3.4.1.4b Percentage mitogenic activity remaining in crude HEp-2 CM and purified FGFs after incubation with anti-FGF neutralising antibodies

Sample	CM		aFGF (2.5 ng/ml)		bFGF (250 pg/ml)		TGFα (1 ng/ml)	
[Antibody] μg/ml	G.E.[a]	% A.R.[b]	G.E.	% A.R.	G.E.	% A.R.	G.E.	% A.R.
None	8.94	100	2.19	100	1.29	100	5.77	100
Anti-Acidic FGF neutralising antibody								
5	9.10	102	1.28	58	0.48	37	N.D.	N.D.
10	10.47	117	N.D.	N.D.	N.D.	N.D.	6.57	114
Anti-Basic FGF neutralising antibody								
5	6.35	71	2.77	126	0.41	32	N.D.	N.D.
10	7.21	81	N.D.	N.D.	N.D.	N.D.	9.93	178
Anti-Acidic and Anti-Basic FGF neutralising antibodies in combination								
5	6.55	73	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10	7.10	79	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a] Absolute growth in sample in excess of the control. This figure was calculated by subtracting the value for the control from that of the treated sample value. [b] % Autocrine activity remaining after treatment. N.D; Not determined.

This data indicated that the anti-acidic FGF antibody was cross-reactive with the anti-basic FGF antibody because the former could neutralise an equivalent amount of the mitogenic response of HEp-2 to bFGF (i.e. 63%). The converse was not true. In the control for antibody function, anti-basic FGF neutralised 68% of the mitogenic response to bFGF but it did not inhibit the stimulation due to aFGF. In fact, an apparent increase in growth was detected when anti-basic FGF was preincubated with aFGF.

Anti-basic FGF antibody alone was capable of inhibiting 27% of the total autocrine stimulatory activity in this lot of crude CM (lot C4) when it was present at 5 μg/ml. The higher concentration tested was less effective (21% of the stimulatory activity was inhibited by 10 μg/ml).

The anti-aFGF antibody was less effective in neutralising the mitogenic response to aFGF than its anti-bFGF counterpart was with bFGF. In the control for function, only 42% of the mitogenic response to 2.5 ng/ml aFGF was inhibited by the antibody. Anti-acidic FGF alone had no inhibitory effect on the autocrine stimulatory activity in crude CM.

A combination of both antibodies (both at 5 μg/ml) inhibited autocrine growth by the same degree as 5 μg/ml of anti-basic FGF alone (i.e by 27%). Inhibition was less with the higher concentrations of both antibodies (i.e. 10 μg/ml of each antibody caused 21% inhibition) but again the magnitude of inhibition was in the same order as anti-bFGF alone.

In the control for non-specific growth inhibition using TGFα, the presence of anti-acidic-FGF antibody, and to a greater extent the presence of anti-basic FGF, resulted in an increase in the level of stimulation detected at 1 ng/ml TGFα.

In summary this experiment indicates that under the conditions used here, aproximately 30% of the total autocrine activity in crude HEp-2 CM was bFGF-like. This data suggests that only the anti-basic FGF antibody can effectively inhibit autocrine activity to any significant degree in crude HEp-2 CM. The results also raise the question of the specificity and efficacy of this particular anti-acidic FGF neutralising antibody.

3.4.1.5 Neutralisation of autocrine stimulatory activity in the HEp-2 CM ultrafiltration fractions R10-30 and R30 with anti-FGF antibodies of known efficacy in the HEp-2 system

The following experiment was designed to confirm the observation (Section 3.4.1.2) that almost all autocrine activity (99%) in the R10-30 fraction could be blocked by an anti-bFGF neutralising antibody. The difference between this experiment and that in Section 3.1.4.2 is that the efficacy of both anti-FGF antibodies used had been determined in the HEp-2 system (Section 3.1.4.3). The effect of both neutralising antibodies on an R10-30 was tested again. An R30 fraction (previously demonstrated to be mitogenic in the HEp-2 autocrine assay) was also assayed for FGF-like autocrine stimulatory activity. The combined effect of the antibodies was also addressed in this experiment. Antibodies were incubated with the samples before assay, as described in Section 2.14.3. The results are shown in Table 3.4.1.5a.

Table 3.4.1.5a Neutralisation of autocrine activity in R10-30 and R30 ultrafiltration fractions of HEp-2 CM with anti-FGF antibodies

SAMPLE	R10-30	R30
[ANTIBODY] μg/ml	Colony ± S.E.M. Area mm² mm²	Colony ± S.E.M. Area mm² mm²
None	2.97 ± 0.31	4.68 ± 0.59
Anti-aFGF 5 μg/ml	2.63 ± 0.32	3.66 ± 1.42
Anti-bFGF 5 μg/ml	1.97 ± 0.40	3.28 ± 0.27
Anti-aFGF + Anti-bFGF[a] (5 μg/ml)	1.99 ± 0.17	2.21 ± 0.33
EXPERIMENT CONTROL (No Antibody)		
Ham's F12 (n=6)	1.34 ± 0.60 mm²	

n=3 unless otherwise stated. [a] 5 μg/ml of each antibody was used in combination.

The overall level of growth in this assay was low. However, autocrine stimulation in both fractions of CM was significantly higher than in the control Ham's F12 medium. In Table 3.4.1.5b the *excess growth over control* for every sample is shown. This data is also expressed as the *percentage activity remaining* after antibody treatment. Activity in CM without antibody was taken as 100%.

Table 3.4.1.5b Percentage autocrine activity remaining in R10-30 and R30 fractions of HEp-2 CM after preincubation with anti-FGF neutralising antibodies.

SAMPLE	R10-30		R30	
[ANTIBODY] μg/ml	Absolute Growth in Excess of Control[a]	% A.R.[b]	Absolute Growth in Excess of Control	% A.R.
None	1.63	100	3.34	100
5 μg/ml Anti-aFGF	1.29	21[c]	2.32	30[c]
5 μg/ml Anti-bFGF	0.63	61	1.94	42
5 μg/ml Anti-aFGF + 5μg/ml Anti-bFGF	0.65	60	0.87	74

[a] This figure was calculated by subtracting the value for the control from that of the treated sample value.

[b] Percentage autocrine activity remaining. [c] These calues are not significant allowing for standard errors.

Only the anti-bFGF antibody was able to significantly reduce autocrine stimulatory activity in the R10-30 fraction. This amounted to 60% inhibition of the total R10-30 mitogenic activity measured in this experiment, compared to the 99% inhibition observed with 5 μg/ml of the first lot of anti-bFGF (Section 3.4.1.2). No more inhibition was detected when anti-acidic FGF was combined with anti-basic FGF, consistent with the results in the previous experiment (Section 3.4.1.4).

Anti-acidic FGF alone did not inhibit the R10-30 autocrine activity. The apparent reduction of 21% was not significantly lower than the untreated R10-30, allowing for standard errors on this determination.

Similarly the apparent 30% reduction in the activity of the R30 fraction in the presence of anti-acidic FGF was within the margin of error. However, the 42% inhibition of the R30 autocrine activity by anti-basic FGF was significant. The level of inhibition was increased by a further 32% in this fraction when anti-acidic FGF antibody was added in combination with anti-basic FGF.

This experiment confirmed that a large portion of the autocrine activity in the R10-30 fraction of HEp-2 CM was bFGF-like. It also indicated that bFGF-like activities and possibly aFGF-like activities may contribute to the autocrine effect in the R30 fraction.

A summary of all the results with neutralising antibodies is presented in Table 3.4.1.5c.

Table 3.4.1.5c Summary of the percentage of autocrine activity neutralised by anti-FGF antibodies in crude and ultrafiltrated HEp-2 CM

[ANTIBODY] μg/ml	PERCENTAGE OF TOTAL AUTOCRINE ACTIVITY NEUTRALISED IN CM		
	CRUDE CM	R10-30	R30
Anti-Acidic FGF Neutralising Antibody			
50	68	66	N.D.
25	N.D.	42	N.D.
10	0	32	N.D.
5	0	21-61	30
Anti-Basic FGF Neutralising Antibody			
50	46	66	N.D.
25	N.D.	46	N.D.
10	19	51	N.D.
5	29	61-99	42

N.D; Not determined.

It can be concluded from these studies therefore that between 30-50% of the total autocrine growth stimulatory activity in crude HEp-2 CM is a bFGF-like species. At least 60% (if not more) of the mitogenic activity in the R10-30 fraction can also be attributed to a similar factor. Approximately 40% of the R30 activity was neutralised by 5 μg/ml anti-bFGF antibody, but the nature of the extra 30% activity neutralised by the presence of 5 μg/ml anti-acidic FGF cannot be ascribed to acidic or basic FGF because of the cross-reactivity shown with the anti-aFGF antibody.

3.4.2 FGF ANTISENSE OLIGODEOXYNUCLEOTIDES

The evidence for aFGF and bFGF-like autocrine activity in HEp-2 CM, provided by the results of experiments with neutralising antibodies in the previous Section (3.4.1), suggested that one or both of these growth factors might play a significant role in the autocrine control of HEp-2 growth.

Basic FGF has been implicated as an autocrine growth regulator for malignant melanoma and gliomas. Further proof for the autocrine role of bFGF in these systems was provided using the recently developed antisense technology. The basis of this technology is the ability to arrest protein translation in a gene specific fashion. Arrest of translation is thought to occur by the hybridisation of the complementary (antisense) oligodeoxynucleotide sequence to mRNA, resulting in its subsequent degradation by RNAses.

The following experiments set out to investigate the potential of antisense bFGF and antisense aFGF oligodeoxynucleotides to inhibit the proliferation of HEp-2. A number of important considerations pertaining to the use of oligodeoxynucleotides in cell culture were addressed in the approach to the experimental design. These included, (1) Oligomer stability; (2) Oligomer uptake and potential toxicity; (3) Quantity of oligomer required/scale of the experiment (i.e. volume of tissue culture media used per determination);
(4) Time of addition of oligomer.

The choice of oligomer sequence and length influence the effectiveness of translation arrest. Fifteenmers (sequences 15 base pairs in length) antisense to the translation initiation start sites for aFGF and bFGF mRNA were chosen as targets on the basis of work published by Becker *et al.*, (1989), and Morrison (1991). Both workers were able to inhibit bFGF-induced cell proliferation using the same oligodeoxynucleotide sequence which is 5′ to and overlapping the initial methionine codon.

In this work a 15 base pair sequence antisense to the sequence beginning at the initiation codon of aFGF was chosen to investigate its potential for inhibition of HEp-2 proliferation. Unmodified oligodeoxynucleotide sequences were used here to avoid potential problems with specific hybridisation (due to chemical modifications).

The sequences used in this work were synthesised commercially by British Biotechnoloty Ltd (see Section 2.15.1 for details, and appendix B for sequence data). The limiting quantities of oligomers available necessitated the use of a miniturised assay system. For this reason the 96-well plate assay was chosen to reduce the scale of the experiment. Concentrations in the 0-200 μM range have been reported in the literature as effective in arresting protein translation in cell culture systems. Sense sequences complementary to the antisense sequences were used as controls for non-specific inhibition (resulting from toxicity of the oligomer preparation et cetera).

To improve oligomer stability, foetal calf serum to be used in the experiment was heated at 65°C for 45 minutes to inactivate endogenous nucleases.

3.4.2.1 Determination of assay parameters for HEp-2 with 1% heat-inactivated serum in the 96-well plate system

The 96-well acid phosphatase HEp-2 assay (described in Section 2.12.5) used 200 μl of culture medium per well. New parameters for HEp-2 growth were investigated for the 96-well plate system in a preliminary experiment designed to optimise growth in 1% heat-inactivated serum. The volume of culture medium was reduced to 50 μl to reduce the quantity of oligos required. This assay looked at the growth of HEp-2 cells at three plating densities, and in two separate batches of serum. These plating densities were higher than those employed in the HEp-2 assay because it is difficult to measure growth inhibition at low cell densities. Proliferation under the same conditions with the same sera untreated was also examined. The results are presented in Table 3.4.2.1.

Table 3.4.2.1 The growth of HEp-2 in 1% heat-inactivated serum and reduced assay volume in the 96-well plate system

CELLS/WELL	1×10^3	2.5×10^3	5×10^3
SERUM BATCH	ABS $\pm$ S.D.[a]	ABS $\pm$ S.D.[a]	ABS $\pm$ S.D.[a]
Untreated Batch AF1201	0.019 $\pm$ 0.007[b]	0.036 $\pm$ 0.014	0.089 $\pm$ 0.024
Heat-inactivated Batch AF1201	0.083 $\pm$ 0.017[c]	0.176 $\pm$ 0.025	0.232 $\pm$ 0.052
Untreated Batch 48	0.105 $\pm$ 0.028[b]	0.211 $\pm$ 0.027	0.257 $\pm$ 0.052
Heat-inactivated Batch 48	0.039 $\pm$ 0.009[c]	0.040 $\pm$ 0.017	0.062 $\pm$ 0.016

[a] Absorbance at 405 nm $\pm$ standard deviation. [b] n=22. [c] n=24. n=30-32 for all other determinations.

Heat-treatment of batch N°AF1201 improved the growth supported by this FCS batch over the range of cell plating densities tested. The converse was true for FCS batch N°48. Batch AF1201 was used therefore in subsequent experiments. In order to ensure a relatively high (readable) absorbance value under these assay conditions the loading density was raised to 10^4 cells per well for the subsequent antisense experiments.

3.4.2.2 Preliminary evidence for the arrest of HEp-2 proliferation using FGF antisense oligodeoxynucleotides

Oligonucleotides were reconstituted in HPLC grade H_2O and quantified spectrophotometrically as described in Section 2.15.3. All dilutions were in DMEM/Ham's F12 (1:1) mixture (plus 1% heat-inactivated serum, 1% L-glutamine and 500 U/ml penicillin/500 μg/ml streptomycin) just before addition in the assay. The assay protocol is described in Section 2.15.5.

The first lots of oligonucleotides were found to be acidic after reconstitution in 100 μl of distilled H_2O (pH 3.5-4.5 as judged by indicator paper). This only became apparent when the top concentration of oligos were diluted in cell culture medium (to 75 μM). In an effort to avoid pH problems these top stock solutions were diluted to 50 μM and subsequent serial dilutions made from these. The assay was read after a 6 day incubation. Briefly, cell culture medium was removed carefully and the cells were washed once with PBS. Acid phosphatase activity was

measured as described in Section 2.12.5.1. The results of this experiment are presented in Table 3.4.2.2(i).

Table 3.4.2.2(i) The effect of Antisense Oligodeoxynucleotides directed against the start sites of acidic and basic FGF mRNAs on the proliferation of HEp-2

[OLIGO] μM	Acidic FGF		Basic FGF	
	Sense	Antisense	Sense	Antisense
	ABS ± S.D.[a]	ABS ± S.D.[a]	ABS ± S.D.[a]	ABS ± S.D.[a]
50	0.006 ± 0.005	0.002 ± 0.002	0.006 ± 0.002	0.014 ± 0.002
30	0.007 ± 0.003	0.012 ± 0.005	0.009 ± 0.005	0.006 ± 0.002
25	0.034 ± 0.007	0.046 ± 0.029	0.064 ± 0.014	0.020 ± 0.005
10	0.233 ± 0.010	0.252 ± 0.021	0.259 ± 0.030	0.216 ± 0.110
5	0.164 ± 0.034	0.189 ± 0.048	0.222 ± 0.059	0.289 ± 0.028
1	0.189 ± 0.020	0.189 ± 0.045	0.217 ± 0.062	0.261 ± 0.016
Control[b]	0.244 ± 0.046 (n=6)		0.275 ± 0.020 (n=6)	

[a] Absobance at 405 nm ± standard deviation. [b] plate control. n=3 unless otherwise stated.

At concentrations of oligo greater than 25 μM HEp-2 growth was inhibited significantly with respect to the corresponding plate control. At 50 μM complete inhibition was seen in all sense and antisense samples, suggesting that this was due to the residual acid. Where growth was measurable (i.e. in the 1-25 μM oligo concentration range), only the bFGF antisense oligodeoxynucleotide at 25 μM appeared to significantly reduce HEp-2 proliferation with respect to the sense oligo. However, as both samples were already reduced with respect to the control a definitive conclusion could not be reached. No reduction in growth was detected at any concentration of antisense aFGF (with respect to the sense control).

Technical problems with the commercial oligodeoxynucleotides were overcome with the purchase of a second lot of oligos which did not contain acid residue. Nevertheless, 1 μl aliquots of all the reconstituted oligos (identical sequences as above) were tested for acidity before dilution in culture medium. The assay was repeated as outlined above, except that the top concentration of oligos assayed was 100 μM, based on the preliminary observation with 25 μM of antisense bFGF. In an effort to minimise plating errors, manual and electronic multichannel micropipettes were compared as described in Section 2.15.4. The electronic model gave the lowest errors on plating and was used in this experiment. The assay was incubated for 4 days and read as before using acid phosphatase as an endpoint. The results are shown in Table 3.4.2.2(ii).

Table 3.4.2.2(ii) The effect of antisense acidic and basic FGF oligodeoxynucleotides on HEp-2 cell proliferation

[OLIGO] μM	Acidic FGF		Basic FGF	
	Sense	Antisense	Sense	Antisense
	ABS ± S.D.[a]	ABS ± S.D.[a]	ABS ± S.D.[a]	ABS ± S.D.[a]
100	0.234 ± 0.187	0.147 ± 0.035	0.608 ± 0.038	0.608 ± 0.021
33.3	0.245 ± 0.082	0.199 ± 0.008	0.610 ± 0.012	0.576 ± 0.036
13.3	0.214 ± 0.049	0.204 ± 0.071	0.586 ± 0.000	0.597 ± 0.059
6.6	0.176 ± 0.097	0.271 ± 0.056	0.617 ± 0.065	0.604 ± 0.068
1.3	0.347 ± 0.146	0.423 ± 0.055	0.541 ± 0.012	0.542 ± 0.040
Control[b]	0.252 ± 0.052 (n=5)		0.377 ± 0.120 (n=6)	

[a] Absorbance at 405 nm ± standard deviation. [b] plate control. n=3 unless otherwise stated.

Toxicity due to oligos was not detected in this experiment. However, despite efforts to the contrary the standard errors remained high. No reduction in HEp-2 proliferation was seen in the presence of antisense bFGF (with respect to the sense control) over the entire concentration range assayed. This contradicts the observation in the previous experiment indicating a reduction at 25 μM. However, because the control level of HEp-2 cell growth was lower in that case the lack of inhibition in this experiment might be explained by a lower ratio of oligo:cell number (i.e.a lower effective dose per cell).

The mean value for cell growth in the presence of 100 μM antisense aFGF suggests a reduction in growth with respect to the control growth and the corresponding aFGF oligo sense control. However, this is only significantly reduced with respect to the plate control because the standard error on the 100 μM aFGF sense oligo control was too large to permit a definitive conclusion.

Because the standard errors in this experiment were unacceptably high, no definitive conclusions could be drawn from the trends which emerged. However, the highest concentration of antisense aFGF did indicate a reduction in HEp-2 growth of 50% (if the standard errors are discounted and only the mean values are compared). No reduction was indicated with the antisense bFGF, but higher cell densities were observed by eye on this plate before the addition of oligodeoxynucleotides — a factor which may support a dose effect as mentioned above.

In conclusion, the experiment did not indicate a role for bFGF in HEp-2 proliferation as implied by the antibody studies, but there were indications that aFGF is involved in this process. However, it should be pointed out that the cell density used in this experiment was 30 fold higher than that in the autocrine assay. Under these conditions the doses of antisense bFGF used may not have been sufficient to significantly arrest translation of bFGF mRNA and/or the timing of the addition of the antisense bFGF may have allowed sufficient time for the expression and deposition of the growth factor in the extracellular matrix which is believed to act as a storage site for this and other growth factors.

3.5 MEASUREMENT OF TRANSFORMING GROWTH FACTOR α IN HEp-2 CM

Transforming growth factor α (TGFα) was shown to be active in stimulating the proliferation of HEp-2 cells under autocrine assay conditions (Section 3.2.6). Two established assays were used in this work to screen HEp-2 CM for TGFα-like activity. The first is based on the ability of TGFα to stimulate the colony forming efficiency of NRK (normal rat kidney) cells in soft agar {Todaro & deLarco (1978)} in a concentration dependant manner.

The second, a radioreceptor assay utilising the human cell line A431, is described in Section 2.12.6 and the results with HEp-2 CM in this assay are presented in Section 3.1.6.3.

3.5.1 The NRK soft agar assay for TGFα

A range of concentrations of TGFα (Genentech) were prepared from a stock solution of 10 μg/ml in serum-free DMEM/Ham's F12 (1:1) plus 1 mg/ml BSA (see Section 2.13.2 for details). Dilutions were made in the same medium as the stock TGFα and were used generate a standard curve in the NRK soft agar assay (described in Section 2.12.3.2). DMEM/Ham'sF12 plus 1 mg/ml BSA was used as a control.

HEp-2 CM sample concentrates (prepared by ultrafiltration using the 30 kDa and the 10 kDa 'cut-off' membranes, Section 2.8.1.1) were assayed together with an unconcentrated CM sample. All of these samples were first shown to be active in the HEp-2 autocrine assay. Mature TGFα with a MW of 6 kDa should have been recovered in the 5-10 kDa fraction if it were present in HEp-2 CM. On the contrary, no stimulatory activity was detected in this fraction of HEp-2 CM (Section 3.2.3.2). Ham's F12 was used as the control for HEp-2 CM samples.

CM from the cell line RPMI-2650 was also assayed in a concentrated form. MEM was used in the collection of RPMI-2650 CM and therefore this medium was included as a control. This experiment was performed in duplicate and the results of both determinations are shown in Table 3.5.1.

Table 3.5.1 Measurememt of TGFα-like activity in HEp-2 CM using the NRK soft agar assay

STANDARD CURVE	EXPERIMENT 1	EXPERIMENT 2
[TGFα] (ng/ml)	% C.F.E. ± S.E.M.[a]	% C.F.E. ± S.E.M.
50.00	27.29 ± 0.83	20.66 ± 0.86
25.00	23.59 ± 1.54	18.89 ± 0.73
10.00	22.58 ± 3.21	16.10 ± 0.62
5.00	19.69 ± 2.20	14.26 ± 1.93
1.00	15.03 ± 0.31	6.68 ± 0.84
0.50	14.89 ± 0.48	5.89 ± 0.73
0.05	1.29 ± 0.49	0.12 ± 0.00
Control Medium + BSA	0.00 ± 0.00	0.04 ± 0.05
CONDITIONED MEDIA		
RPMI-2650 R1 (10x)	7.95 ± 1.47	1.18 ± 0.19
RPMI-2650 R1 (5x)	3.23 ± 0.29	0.39 ± 0.06
RPMI-2650 R1 (1x)	0.67 ± 0.07	0.00 ± 0.00
Control MEM	0.00 ± 0.00	0.04 ± 0.05
HEp-2 R30 (10x)	0.00 ± 0.00	0.00 ± 0.00
HEp-2 R10-30 (10x)	0.00 ± 0.00	0.00 ± 0.00
HEp-2 Unconcentrated	0.00 ± 0.00	0.00 ± 0.00
Control Ham's F12	0.00 ± 0.00	0.00 ± 0.00

[a] Percentage colony forming efficiency ± standard error of the mean. n=3 for all determinations.

Transforming growth factor α was detected in the NRK assay at the lowest concentration tested, 0.05 ng/ml (≡ 50 pg/ml). No TGFα-like activity was detected in any of the HEp-2 CM samples. Maximal stimulation with TGFα in the autocrine assay was at 500 pg/ml, but lower concentrations of the growth factor were active depending on the assay conditions (see Section 3.2.6.1).

Concentrated CM from the cell line RPMI-2650 contains a TGFα-like activity which is dilutable.

4.0 DISCUSSION

4.1 PRIMARY CELL CULTURE

Successful establishment of primary cell cultures and continuous cell lines from commonly occuring carcinomas would have important clinical and scientific applications. However, establishment of continuous human cell lines is a rare event, and the phenotypic and/or the genetic alterations that may be necessary to predispose a tumour to this potential are not yet clearly defined. Genetic selection in tumour populations favouring *in vivo* metastasis may also confer growth advantages on such cells in *vitro* and facilitate their establishment as continuous cell lines.

Earlier work in this field suggests that no universal strategy can be applied to optimise the *in vitro* culture of all tumours or even tumours of the same histological type {Dietal *et al.*, (1987); Leibovitz (1986); Fogh (1975)}. In this project, efforts to establish primary cell cultures from thorasic tumours (predominantly of lung origin), support this consensus.

Mechanical disaggregation of tumour tissue proved more successful in releasing epithelal cells than the use of proteolytic enzymes. This was consistent with the findings of Dietal *et al.*, (1987) who also reported that collagenase/dispase was superior to trypsin in cases where mechanical dissaggregation alone gave insufficient cell numbers. A frequent observation with primary cultures from different tissues was the better success, in terms of growth performance, achieved with small cell clumps released during mechanical disaggregation {Freshney (1987)}. This observation was noted with samples processed in this project too. However, the explant technique was found to be of limited use in establishment primary epithelial cultures.

In agreement with the findings of Miyazaki *et al.*, (1984) working with bronchogenic squamous cell carcinomas, incubation in collagenase for prolonged periods also resulted in higher yields of fibroblasts in this study.

In the majority of primary cultures of malignant epithelial cells handled in this work, it was not possible to prevent the eventual loss of these cultures through senescence and/or terminal differentiation, despite vigilant attempts to remove fibroblasts. No obvious benefits accruing from the presence of even small numbers of fibroblasts for the growth of epithelial cells were observed. There were preliminary indications (n=3) that substituting foetal calf serum with autologus serum resulted in reduced numbers of fibroblasts. However, no significant benefit for tumour cell growth was observed in this small set of samples and the routine use of donor

human serum from cancer patients was not a practical consideration for ethical reasons.
The use of growth-arrested 3T3 feeder layers was not found to be a satisfactory technique for the inhibition of fibroblast overgrowth in primary lung epithelial cultures. This was consistent with the findings of others {Jorissen *et al.*, (1991)}. In contrast, a more recent publication by de Jong *et al.*, (1993) reported the successful use of such feeder layers for limiting fibroblast growth and establishing succesful cultures from explants *in vitro* of normal human bronchial epithelial cells. These workers used a DMEM/Ham's F12 basal medium (3:1) supplemented with insulin, hydrocortisone, L-isoproterenol, as well as 5% foetal bovine serum. A possible explanation for the inability of 3T3-feeder layers to prevent the overgrowth of malignant lung epithelial cells by their stromal counterparts in primary tumours, is the ability of the former to stimulte the latter by producing the potent fibroblast mitogen, platelet-derived growth factor (see Section 1.4 above). Similarly collagen-coating of culture flasks was ineffective as a method of reducing fibroblast proliferation.

Differential removal of fibroblasts was attempted using different formulations of the standard trypsin/EDTA mixture routinely used to subculture cell lines. This approach was met with some success by other workers in this laboratory {Mc Donnell, Ph.D thesis, National Institute for Higher Education, Dublin [NIHED] (1987)} and further improved upon here. Selective trypsinisation at 37°C proceeded rapidly and often the interval between the removal of fibroblasts and epithelial cells was too short in practice to stop the reaction with growth medium. However, by incubating at 4°C better control of the detachment process was obtained. In agreement with the findings of Martin, Ph.D thesis [DCU], (1992), the most effective mixture for the removal of fibroblasts was 0.01% trypsin/0.04% EDTA at 4°C. However, it was also found that 0.25% trypsin alone (also at 4°C) was effective in selectively removing epithelial cells. The use of 0.25% trypsin at low temperature may prove to be more generally useful in the selective selection of epithelial cells from fibroblasts given that Hodges *et al.*, (1973) found that entry of the protease into cells at 4°C was negligible, but the enzyme still had some effect on the cell surface at this temperature.

The results obtained with primary cultures of non-small cell lung carcinomas was disappointing in the context of long-term survival and propagation of tumour cells *in vitro*, with only 9.5% of all samples classes received being subcultured for at least one passage after initiation of the primary culture. In agreement with the findings of Martin {Ph.D thesis, 1992} small-sized samples taken at bronchoscopy or mediastinoscopy were the least reliable source for successful

primary lung tumour cells *in vitro*. Compared to an 65% 'take' with the generally larger resection samples from lobectomies and pneumenectomies, only 30% of all bronchoscopy/mediastinoscopy samples yielded epithelial cells in culture after tissue processing. Of this 30%, none were subcultured further, compared to 20% of the initially succesful lobectomy/pneumonectomy samples (equivalent to 13% of all samples processed in this category, i.e lung tumours).

Although fewer oesophageal samples were cultivated, these tumour types appear to establish with greater facility as *in vitro* cultures (in 4/6 cases, epithelial cells were obtained from primary oesophageal tumours) than the lung tumours processed. This may be a reflection of the inherent biolgical make-up of these tumours and/or the nature of the samples obtained (large resection as opposed to smaller biopsy-sized samples).

Consistent with the observation that cell lines tend to establish *in vitro* more easily from metastatic tumours than from the same tumours at their primary sites {Shaw *et al.*, (1993)}, and from poorly differentiated rather than well differentiated NSCLC tumours {Liu & Tsao (1993)}, a metastatic poorly differentiated squamous cell lung carcinoma was established as a continuous cell line in this work. A second permanent cell line was established from a culture derived from a malignant effusion associated with a metastatic breast adenocarcinoma. The better success rates for epithelial cultures seen with the effusion-derived cells (5/8 gave epithelial growth excluding one which was established as a permanent cell line) also supports the observation that metastatic carcinomas are easier to establish in culture, most likely due to the their reduced requirements for external growth stimuli.

4.1.1 CELL LINE CHARACTERISATION

4.1.1.1 DNA fingerprinting

The integrity of the newly established cell lines DLRP and BAC was verified by DNA fingerprint analysis performed commercially by Cellmark Diagnostics. In the case of DLRP, single locus probes were used to distinguish this cell line from all of the other cell lines in use at the time of its inception (see figure 3.1.13). BAC was matched to its donor using multilocus probes to screen the fingerprint patterns of cell line DNA with that of cells harvested from the original effusion. These were found to be identical (figure 3.1.14).

4.1.1.2 Cytogenetic and histological analysis

In work carried out in this laboratory, Gilvarry *et al.*, (1990) found that the presence of abnormal mitoses could be used to distinguish normal epithelial from tumour cells in early passage primary cultures. In this study, which also included DLRP, no abnormal mitotic indices were detected in a variety of normal epithelial cells during mitosis. However, early passage DLRP cells were shown to exhibit tripolar and multipolar mitoses — a phenomenon characteristic of some malignant cell divisions (see figure 3.1.15).

Cytogenetic analysis of DLRP by Law *et al.*, (1992) revealed that this cell line had eight marker chromosomes, including two deletions of chromosome 3. Three of the additional markers del(1)(q11), del(2)(p11.1) and del(2)(q11.1) are shared with another squamous cell lung carcinoma cell line established in this laboratory, DLKP, but otherwise the chromosomal karyotypes were markedly different. A prominent feature in metaphase chromosome spreads of DLRP was the detection of telomeric association. This feature was not observed in DLKP. The frequent chromosome breakage found in DLRP metaphases made it difficult to analyse cytologically, but a modal number of 66 was ascribed to this cell line on the basis of a composite chromosome profile.

4.1.1.3 Immunohistochemical analysis

Epithelial-specific antibodies

Relative to the positive staining pattern demonstrated by SCC-9 (a squamous cell carcinoma of the tongue), and the negative staining pattern of the human embryonic lung fibroblast cell line MRC-5, DLRP stained very weakly with the Ep-16 antibody directed against a surface epithelial antigen (figures 3.1.16. 3.1.17, and 3.1.18 respectively). However, using the anti-cytokeratin Nº 18, antibody a more defined cytokeratin staining pattern was seen with DLRP (figures 3.1.21 & 3.1.22) confirming its epithelial origin. This staining pattern is expected for epithelial cells (see SCC-9 figure 3.1.19), which unlike fibroblasts (e.g. MRC-5 figure 3.1.20) incorporate keratin into the intermediate filaments of their cytoskeleton. BAC cells produced a particularly strong staining pattern with anti-cytokeratin Nº 18, demonstrating well defined filaments and confirming the epithelial origin of these cells (figures 3.1.23 &3.1.24).

NSCLC-specific antibodies

The immunoreactivity of two murine monoclonal antibodies, 703D4 and 704A1, recognising the same epitope on NSCLC, but not SCLC cells, were used to confirm the NSCLC phenotype of the newly established DLRP. The staining pattern of BAC with these antibodies was also determined.

As expected, 704A1 did not demonstrate reactivity with the SCLC cell line HTB 120 (i.e. the negative control) but a positive staining pattern was demonstrated with the human NSCLC cell line SK-MES-1, a squamous cell carcinoma used as a positive control (figures 3.1.31 & 3.1.32 respectively). By comparison, DLRP also stained positively with 704A1, but MRC-5 fibroblasts were negative for this epitope (figures 3.1.33 & 3.1.34 respectively).

The positive staining of the breast adenocarcinoma cell line BAC with 704A1 was unexpected in view of the original report that another breast carcinoma cell line (MCF-7) did not stain positively with this antibody {Mulshine *et al.*, (1983)}. However, the positive reaction with BAC is unlikely to be artifactual because the second NSCLC-specific antibody used, 703D4 recognises the same epitope as 704A1, and this too recognised an epitope on BAC cells (figure 3.1.30).

DLRP also stained positively for 703D4 (figure 3.1.29) compared to the negative pattern for HTB 120 (figure 3.1.26). The NSCLC control cell lines used with this antibody, SK-LU-1 (adenocarcinoma) and SK-MES-1, were also shown to stain positively (figures 3.1.25 & 3.1.28 respectively).

In conclusion, these antibodies were useful in establishing the epithelial origin of DLRP and BAC. The positive reactive patterns of both cell lines to the tumour-specific epitope expressed by NSCLC but not SCLC cells confirmed the 'non-small cell status' of DLRP.

4.1.1.4 Mycoplasma status of new cell lines

Given the ubiquitous distribution of mycoplasma species, and the nature of the primary sample collection protocol, together with the fact that certain mycoplasma species are endemic to the human lung, it was not surprising that a cell line established from such a source proved to be mycoplasma positive at an early passage number. DLRP cultures were quarantined until they had been successfully treated with an anti-mycoplasma agent and tested negative. The human

breast adenocarcinoma cell line BAC, established from a sterile effusion, was mycoplasma negative at the earliest passage tested.

4.1.1.5 Clonogenicity in soft agar

The ability of tumour cells, but not normal cells, to form colonies from single cells when grown in semi-solid agar medium has been used as a criterion for defining the malignant phenotype of tumour cells {Bergh *et al.*, (1981); Luster *et al.*, (1985)}. The ability of DLRP to form colonies in soft agar also confirmed the malignant character of this cell line. Determination of DLRP colony forming efficieny at various plating densities indicated that the percentage colony forming efficiency was directly related to cell density. Increasing the cell number per plate resulted in increased %CFE up to a maximum level between 1 and 2 $\times$ 10^5 cells/plate in the 35mm system used. A lower limit to plating density was detected between 1 and 5 $\times$ 10^4 cells/plate, below which there was zero colony formation. The cut-off point for colony formation with DLRP was matched by a similar limit to colony formation at reduced plating densities with another human lung squamous cell line, DLKP. The cell density-dependence of colony formation in soft agar may be indicative of autocrine growth factor production by these cell lines. However, at a plating density higher than that required for maximal colony forming efficiency with DLRP, a reduction in the %CFE was noted. This may be indicative of the production of an autocrine inhibitory species by these cells at high cell densities. This inhibitory effect was not matched by DLKP at high density where the increase in colony number was such that it made colony counting impractical.

4.1.1.6 Growth factor responsiveness of DLRP and DLKP

Although TGFα has been implicated as an autocrine growth factor in lung squamous cell carcinomas, it did not display mitogenic activity on DLRP and DLKP; two cell lines derived from poorly differentiated SCC lung tumours in this laboratory (Section 3.1.7.1). This was in spite of the evidence suggesting that DLRP may secrete a TGFα-like species into its culture medium (Section 3.1.6.3). An explanation for the lack of activity with exogenously supplied TGFα in this case, may be that the receptors for this growth factor were already saturated and/or down-regulated with endogenously produced ligand. This would render the cells insensitive to further stimulation by exogenous TGFα.

Another possible explanation is that the combination of assay parameters used (low plating density and low serum concentration) may not have provided sufficiently stringent growth

conditions with which to detect the mitogenic response to TGFα. An inappropriate combination of assay parameters may also explain the lack of an autocrine growth response by DLRP in monolayer culture, to DLRP-conditioned medium (Section 3.1.8.1), even though the growth of this cell line in agar indicated the possible production of such a species. Similarly, there was no evidence for the production of positive autocrine growth factor activity by DLKP in monolayer culture under the conditions assayed. On the contrary, there was evidence for an autocrine growth inhibitory species in medium conditioned by DLKP (Section 3.1.8.2).

DLRP and DLKP demonstrated markedly different respones to acidic and basic FGF (Section 3.1.7.1). When the fold stimulation in DLRP growth was calculated with respect to the experimental control mean, a maximal response to acidic FGF of almost three fold (i.e, 2.85 fold) was detected. The maximal mitogenic response to basic FGF (at 1 pg/ml) was 1.7 fold, and this was measured at a 250-fold lower concentration than that required to induce maximal stimulation with aFGF. In addition, no obvious dose response was seen with either FGF, but this may be attributable to the narrow concentration range for mitogenic activity reported with these growth factors. The differential in concentrations of growth factor required to elicit maximal stimulation with acidic and basic FGF in DLRP cells is also consistent with the effects of these FGFs in other cell types *in vitro* where bFGF has been reported to be 10- to 30-fold more potent than aFGF {Gospodarowicz (1987); Joseph-Silverstein & Rifkin (1987)}.

There was some evidence for weak stimulation of DLKP growth in the presence of 0.25 ng/ml aFGF. However, unlike DLRP, increasing concentrations of this growth factor above 0.5 ng/ml were progressively more inhibitory to DLKP proliferation (Section 3.1.7.2). A similar pattern of inhibiton was detected with bFGF at concentrations in excess of 10 pg/ml, but growth stimulation of DLKP was indicated at 1 and 5 pg/ml bFGF, with fold stimulation measured as 1.56 and 1.87 at these concentrations respectively.

Interleukins-1α and -1β were both stimulatory for DLRP. IL-1α induced a two-fold increase in cell growth between 100 and 250 pg/ml, but IL-1β was mitogenic only at a concentration of 250 pg/ml, where the fold induction in growth (at 1.68 fold), was less than that achieved in response to IL-1α at the same concentration. In contrast, DLKP was unresponsive mitogenically to IL-1α, but IL-1β induced a 1.92 fold increase in growth at 5 pg/ml.

4.1.2 NEGATIVE REGULATORS OF NSCLC GROWTH

4.1.2.1 Transforming growth factor β

Although TGF-β1 inhibits normal bronchial epithelial cells, a direct role for TGF-β1 as an inhibitor, and/or inducer of terminal differentiation in NSCLC cells *in vitro* is not clearly established. Moroever, the results of studies with TGF-β on cell lines may not be representative of the potentially more complex interactive network of growth factors found in the mixed population of cell types present in primary tumour cultures. It is well known that many of the biological effects of TGF-βs are mediated indirectly, by the antagonism of other growth factor signalling pathways, and that the eventual cellular response to TGF-β may depend on its context with respect to other growth factors {Sporn & Roberts (1988)}. In support of this concept, Matsumoto *et al.*, (1992) found that TGFβ (and glucocorticoids such as hydrocortisone) suppressed the expression of the HGF/SF gene in the human lung embryo fibroblast cell line MRC-5. By analogy with the actively dividing embryonic lung tissue, it is possible that 'stimulated' fibroblasts which are actively dividing in lung tumours, produce TGFβ which may exert a negative paracrine effect on the autocrine production of autocrine HGF/SF by the malignant epithelial component in these tumours (see Section 1.6). The ability to inhibit the production of an autocrine stimulator for NSCLC may be one mechanism whereby TGFβ can indirectly modulate tumour cell proliferation.

A recent publication by Kirk *et al.*, (1993) would support this idea. These authors have presented evidence suggesting that the inhibition of many tumour cell types may be mediated by TGF-β1 secreted by fibroblasts. There are a number of other possible sources of TGF-β in culture systems for NSCLCs. Apart from fibroblasts, tumour cells are known to produce TGF-β {Derynck *et al.*, (1985)}, and high levels of TGF-β are also found in serum {Masui *et al.*, (1986)}. Although the tumour- and serum-derived TGF-β (from platelets) may be present in an inactive form, physiologically relevant processing mechanisms, such as proteolytic activation by plasmin, may be operative in primary cultures.

4.1.2.2 Antiproliferative effects of interleukins

The activity of cytokines in solid tumour malignancies has recently come to light. When tested on DLRP cells in a monolayer assay, interleukins 1α and 1β were mitogenically active to varying degrees on these cell lines. Production of IL-1-like activity by a variety of different carcinomas, including NSCLC cell lines, has been documented in a study by Miyauchi *et al.*, (1988). Contrary to the results obtained with DLRP, Nakane *et al* ., (1990) reported that IL-1

had a negative effect on thymidine uptake and cell proliferation in the lung adenocarcinoma cell line A-549. However, other investigators have reported both stimulatory and inibitory effects on the growth of primary NSCLC tissue samples using recombinant human IL-1α {Hanauske *et al.*, (1992)}.

Another cytokine which may have a negative effect on the proliferation of NSCLC cells *in vitro* and *in vivo* is interleukin-4. In a comparative study by Tungekar *et al.*, (1991), the IL-4 receptor was detected in both normal bronchial epithelium and NSCLC tissue sections, but was undetectable in SCLC and carcinoid lung tissues. In this study, coexpression of IL-4 receptors together with EGF receptors was reported in 10/29 lung squamous cell carcinomas and 6/17 adenocarcinomas. These findings were underscored by a more recent report demonstrating the antiproliferative effect of recombinant human (rh) IL-4 on NSCLC cell lines *in vitro* and *in vivo* {Topp *et al.*, (1993)}. In this study, rhIL-4 inhibited the growth of two human NSCLC cell lines — CCL 185 and to a lesser extent HTB 56. The *in vitro* inhibition correlated with the level of IL-4 receptor mRNA expression and protein levels in these cell lines, and it could be blocked by the addition of anti-rhIL-4 neutralising antibody. In the same study, the *in vitro* growth of two SCLC cell lines, HTB 119 and HTB 120, was unaffected by rhIL-4. In the absence of evidence for IL-4 production by nonhematopoietic cells, a role for this cytokine in the negative regulation of NSCLCs *in vitro* can only be guessed at. However, in view of the evidence for receptor expression on NSCLC cells, the prospect of paracrine and/or autocrine production of IL-4 cannot be ruled out.

Another recent publication suggests that the multifunctional cytokine IL-6 can also act as a negative autocrine growth regulator for NSCLC cell lines *in vitro* {Takizawa *et al.*, (1993)}. These workers showed that BEAS-2B, a noncarcinogenic transformed bronchial epithelial cell line, and the adenocarcinoma cell line A-549, were inhibited by exogenously added IL-6. Three other NSCLC cell lines examined, RERF-LC-MS (adenocarcinoma), VMRC-LCP and LC-1 sq (both squamous cell carcinomas), were also inhibited by this cytokine — but to a lesser extent. In the same study, A-549, VMRC-LCP and BEAS-2B expressed specific cell surface IL-6 receptors, and all the NSCLC lines expressed and released immunoreactive IL-6. However, significantly more IL-6 was detected in A-549 conditioned medium and this cell line was also more sensitive to IL-6-induced inhibition than the other NSCLC cell lines. The inhibition induced by IL-6 could be neutralised by an anti-IL-6 specific antibody, and the growth of A-549 cells plated at higher plating densities was accelerated in the presence of anti-IL-6 antibody.

TGFβ also inhibited the proliferation of A-549, but an anti-TGFβ antibody did not affect the growth-inhibitory effect of IL-6. In addition, the effects of TGFβ and IL-6 were additive, suggesting that these cytokines act through different signalling pathways. The noncarcinogenic transformed bronchial epithelial cell line used in this study was also more sensitive to inhibition by IL-6 than any of the NSCLC cell lines. This confirmined previous studies by this group {Takizawa *et al.*, (1992)}, which indicated that normal bronchial epithelial cells were inhibited by IL-6, and led the authors to suggest that the relative loss of responsiveness to an autocrine IL-6 inhibitor may contribute to the carcinogenic process in the human lung {Takizawa *et al.*, (1993)}.

In another report using 64 different malignant cell lines including HEp-2, and four of the cell lines used in the study of Takizawa *et al.*, (1993) {i.e the three NSCLC cell lines A-549, RERF-LC-MS, VMRC-LCP and the vulval squamous cell carcinoma line A-431}, all of the cell lines examined secreted an IL-1-like activity into their respective culture supernatants {Miyauchi *et al.*, (1988)}. Among the IL-6 producing NSCLC cell lines identified by Takizawa *et al.*, (1993), the levels of IL-1-like activity secreted by the squamous cell carcinoma cell lines VMRC-LCP and A-431, were significantly higher than those of the adenocarcinoma cell lines A-549 and RERF-LC-MS {Miyauchi *et al.*, (1988)}. The observed induction of steady state levels of IL-6 and IL-6 receptor mRNA levels in A-549 by exogenously added IL-1α {Takizawa *et al.*, (1993)} were consistent with previous demonstrations that IL-1 can enhance the expression of IL-6 in some cell types {Seghal *et al.*, (1987)}. The relatively higher levels of IL-1-like activity detected by Miyauchi *et al.*, (1988) in the lung squamous cell carcinoma line VMRC-LCP, may induce levels of endogenous IL-6 production sufficiently to result in the down-regulation of the IL-6 receptor in such cells. This might explain why these cell lines were less sensitive to inhibition by exogenous IL-6 in the study of Takizawa *et al.*, (1993).

4.1.2.3 Growth regulatory peptides for NSCLCs

Another explanation for the poor growth performance of NSCLCs *in vitro* may be provided by recent reports in the literature concerning the sensitivity of these cells to peptides with growth inhibitory effects, and the possible autocrine production of such peptides by NSCLCs. An example is the 28-amino acid peptide thymosin α1, which has been implicated as a negative growth regulator for NSCLCs. This peptide, originally isolated from a crude bovine thymic extract {Goldstein *et al.*, (1977)}, is derived from a 113-amino acid precursor protein called prothymosin α, whose NH_2-terminal sequence is identical to that of thymosin α1 {Eschenfeldt

& Berger (1986); Grangou-Laxiridis *et al.*, (1988)}. In a recent report, Moody *et al.*, (1993b) were able to inhibit the *in vitro* and *in vivo* growth of human cell lines representative of each of the three main types of NSCLC using this peptide. The inhibitory effects were dose dependent in both circumstances, and were reversible *in vivo*. Unpublished observations {by Moody, T.W.} in the same report, stated that thymosin-like peptides were detected by radioimmunoassay in NSCLC extracts and conditioned medium from these cells. However, a definitive autocrine inhibitory role for these peptides in NSCLC remains to be established.

Thymosin α1 shares sequence homology with a peptide called vasoactive intestinal polypeptide (VIP), which has been shown to stimulate the growth of several NSCLC cell lines examined by Moody *et al.*, (1993b). An earlier publication by these authors reported that almost all the NSCLC cell lines examined in that study had high affinity receptors for VIP {Moody *et al.*, (1993a). Unpublised results in Moody *et al.*, (1993b) indicated that thymosin α1 can compete with ^{125}I-VIP for binding to NSCLC cells, albeit with low affinity, and that the *in vitro* stimulation of NSCLC cell lines in soft agar by VIP was blocked in the presence of thymosin α1.

4.1.3 ABSENCE OF POSITIVE AUTOCRINE/PARACRINE GROWTH FACTORS IN NSCLC CULTURES *IN VITRO*

Insulin-like growth factors are known to be mitogenic for human small cell lung cancer *in vitro* {Nakanishi *et al.*, (1988)}. However, unlike SCLCs, evidence to support an autocrine role for IGF-like factors in NSCLC tumours has been scarce and conflicting. Minuto *et al.*, (1986) found that IGF-I levels were 1.4 - 7.0 fold higher in the NSCLC tissue than in uninvolved lung tissue from the same donors, but Soderdahl *et al.*, (1988) were unable to detect IGF-I mRNA in NSCLC cell lines. Another study detected immunoreactive IGF-I secreted by the human lung adenocarcinoma (NSCLC) cell line CaLu-6 {Minuto *et al.*, (1988)}, but in a subsequent report IGF-I was not detected in extracts of NSCLC cells {Reeve *et al.*, (1990)}.

IGF-like species have been partially purified from serum-free culture medium conditioned by human foetal lung fibroblasts {Stiles & Moats-Staats (1989)}, suggesting a paracrine role for IGFs in the regulation of lung epithelia. A more recent publication by Ankrapp & Bevan (1993), also supports a paracrine role for IGF-I in NSCLC. In this study, IGF-I receptors were detected on the three lung adenocarcinoma cell lines, A-549, A-427, and SK-LU-1. IGF-I stimulated the serum-free proliferation of these lines (SK-LU-1 at 0.25% FCS) seeded at low density, but no IGF-I could be detected in their respective conditioned media. In the same study, a human adult

fibroblast cell line (CCD-19Lu), was shown to produce an IGF-I-like protein which stimulated an increase in the tyrosine kinase activity of detergent-solubilised IGF-I receptors from A-549. The CCD-19Lu conditioned medium also stimulated A-549 cell growth, and both effects were neutralised by anti-IGF-I antibody, suggesting a paracrine role for IGF-I in NSCLC.
The possible involvement of IGFs in the growth control of squamous cell lung carcinomas did not come to light until recently.

The results of comparative studies performed by Kaiser *et al.*, (1993), using monoclonal antibodies against the receptors for IGF-I and IGF-II, found that while the IGF-I receptor was detectable in both normal lung and malignant lung, it was particularly prominent in squamous cell carcinomas. IGF-II staining however, was weaker in lung cancer than in normal lung.

Although the precise growth requirements for NSCLCs have not been defined, it is becoming increasingly clear that growth factors from several classes are important for the maintenance of proliferative potential for these cell types *in vitro*, particularly for squamous cell carcinomas. An example of the range of growth factors which may be involved in autocrine growth control in NSCLCs *in vitro* is given by the results of a recent study by Occleston & Walker (1993). These workers found that 1PT, a NSCLC cell line derived from an undifferentiated human bronchial carcinoma, secretes multiple growth factors into its CM. These included species which were immunoreactive to bFGF, IGF-I and IGF-II, EGF and TGFα as well as latent TGF-β2. In the same study, PDGF, TNFα and TNFβ could not be detected.

4.1.4 PROSPECTS FOR IMPROVING *IN VITRO* GROWTH PERFORMANCE OF NSCLC-DERIVED PRIMARY CULTURES

One of the outstanding difficulties in the primary culture of NSCLCs *in vitro* is the loss of successful cultures through growth arrest and differentiation. The factors regulating this process need to be more clearly defined if the routine establishment of long-term NSCLC cultures is to be realised. The role of TGF-β1 as a negative regulator in these tumours needs to be better understood if its effects are to be abrogated. However, Takizawa *et al.*, (1993) recently reported that an anti-TGFβ did not affect the *in vitro* proliferation of the NSCLC cell line A-549.

The principal sources of TGF-β would seem to be the stromal component of the tumour tissue, but in serum-supplemented media, TGF-β arising from platelets is another source of this inhibitor. The use of a selective serum-free media system is an attractive alternative to serum-

supplementation, but as yet, this remains an unrealised goal for all NSCLC cultures. In addition, the wisdom of attempting to remove fibroblasts from primary NSCLC tumour cultures, as a measure designed to improve their growth potential *in vitro*, remains to be validated. This is supported by the emerging role of stromally elaborated positive-acting paracrine growth factors such as KGF and HGF/SF which may stimulate NSCLC proliferation *in vivo* and *in vitro*. However, fibroblasts are known to express IL-6 mRNA and protein constitutively {Kohase *et al.*, (1986)} and this cytokine may be responsible for the growth inhibition of lung epithelial cells associated with the presence of stromal cells in primary cultures. As mentioned above, TGF-β1 was also found to have a negative effect on the expression of HGF/SF in a normal human embryonic fibroblast cell line {Matsumoto *et al.*, (1992)}.

A greater understanding therefore, of the role of autocrine and paracrine negative growth regulators, should identify the important inhibitors of NSCLC growth and/or differentiation, and suggest ways to overcome their limiting effects on NSCLC growth *in vitro*. One possibile approach might be the use of neutralising antibodies to block the effects of the two recently identified inhibitors of NSCLC growth, IL-6 and the peptide thymosin α1. However, simply blocking the arrest of cell growth would be unlikely to result in growth promotion, given the paradigm for growth regulation in more defined cell systems. *In vitro* growth stimulation of NSCLCs therefore, will undoubtedly require the supplementation of NSCLC growth media with additional positive growth stimulators.

Evidence that platelet-derived growth factor may be important in the *in vivo* progression of NSCLC was discussed earlier. The use of PDGF for *in vitro* culture of primary lung tumours was not considered in this work because of its known mitogenic effect on fibroblasts. However, in the context of a more defined medium, this growth factor may prove useful for *in vitro* cultures of squamous cell carcinomas and other NSCLCs.

The inclusion of such factors as VIP might also contribute to the improvement of NSCLC growth potential *in vitro*, and perhaps too, the squamous cell carcinoma subtype. Neither IGF-I or IGF-II was tested for mitogenic activity on DLRP or DLKP, but this experiment should now be done in the light of recent publications detecting the IGF-I receptor in squamous cell lung carcinomas {Kaiser *et al.*, (1993)}.

The detection of bFGF in CM from an undifferentiated human bronchial carcinoma cell line (1PT) {Occleston & Walker (1993)}, may indicate a regulatory role for this growth factor in NSCLCs. The mitogenic response of DLRP to acidic and basic FGF (and to a lesser extent DLKP) supports this hypothesis and suggests that FGFs might be a useful supplement in the development of a selective medium for lung SCCs. It is noteworthy that in a recent study by Thomassen (1993), only BSA and bovine pituitary extract (BPE) were required to support the serum-free growth of neoplastically transformed rat tracheal epithelial cells. BPE is known to contain high levels of fibroblast growth factor activity and has been used as a source of these factors in their purification to homogeneity {Gospodarowicz *et al.*, (1985)}.

The presence of FGFs in DLRP CM was not measured here, and there was no evidence for secretion of autocrine growth factors by DLRP and DLKP. However, given the evidence for the existence of 'private' FGF autocrine stimulatory loops in other cell lines, the existence of similar control mechanisms in DLRP cannot be ruled out. Even if FGF-like growth factor(s) are secreted by squamous cell lung carcinoma cell lines such as DLRP, rapid sequestration by heparan sulphate proteoglycans of the extracellular matrix by cell surface proteoglycans may ensure that these growth factors remain tightly cell-associated {Rapraeger *et al.*, (1991); Vlodavsky *et al.*, (1991)}.

Two other mitogens which may also prove useful as growth promotors of NSCLC *in vitro* are, KGF and HGF/SF. Highly purified human recombinant HGF/SF is now available commercially.

4.1.5 GROWTH FACTOR SPECIES IN MALIGNANT EFFUSIONS

Summary of results with malignant effusions

Preliminary results with a malignant effusion (ME) taken from a patient with ovarian carcinoma displayed both inhibitory and stimulatory activity on low density cultures of three human sqaumous cell carcinoma cell lines *in vitro* (DLRP, DLKP and SK-MES-1). Both activities were concentration dependent with stimulation at greater dilutions, and inhibition at lesser dilutions. Inhibition of all three cell lines was absolute down to a 1/5 dilution of the ME (equivalent to a final concentration of 10% v/v ME in the assay). However, further dilution of the ME (between 1/10 and 1/20), resulted in an average ten-fold increase in growth, with respect to the control in the growth assays for DLRP and SK-MES-1. In the case of SK-MES-1 at least, this

mitogenic activity was dilutable, as indicated by the loss of stimulatory activity at a 1/40 dilution. DLKP was much less sensitive to the growth-promoting effect of ME than DLRP or SK-MES-1 but lesser dilutions of ME were also inhibitory for DLKP.

A similar growth sensitivity pattern was seen with HEp-2, but in this case the order of dilution required to abolish the inhibitory activity (and manifest the mitogenic activity), was less (at 1/5). Stimulation of HEp-2 cells established that the growth-promoting activity in this ME was not specific to lung carcinoma cell lines.

Using HEp-2 cells, a variety of MEs associated with different malignancies were screened for growth-promoting activity. The samples assayed included MEs from breast, lung, kidney, and ovarian carcinomas. In addition, effusions from a patient with the more benign disease non-Hodgkins lymphoma, and another with a non-malignant proliferative disorders were also tested.

A number of observations were made in this series of exploratory experiments.

1. The inhibitory activity noted in two ovarian MEs, and a non-Hodgkins lymphoma-associated ME sample, appeared to become more potent with longer storage time at 4°C {compare 'ovarian 1' sample results in tables 3.1.10(i) and 3.1.10(ii)}.

2. The growth-promoting activity in effusions was not restricted to those associated with malignant disorders {see Table 3.1.10(i)}.

3. Lung-derived MEs contained the lowest levels of growth-promoting activity relative to the high levels seen in kidney- and breast-associated MEs {see Table 3.1.10(iii)}.

4. Preliminary purification of an ovarian-associated ME using the ammonium sulphate technique (with a view to further characterising the growth regulatory species in this sample), isolated growth-promoting activity in two fractions, the majority of which was found in the 20-30% cut, but there was also significant activity in the 30-50% cut {see Table 3.1.10.1}.

5. Comparative studies using serum and/or plasma with MEs from the same donor patients indicated that the order of growth stimulatory activity over a range of dilutions was the same in autologous plasma or serum, as in the corresponding effusion samples. However, although insufficient serum or plasma remained for protein determination after bioassays, the expected total protein concentration in normal plasma is 66-87 g/L. When this is compared to the protein concentration of 31 g/L in the ME sample 'breast 3', (one of the autologous 'paired ME samples' used) {see table 3.1.10(iii)}, then the specific activity of the growth-stimulatory activity in this effusion sample might reasonably be expected to be higher than in the serum from this patient.

The original idea of screening MEs for growth factor activities on NSCLC *in vitro* was re-evaluated after the findings of these pilot experiments. The comparatively similar growth-stimulatory profiles for MEs and serum or plasma from the same donors suggested that the effusion-derived growth-promoting species might represent a serum-derived mitogen. This was considered likely in view of the fact that a 'vascular permeability factor' (VPF) is produced by many tumour cells, and is present in high concentrations in tumour ascites {Senger *et al.*, (1983)}. It was suggested by these authors that VPF (subsequently identified as the mitogen VEGF) might be important in the accumulation of malignant fluid in body cavaties by causing blood vessel endothelial cells to leak plasma proteins.

In order to avoid the complex task of trying to purify 'one among many mitogens' (which can conceiveably include attachment factors) from what was potentially just a more dilute fraction of human serum, it was hoped to establish whether or not the active species was solely tumour-derived. To do this, it would have been necessary to assay 'paired samples of effusions and serum' from a set of indivduals with non-proliferative disease (as well as extending the sample size of the 'malignant effusion/serum' pairs tested) before a conclusion could be reached on the merits of further purification. Unfortunately it did not prove possible to obtain any clinical samples from individuals presenting with non-proliferative disease-related effusions within the time frame of this study. Other laboratories have been able to do similar comparative studies on a limited number of samples {see Wilson *et al.*, (1991) discussed below}.

An alternative direction was taken therefore, which involved the elucidation of the autocrine growth regulatory system which had come to light in preliminary experiments with the cell line HEp-2 (Section 3.2.1.2).

In the context of evidence for the production of autocrine growth factor activity by HEp-2, a number of purified growth factors were tested for mitogenic activity under the stringent growth conditions employed in this assay (Section 3.2.6). Some of these were found to be mitogenically active for HEp-2, and have also been detected in the malignant effusions from patients with various malignancies.

TGFα was a powerful mitogen for HEp-2, and displayed bifunctional regulation of these cells under autocrine assay conditions; high doses were inhibitory (> 5 ng/ml), but at lower concentrations (0.5-5.0 ng/ml) the effect of TGFα was stimulatory. This was reminiscent of the

effect of ovarian MEs on HEp-2 proliferation, which demonstrated a similar bifunctional, concentration-dependent modulation of HEp-2 growth (Section 3.2.6.1). The active species for HEp-2 in ovarian MEs may well be TGFα because this growth factor has been detected immunologically in effusions from patients with disseminated ovarian cancer {Artega *et al.*, (1988)}. Mills *et al.*, (1988) had presented evidence for the presence of an ovarian-specific growth factor in ascites fluid from patients with ovarian cancer, and this appeared to be distinct from a panel of known growth factors, including EGF. However, the possible contribution of the homologus TGFα (to the ovarian-specific growth factor activity observed) was not eliminated in this study. Although EGF and TGFα bind to the same high affinity receptor, they have been reported to exhibit differential biological responses in some cell systems {Barrondon & Green (1987); Pittelkow *et al.*, (1989)}. The similar potencies of EGF and TGFα in the HEp-2 assay (Section 3.2.6.1) make it unlikely that such a differential in activities would be detected in the HEp-2 assay.

In contrast to the findings of Artega *et al.*, (1988), a subsequent study by Wilson *et al.*, (1991) did not detect EGF/TGFα in malignant ovarian fluids. In this comparative study using ascitic fluids, cyst fluids, and peritoneal fluids from patients with benign ovarian tumours and non-tumour related gynaecological conditions (e.g. fluids from patients with cirrhotic liver disease), these authors assessed the ability of such fluids to promote the anchorage independent growth of three cell lines. The species responsible for the induction of colony formation with the normal cells, NRK-49F (a clone of the normal rat kidney fibroblast cell line), and 58MC (a mesothelial cell line established from the ascites of a patient with ovarian cancer), was designated 'transforming activity' (TA). The stimulation of colony formation in the ovarian tumour cell line, OAW-42 was called 'colony stimulating activity (CSA). CSA was found in benign and malignant effusions, and was not therefore specific to tumour-related fluids. However, the induction of colony formation in normal cells, measured as 'transforming activity', was associated with the tumour-related fluids, indicating that it was a tumour-derived factor rather than a systemic species, produced as a reaction by the host to the disease. The CSA and TA were also distinguished by the observation that added heparin inhibited the CSA in tumour cells but not the TA in normal cells. This suggested that the CSA was a coagulation factor such as fibrin. The 'transforming activity' was unaffected by the addition of heparin, but it only detected using normal cells. Transforming growth factor β levels of 10-100 pg/ml were detected in these fluids, and TGFβ alone at these concentrations was capable of enhancing the growth of 58MC in agar. EGF had a similar effect in the 0.1-10 ng/ml concentration range.

Attempts to characterise the nature of 'transforming activities' in other human malignant effusions have found that TGFα and TGFβ-like growth factors appear to account for much of this activity in many malignant and non-malignant effusions {Seo *et al.*, (1988)}. While preliminary work has often suggested the presence of tumour-specific production of transforming activities the absence of subsequent reports detailing the purification and cloning of these putative novel growth factors implies that it has not been possible to purify them, or more likely, that they have been found to be identical to previously characterised growth factors.

Watson *et al.*, (1990) have also measured significant levels (> 3ng/ml) of the cytokine IL-6 in ascites fluid from ovarian cancer patients. A role for IL-6 in the control of NSCLC proliferation was not suspected at the time the ovarian MEs were assayed. However, the emergence of evidence for the more widespread involvement of IL-6 in solid tumours has heralded the publication of a recent paper reporting the functional expression in HEp-2 cells of IL-6, its receptor, and the IL-6 signal transducing glycoprotein gp130 {Guillaume *et al.*, (1993)}. The contribution of IL-6 to the autocrine growth control of HEp-2 cells is discussed in Section 4.2.3.2). The detection in ovarian MEs of IL-6 may also explain the inhibitory effect of one such fluid on the growth of the NSCLC lines examined here, given that others have demonstrated an inhibitory role for IL-6 in this cell type *in vitro* {Takizawa *et al.*, (1993)}. The presence of IL-6 in HEp-2 CM may also explain the inhibitory effect of unconcentrated (crude) HEp-2 CM on the growth of the NSCLC cell line, DLRP (data not shown). While this was a dissapointing result, in view of the original aim of this thesis, it did not preclude the possibility that a highly purified fraction of HEp-2 would be found with NSCLC-promoting activity. This was supported by the observation that two NSCLC cell lines used in this work responded to two heparin-binding mitogens, acidic and basic FGF, and that HEp-2 CM contained an autocrine activity which resembeled bFGF. Moreover, evidence for the production of several autocrine growth regulatory species in the HEp-2 system was an interesting observation in itself.

HEp-2 cells also responded mitogenically to exogenously supplied acidic and basic FGF (Section 3.2.6.4). Until recently however, basic FGF had not been detected in biological fluids such as serum {Baird *et al.*, (1986); Vlodavsky *et al.*, (1987)}. By using more sensitive EIAs, a number of authors have detected this growth factor in urine and serum from cancer patients. Fujimoto *et al.*, (1991) reported elevated levels of bFGF in the serum of renal cancer patients using a new sandwich EIA for bFGF, and with the same system Watanabe *et al.*, (1992) found

abnormally high levels of this factor in serum from breast cancer patients. Elevated urinary levels of bFGF were reported by Nguyen *et al* ., (1992) in patients with a variety of neoplasms. Another improved enzyme immunoassay system for human bFGF was employed by Ii *et al.*, (1993), and these authors reported average serum concentrations of bFGF at 5.9 pg/ml in 25 normal volunteers. By comparison, patients in the same study with renal, lung and brain tumours demonstrated elevated serum concentrations of bFGF. While these studies provided indirect evidence for secretion of bFGF by tumour cells, Soutter *et al.*, (1993) have recently provided more direct evidence to support the idea that the bFGF detected in biological fluids of cancer patients is tumour-derived. These investigators presented clear evidence that bFGF in tumour-bearing mice arose from the tumour cells, as distinct from other cells in the hosts. Using a mouse model system described by Hori *et al.*, (1991) {discussed, Section 4.3.5} nude mice were inoculated with tumour cells expressing a mutant form (mutein) of bFGF. By applying specific immunoassays for the detection of native and the mutein bFGF, these authors were able to distinguish between tumour-derived mutein bFGF, and host-derived native bFGF.

To date, no evidence for tumour-derived bFGF has been reported in MEs but it is not unlikely that bFGF will yet be detected in these fluids given that many tumour cells produce the vascular permeability factor (VPF), now know to be identical to vascular endothelial growth factor (VEGF). An alternative source of FGFs in MEs is suggested by the detection of immunoreactive FGF in macrophages from peritoneal exudate {Baird *et al.*, (1985)}.

Despite the high levels of VPF/VEGF reported in ascites fluids, VEGF was not considered to act as a mitogen for HEp-2 cells. This was because its target cell specificity seemed to be restricted to endothelial cell progenitors and monocytes {Terman *et al.*, (1991); Millauer *et al.*, (1993)}. This situation has been reappraised in the light of a recent report which documented the detection of VEGF receptors in other cell types, including HeLa cells {Gitay-Goren *et al.*, (1992)}. Given that HEp-2 is considered to be a variant of HeLa, the possibile existence of a functional VEGF autocrine loop in HEp-2 cells is discussed further, in the context of the properties of the partially charcterised HEp-2 autocrine species (Section 4.3.1.2).

The mitogenic response of HEp-2 to the VEGF-related PDGF was barely significant at the concentrations tested (Section 3.2.6.6). The relatively low growth-promoting activity for HEp-2, detected in two pleural effusion samples associated with NSCLCs, is consistent with the findings of Safi *et al.*, (1992). These workers reported higher PDGF levels in pleural effusions

associated with lung adenocarcinomas than in those from SCLC or non-malignant pleural effusions.

The above are but a few of the possible serum-derived mitogens which may be found in malignant effusions. Other mitogenic factors in the HEp-2 system include the insulin-like growth factors. The detection of an IGF-II-like factor in bovine serum {Li *et al.*, (1990)} and the existence of several carrier proteins for IGFs in human serum {Baxter & Martin (1989)}, argue strongly that these species may yet be found in malignant effusions.

4.2 THE HEp-2 AUTOCRINE SYSTEM

4.2.1 Evidence for autocrine growth factor production by HEp-2 cells

The ability of HEp-2 cells to proliferate at very low densities (200 cells/cm^2) with reduced serum supplementation, suggested the production of autonomous growth stimulators by this cell line. More direct evidence for autocrine growth factor production was provided by the finding that serum-free medium conditioned by HEp-2, also stimulated their own growth at low cell density, and reduced serum concentration (Section 3.2.1.2). Indirect evidence to support the existence of autocrine stimulation came from the observation that cultures of HEp-2 in log phase growth, continued to proliferate until confluency when these were transferred to serum-free medium (Section 3.2.1.5). It was established that the autocrine effect was independent of the basal medium and the serum type used, although at low plating densities the sensitivity of cells to autocrine stimulatory activity demonstrated a reciprocal relationship between cell density and the level of serum-supplementation (Section 3.2.1.2).

An essential part of the pretreatment protocol for the HEp-2 autocrine assay required that the cells were harvested from exponentially dividing cultures grown in 10% (v/v) serum. Reducing the level of serum substitution in the pretreatment cultures resulted in a complete lack of cell growth at the low cell density used in the 24-well autocrine assay (i.e. 4×10^2 cells/well). This probably reflects a lower 'growth fraction' in cultures grown in reduced serum, because such populations are more likely to contain fewer numbers of actively dividing cells (i.e. as a percentage of the total population).

Cell growth in the HEp-2 monolayer assay was quantified by measuring colony area. This method was found to give a linear growth response to increasing serum stimulation, with concentrations of serum up to 10% (v/v). The size of colonies measured in monolayer is an index, not just of cell proliferation, but also of the outward migration of cells from the colony edge. Therefore, using colony area as an endpoint to quantify the mitogenic respsonse to growth factors may incorporate the contribution of one, or both, of these growth factor properties. In a study examining the roles of EGF and TGFα on keratinocyte growth in monolayer, cell migration did not become a limiting factor in colony expansion until eight days after seeding {Barrandon & Green (1989)}. The growth of keratinocyte colonies was exponential up to that point, but thereafter the rate of increase in colony radius was the same in the presence, and absence, of either EGF or TGFα. This suggested that in large colonies, the advance of the

colony perimeter is dependent on the fixed rate of cell migration. The duration of the HEp-2 autocrine assay was seven days, so it is unlikely that cell migration became a limiting factor in this system in that time frame.

An independent index of cell growth was used to confirm the autocrine effect determined by the colony area endpoint method. To this end, increases in CM-stimulated HEp-2 cell growth were quantified by measuring the level of cellular acid phosphatase. This qualitatively different endpoint for the quantification of cell number has been validated in this laboratory by Martin *et al.*, (1993), and a linear correlation was demonstrated between acid phosphatase activity and cell number.

4.2.2 Characterisation of the HEp-2 autocrine activity

The autocrine activity in unconcentrated (crude) CM was dilutable. On the basis of size fractionation studies, using ultrafiltration membranes of varying pore size, the active autocrine stimulatory species could be separated into two molecular weight fractions. The first was recovered in the 10-30 kDa MW range, and designated 'R10-30'. The second, designated 'R30', was found in a fraction containing molecular species with MWs greater than 30 kDa. In addition, evidence for an autocrine inhibitory species was found in a low molecular weight fraction. This inhibitory species, with a molecular mass in the range 1-5 kDa, was only manifest at concentrations in excess of those found in crude HEp-2 CM (see figure 3.2.3.2). The 1-5 kDa inhibitory species was not further characterised in this work because the limiting cell density used in the HEp-2 autocrine assay was not suited to measuring growth reduction. However, independent studies also performed in this laboratory (Angela O'Toole, unpublished), confirmed the autocrine inhibitory activity in this fraction. Using HEp-2 plated at 5×10^3 cells/well (also in 24-well plate system) and in a 1.25% (v/v) serum background, maximal inihbition equivalent to a 40% reduction in growth was measured in the 10x concentrate of the R1-5 fraction.

In the work described in this thesis, an autocrine inhibitory species was also recovered from HEp-2 CM after separation on a cation exchange resin (Section 3.3.4.1). The effect of this inhibitory species, (eluting at 0.75M KCl) was masked when it was reconstituted with a stimulatory activity from the same separation (eluting at 0.25 M KCl). This suggests that the inhibitory activity responsible for the 0.75 M KCl effect is only active in an isolated (and possibly concentrated) form. In this respect, it similar to the concentration-dependent inhibitory

activity detected in the R1-5 ultrafiltration fraction.

The autocrine stimulatory activity (ASA) in the R10-30 fraction was maximally active at a five-fold greater concentration than that found in crude CM. There were indications that this activity might be active at lower dilutions, but the siginficance of R10-30 stimulation, at concentrations normally found in crude CM, remains to be verified. However, loss of the R10-30 ASA upon dilution may reflect the lability of the active species is this fraction to dilution. In support of this idea, the R10-30 ASA was found to be much less stable to storage at 4°C, compared to the correpsonding activities in crude or R30 fractions.

The R30 stimulatory species displayed a concentration-dependent inhibitory effect, manifest only at a 10x concentration. Dilution of the concentrated R30 fraction revealed the presence of a stimulatory activity which, unlike the R10-30 species, was stimulatory at dilutions down to 1x. Preliminary gel-filtration analysis using a concentrated HEp-2 CM fraction, did not support the existence of a separate high MW inhibitory species. Loss of the R30 inhibitory species (but not the stimulatory activity) after storage at 4°C, is consistent with the idea of an R30 bifunctional modulator. Detection of a low MW inhibitory species in the gel-filtration separation of CM seems to support the existence of a concentration-dependent activity in the 1-5 kDa ultrafiltration fraction (Section 3.3.1).

Separation of the ASA in HEp-2 CM by gel-filtration (figure 3.3.1,B) confirmed the findings of the ultrafiltration studies. The activity in crude CM was separated into several MW species by gel-filtration, and indicated that one large (close to, or greater than, the MW exclusion limit of the gel, i.e $\sim$30 kDa), and at least three intermediate MW stimulators (within the 3-30 kDa MW range resolved by the gel) could be recovered by molecular sieving. A small amount of stimulatory activity which eluted early in the chromatogram (possibly in the void volume) might be any one of a number of species. Extracellular matrix-attachment proteins such as laminin (MW $\sim$ 900 kDa) are capable of promoting cell growth in some cell systems {Rizzino (1984)} and the large MW ECM heparan sulphate proteoglycans (MW $>$800 kDa) are also known to bind growth factors (see discussion Section 4.3). This large MW species was not investigated further.

Analysis of the R10-30 fraction by hydrophobic interaction chromatography, separated four mitogenic species in this fraction. However, the appearance of an inhibitory activity, eluting

under hydrophobic conditions, could not be confirmed because of a persistant problem with inhibition arising from a dialysis step. This problem was also encountered with dialysis steps performed in conjunction with the other chromatographic separations used in these studies (Section 3.3.6). Repeated attempts to remove the dialysis-related inhibitor, by progressively more thorough washing of dialysis tubing, were unsuccessful (Section 2.8.2).

Physicochemical stability studies performed on crude HEp-2 CM, and two of the active stimulatory fractions, R10-30 and R30, were designed with the knowledge that mitogenic species in HEp-2 had various degrees of affinity for immobilised heparin (see batch elution of concentrated HEp-2 CM, Section 3.3.2.1). As mentioned earlier, heparin is reported to stabilise acidic and basic FGF to denaturation by heat, proteinase and acid inactivation. It was used here to assess its potential to stabilse the HEp-2 ASA against these treatments.

The pH stability profiles for the three CM fractions tested were not informative in the acid pH range. This was due to an unexplained inhbitory effect resulting from the acidification process. High osmolarity was ruled out as the major cause of this inhibition, on the basis of conductivity measurements taken which demonstrated a lack of correlation between high osmolarity and inhibition. The prospect of a chemical change in the basal medium is therefore likely, and may be informative in the context of some published data, and the evidence presented here for a bFGF-like growth factor activity in HEp-2 CM. Rapraeger *et al.*, (1991) have reported that the presence of chlorate ions (at 30 mM) inhibit the sulphation of heparan sulphate proteoglycans in myoblasts, thereby rendering these cells insensitive to the biological effects of bFGF. The concentrated HCl used here for the pH adjustment of medium samples may contain trace quantities of chlorate ions, or they may be produced in a by-reaction with one of the basal media components in Ham's F12. The inhibition of the formation of low affinity cell surface binding sites, essential for FGF binding and signal transduction, is only one possible explanation for the effect of acid-treated Ham's F12. This hypothesis remains to be tested.

The following table summarises the stability properties of the respective HEp-2 CM fractions containing ASA, and compares these samples to the percentage acidic and basic FGF-like autocrine activity which could be neutralised in these samples.

Table 4.2.2 Relative stabilities of the autocrine activities in crude HEp-2 CM and two ultrafiltration fractions, R10-30 and R30: comparison to the percentage autocrine activity neutralised by anti-FGF neutralising antibodies

SAMPLE	CRUDE CM	R10-30	R30
% ACTIVITY OF TOTAL AUTOCRINE REMAINING AFTER TREATMENTS			
Acid	30[a]	0[a]	0[a]
Acid + heparin	30	N.D.	N.D.
Base	60-70	100	0
Base + heparin	70-100	N.D.	N.D.
Trypsin (crude)	10-30[b]	N.D.	N.D.
Trypsin (pure)	40-90[b]	25	100
Trypsin (pure) + heparin	30	N.D.	N.D.
Trypsin Inhibitor	100	0	35
Heat, 65°C	70-100	26	77
Heat, 65°C + heparin	100+[c]	36	65
Heat, 100°C	30-50,(0-70)	9	N.D.
Heat,100°C + heparin	70-85	0	90
Percentage of total autocrine activity remaining after incubation with FGF neutralising antibodies			
Anti-basic FGF	54	1-39	58
Anti-acidic FGF	32	39-79	70[d]

N.D; not determined. [a] Inconclusive result, due to the limitations described in Section 3.2.4.1. [b] Differences in the heat stabilities of 'early' versus 'late' CM collections from the same cultures were observed but not confirmed. The different heat stabilities were reflected in the tryspsin sensitivities. [c] Increasing treatment time at 65°C in the presence of heparin augmented the stimulatory activity up to a maximum of 219% after 20 mins, (see Section 3.2.4.5, table 3.2.4.5(i)b). [d] The percentage activity remaining in this fraction may be closer to 100% (Section 3.4.1.5, table 3.4.1.5b).

The existence of several HEp-2 autocrine growth factors activities in the 10-30 kDa MW range, suggested from the results of chromatographic separations, make it impossible to draw definitive conclusions based on the stability data for ASA in either crude or the ultrafiltration fractions. This is because the overall activity remaining after treatment may represent the effect on the stability of one or more different growth factors. In theory, these may act synergictically or antagonistically to control the growth of HEp-2 cells. Some general distinctions are possible

however, and these serve to distinguish the 10-30 kDa activities from those in the higher MW fraction.

The R10-30 activity is more sensitive to heat treatment and trypsin exposure than the R30 stimulatory species, raising the question of whether the larger sepcies is a protein or not (table 4.2.2 above). The R30 activity appeared to be completely abolished by acid and base treatments, using the same conditions employed for pH treatment of the R10-30 fraction. Despite the concomitant reduction in control growth in that experiment, the R10-30 stimulatory activity remained stable at high pH (8.5-10.5) but the R30 activity was abolished. This may suggest a differential in pH sensitivity between the two fractions, at least in the alkaline pH range.

The results of the trypsin treatments of unfractionated (crude) CM were varaible. The early collection of CM from HEp-2 cultures appeared to contain more trypsin-resistant ASA relative to a later collection from the same culture. Higher concentrations of a less pure grade of trypsin abolished some of this 'resistant' autocrine activity, but a residual 30% was refractory to further degradation by the proteinase (3.2.4.4). The possibility of carry over of serum-derived growth factors is unlikely because cultures were rinsed extensively before CM preparation and the first collection was discarded in all cases.

Another interesting finding in these experiments was the ability of the soyabean trypsin inhibitor to abolish the R10-30 ASA, and to a lesser but nonetheless significant extent, the ASA in the R30 fraction. This suggests a role for a trypsin-like proteinase in the mediation of the autocrine activity in both fractions. Such an hypothesis is supported by the detection of serine proteinase activity in HEp-2 CM {O'Leary *et al.*, (1991)} and is discussed further in Section 4.3.2.

Many of the growth factors identified to date have molecular weights in the 10-30 kDa range and several highly purified preparations of these growth factors were mitogenically active on HEp-2 cells. This indicates that HEp-2 express the cognate receptors for these growth factors, and raises the possibility that autocrine loops involving such mitogens may be operative in HEp-2 cells.

The most potent growth factor tested in the HEp-2 assay system was TGFα. Maximal stimulation, equivalent to a three fold increase in growth over control, was measured at doses

between 0.25 and 0.50 ng/ml, but higher concentrations (> 5.0 ng/ml) were inhibitory to HEp-2 growth. Despite evidence for TGFα-induced stimulation of HEp-2 (Section 3.2.6.1), no TGFα-like biological activity was detected in HEp-2 CM, or in either of the active HEp-2 CM fractions tested using the NRK soft agar assay (Section 3.5.1). Similarly, no EGF-like activity was detected in a 50x concentrate of HEp-2 CM using a radioreceptor assay for competivitive binding to the EGF receptor (3.1.6.3). The size of the mature TGFα (6 kDa) and the absence of detectable TGFα-like biolgical activity in HEp-2 CM, make it an unlikely contributor to the observed HEp-2 autocrine effect. A similar argument applies to the candidacy of EGF in this autocrine system.

In some proliferation-dependent assays, TGFα has been found to be a more potent stimulator of growth than EGF. The colony formation of human keratinocytes in monolayer for example, has been reported to be induced more by TGFα than EGF {Barrandon & Green (1987); Pittelkow *et al.*, (1989)}. No such differential was observed in the HEp-2 system.

Transforming growth factor β (TGFβ) had no significant effect on HEp-2 growth even though HEp-2 were found to secrete relatively high levels of TGFβ activity into their culture medium (Dr. Margaret Dooley, unpublished results from TGFβ bioassay). This may suggest that the latent form of the inhibitor is secreted by HEp-2, as is the case with many cell lines in culture.

The IGFs also displayed potent mitogenic activity in the HEp-2 system, with maximal fold increases over control of 2.7, 2.0 and 1.8, induced by IGF-I, Insulin, and IGF-II respectively. Despite the relatively small size of these growth factors, the existence of larger binding molecules for this class of mitogen, and the indication that 30% of autocrine stimulatory activity might be 'acid resistant' does not permit their elimination as potential contributors to the autocrine effect.

Interleukins were weaker stimulators of HEp-2 growth than the other growth factors tested. Moreover, the sensitivty of cells to these cytokines varyied by a factor of 10 between replicate experiments (Section 3.2.6.5), suggesting perhaps that the effect of IL-1 on HEp-2 growth is modulated by other factors.

PDGF stimulated a low level fold stimulation over the control (i.e x 1.41 fold) at only one concentration, but at higher concentrations than this it was inhibitory, suggesting that it may act

as a bifunctional regulator of HEp-2 growth. Evidence for an intracellular heparin-binding mitogen with low affinity for immobilised heparin-Sepharose is consistent with the value of 0.5 M NaCl reported to elute PDGF from this matrix {Klagsbrun (1989)}. PDGF has also been implicated in 'private' autocrine mechanisms of cell stimulation.

The stimulatory efffect of bombesin/GRP in the HEp-2 assay was not dose dependent, suggesting either an indirect effect on cell growth, or perhaps that the threshold for maximal stimulation with this peptide was exceeded at the lowest concentration tested. No stimulatory activity was detected in the low MW fractions of HEp-2 CM to suggest autorine production of bombesin/GRP in Hep-2.

Acidic and Basic fibroblast growth factors were stimulatory for HEp-2 over very narrow concentration ranges, consistent with the mitogenic activities of these growth factors in other cell line systems. Basic FGF, unlike many of the known growth factors, including acidic FGF, is labile in acid pH. The mitogenic response of HEp-2 to acidic FGF and basic FGF, together with the apparent acid sensitivity of a larger portion of the ASA in the R10-30 and crude HEp-2 CM fractions, prompted the use of a heparin-Sepharose chromatography system, both as a means of characterising the autocrine species, and as a potential step in its purification.

4.2.3 Purification of HEp-2 autocrine activity

Effect of dialysis

The presence of high salt concentrations in the elute of heparin-Sepharose (HS) separations necessitated the dialysis of fractions before these could be assayed in the proliferation-based HEp-2 autocrine assay. However, this process was found to create problems with inhibition of HEp-2 cells (Section 3.2.3.1). The reason for this was unclear, but trace levels of numerous impurities can be toxic to animal cells *in vitro,* and it is theoretically possible that toxic residues remained in the particular batch of the dialysis tubing used in these studies, despite the pretreatment protocol used.

The problem with dialysis posed a number of difficulties in the detection and interpretation of growth regulatory activities eluting during chromatographic separations. Under these circumstances, stimulatory activity may fail to be detected because of the masking effect of the dialysis-related inhibitory species. It is also impossible to discriminate between what may be a

'real' inhibitory activity, and the artifactual inhibition associated with the separation and/or dialysis process. Greater dilutions of column eluates were employed to overcome the inhibitory effect of dialysis and/or high salt concentrations. However, at the higher dilutions used, it is possible that some activities may not have been detected due to sub-optimal doses or instability arising at low concentrations.

Other workers have also reported loss of biological activity due to dialysis, as for example, in the purification of fibroblast growth factors {Klagsbrun *et al.*, (1987)}. The loss of FGFs during dialysis may be caused by adsorption to the dilaysis tubing. Acidic FGF for example is reported to be a 'sticky' polypeptide, especially at neutral pH and low salt concentrations {Kan *et al.*, (1991)}. Nauro *et al.*, (1993) reported similar problems in the purification of FGF-9. Dialysis has also resulted in the loss of other mitogenic activities. A variable loss of partially purified IL-3 activity from the myelomonocytic leukemia cell line WEHI-3 was associated with a dialysis step {Bazil *et al.*, (1983)}, and a similarly GM-CSF was lost after dialysis during its purification from T19.1 cells {Burgess *et al.*, (1980)}.

In this project, the problem with dialysis appeared to be one of toxicity. This is suggested by the fact that inhibition could be diluted out.

Many investigators in this field avoid the problems associated with proliferation based bioassays by using different indices for measuring the response to growth factors. A popular alternative detection system is based on the incorporation of[^{3}H]thymidine into acid insoluble materials. This assay relies on the quantification of DNA synthesis (as determined by [^{3}H]thymidine uptake into cells), following mitogen-induced cell division. [^{3}H]thymidine incorporation has been widely used to measure both positive and negative growth regulation in various cell lines, including the detection of FGF activity in the mouse fibroblast cell line 3T3 {Klagsbrun *et al.*, (1987)}. By quantifying a more immediate response to growth factors, use of the [^{3}H]thymidine incorporation assay gives a 'readout' before the delterious effects of high salt etc. on cell growth are manifest. As a technique however, it is not without limitations. Principal among these, is the fact that thymidine uptake by cells reflects not only its incorporation into nascent DNA, but also changes in thymidine transport across the plasma membrane. Differences in the phosphorylation of thymidine by thymidine kinase can also influence the results obtained with this method {Rozengurt (1991)}.

More direct methods for quantifying cell number, such as the proliferation-based assay employed here, have the advantage of detecting real changes in the 'bulk' of the culture. Therefore, perturbations in cellular biochemistry, such as those which cause aberrations in thymidine transport/uptake, are not translated into spurious measurements of cell number. A disadvantage of the HEp-2 assay system however, is the minimum requirement that test samples are in biologically neutral solutions, or at least that such test solutions do not contain inhibitors for the 'indicator' cell line. This aspect of the assay becomes a limiting factor when large numbers of column fractions need to be assayed (e.g when dialysis of every fraction is required).

4.2.3.1 Heparin-Sepharose chromatography

The heparin-binding ASA detected in HEp-2 CM and cell extracts were classified on the basis of the NaCl concentration required to elute them from heparin-Sepharose (HS). 'Weak' binding species eluted between 0-0.5 M NaCl, while those recovered in the 0.5-1.0 M NaCl range were considered to display 'moderate' binding for the matrix. Species with 'high affinity' eluted in the 1-2 M NaCl concentration range, and any activity recovered above 2 M NaCl was considered to have a 'very high affinity' for heparin. Table 4.2.3.1 summarises the activity profiles for stimulatory species recovered in HEp-2 CM and cell extracts.

Table 4.2.3.1 Affinity of autocrine stimulatory species in HEp-2 CM and cell extracts for the heparin-Sepharose (HS) matrix

HEPARIN AFFINITY - Chromatographic elution system used	Weak	Moderate	High	Very High
	NaCl elution profiles (Molar) of autocrine stimulators on HS			
Concentrated crude HEp-2 CM - Batch elution, 5 ml column	NONE	NONE	1.0; 1.5; 2.0;	5.0;
Concentrated crude Hep-2 CM - Gradient elution, 10 ml column	NONE	0.7;	1.0-1.9; 2.1;	2.45-2.50;
0.25 M KCl eluate of unconcentrated crude CM from cation-exchange column - Gradient elution, 10 ml column	0.25;	NONE	1.05-1.28; 1.46-1.66; 1.89-2.07;	2.7;
HEp-2 'high salt' cell extract - Gradient	0.1-0.40;	0.70-0.85;	1.78-2.0;	3.0;
HEp-2 'low salt' cell extract - Gradient	0.0-0.50;	NONE	1.8-2.4;	NONE

4.2.3.2 Purification of ASA using cation-exchange and heparin-Sepharose chromatography

Preliminary evidence for heparin-binding ASA in HEp-2 CM with HS affinities in the same order as those reported for acidic and basic fibroblast growth factors, suggested that a similar purification strategy might be useful for the purification of the active HEp-2 autocrine factor(s). This protocol employed the use of a cation-exchange step followed by HS chromatography, and has been used to purify aFGF and bFGF to apparent homogeneity {Shing *et al.*, (1983 & 1984)}. In this work, the ASA in one of two separate fractions (i.e the 0.25 M KCl fraction) purified from crude HEp-2 CM by cation exchange chromatography, was then separated on a HS matrix. This separation demonstrated that the greater portion of the total protein therein was non-heparin binding. Some autocrine stimulatory activity was associated with this non-bound protein.

Non heparin-binding ASA

A possible candidate for the non-heparin binding activity was interluekin-1. This cytokine does not bind to immobilised heparin {Besner & Klagsbrun, unpublished observations in Klagsbrun (1989)} and an interleukin-1-like activity had been reported in HEp-2 culture medium by Miyauchi *et al.*, (1988), as mentioned above (Section 4.1.2.2). Evidence from the results of experiments with IL-1 in this project indicated that IL-1 is mitogenic for HEp-2 cells, supporting the idea that this cytokine may be active via an autocrine loop in these cells.

The potential benefit of IL-1α and IL-1β as mitogens for the lung SCC cell lines used in this work, was ambiguous (Section 4.1.16 above). This observation has also been substantiated by a report in the literature {Hanauske *et al.*, (1992)}. The characterisation of the non-heparin binding HEp-2 autocrine activty therefore, was deferred in preference to isolating the heparin-binding species. This was because preliminary results with two of the heparin-binding FGFs (acidc and basic FGF) had demonstrated signs of mitogenic activity for the NSCLC cell line DLRP. Indirect support for an FGF-like autocrine activity in HEp-2 cells was presented in a report by Moscatelli *et al.*, (1986). These authors had detected a bFGF-like species in the extracts of the HEp-2-related HeLa cell line. However, the basis of this detection was indirect, (i.e. based on the ability of bFGF to induce plasminogen activator production in bovine capillary endothelial cells). Moreover, these authors were unable to detect any bFGF activity in medium conditioned by these cells.

Interleukin 6 as an autocrine growth regulator in HEp-2

A second candidate for the non-heparin-binding ASA in HEp-2 CM is IL-6. This stems from the findings of a recent study of nonhematopoietic malignant cell lines which included HEp-2 {Guillaume *et al.*, (1993)}. The coexpression of IL-6 and a functional IL-6 receptor system were detected by these investigators in HEp-2. Moreover, significant levels of IL-6 protein (225 ng/ml) were measured in HEp-2 supernant culture medium by bioassay using the IL-6-dependent murine hybridoma 7TD1. Expression of IL-6 protein was corroborated by the detection of IL-6 gene expression using PCR amplification. HEp-2 were also responsive to stimulation by exogenously supplied human recombinant IL-6, as determined by the [^{3}H]thymidine incorporation assay. At concentrations of 20, 100, and 500 ng/ml, the respective fold induction of DNA synthesis in HEp-2 cells was 1.46, 1.65 and 1.36 respectively. These findings suggested the existence of a functional IL-6 autocrine loop in HEp-2 cells.

The possibility of a functional IL-6 loop in HEp-2, is interesting in the context of evidence presented here for a bFGF-like autocrine loop in the same cells, and a report which documents a modulatory role for IL-6 in a bFGF autocrine system. Araujo & Cotman (1992) presented evidence supporting a bFGF autocrine loop in astroglial, microglial and neuronal cells.
These workers found that IL-6, but also IL-1 and EGF, enhanced the release of bFGF from these cells. It is conceivable therefore that IL-1 and/or IL-6 may mediate their actions on HEp-2 cells through modulation of the release of the bFGF-like autocrine activity.

Also in the report of Guillaume *et al.*, (1993), GM-CSF and IL-3 were not detected in HEp-2 medium by ELISA. Similarly, there was evidence no evidence for the expression of GM-CSF or IL-3 genes in HEp-2, as determined by PCR analysis. Nevertheless, HEp-2 cells were found to express the specific α subunit and the β receptor subunit utilised by GM-CSF in the formation its high affinty receptor complex. Despite a lack of evidence for HEp-2 stimulation by GM-CSF in the report of Guillaume *et al.*, (1993), an autocrine role for this cytokine, under the stringent growth conditions used in the HEp-2 autocrine assay, cannot be ruled out for a number of reasons. These authors found a lack of evidence for GM-CSF gene expression in HEp-2 grown in serum-supplemented medium, however, GM-CSF gene expression might occur in the serum-free conditions employed for the collection of HEp-2 CM in this work. Moreover, the absence of detectable immunoreactive GM-CSF in HEp-2 culture medium need not imply that the protein is not expressed by these cells. GM-CSF is known to have an affinity for heparan sulphate proteoglycans in the extracellular matrix (ECM) {Roberts *et al.*, (1988)}, and

as such may remain bound to the ECM under the conditions used by Guillaume *et al.*, (1993). Furthermore, these investigators did not detect stimulation of HEp-2 with exogenously supplied recombinant GM-CSF, which in the face of evidence for the expression of the GM-CSF receptor genes, they attribute to either ineffective receptor peptide assembly, or dysfunctional signal transduction by the receptor complex. However, an alternative explanation (admitted by these workers), is that the detection of GM-CSF activity in HEp-2 may have been limited by the assay conditions used. In this thesis, the sensitivity of HEp-2 to the autocrine stimulatory effects of HEp-2 CM were reduced when the serum concentration or the platining density was raised (Section 3.2.1.2). The relatively high plating density (10^5 cells/ml) and background serum concentration (3% v/v) used by Guillaume *et al.*, (1993) therefore may render HEp-2 cells insensitive to GM-CSF. If such is the case, then perhaps IL-6 would also be more active mitogenicllly in the HEp-2 assay employed here.

Evidence also exists to support an intracrine model for autocrine growth stimulation by GM-CSF in some hematopoietic cells {Browder *et al.*, (1989)}. In this context, the detection of an intracellular stimulatory activity in HEp-2 cells, with a similar affinity for heparin as that reported for GM-CSF (see table 4.2.3.1 below) would be consistent with an a 'private' autocrine GM-CSF loop in HEp-2 cells.

Heparin-binding ASA

A high order of purification following the two-step protocol used here, was suggested by the fact that the heparin-binding ASA separated from the bulk of protein in this fraction. Although the level of autocrine stimulation recovered after this second step was low ($\sim$1.4 fold average in active fractions from heparin-Sepharose), it was nonetheless significant. This apparently low level of activity may reflect a combination of the instability of the active species (in a more highly purified form), and the high dilution required for use in the assay. Two of the 'high affinity' stimulatory activities (eluting between 1 and 1.3 M NaCl, and at 1.4 - 1.7 M NaCl respectively) correspond with those reported for acidic and basic FGFs respectively. The possibilty that these are in fact aFGF and bFGF proteins would be consistent with the reported instability of pure acidic and basic FGF in solution {Gospodarowicz *et al.*, (1985)}. However, other growth factors have subesequently been shown to have high affinity for immobilised heparin, and included among these are the non-FGF growth factors. A list of the known heparin-binding mitogens is presented in table 4.2.3.2 below.

Table 4.2.3.2 Comparison of the salt elution profiles, molecular masses, and pH stabilities of the major heparin-binding growth factors classes

GROWTH FACTOR (Alternative name)	[NaCl]^a required to elute from heparin-Sepharose	SIZE (kDa)	STABILITY Heat/Acid
FGF GROWTH FACTOR FAMILY			
FGF-1 (acidic FGF)	1.0-1.2	16-18	L/S^b
FGF-2 (basic FGF)	1.5-2.0	18	L/L
FGF-3 (*int*-2)	N.R.^{c}	30.5-31.5	N.R.
FGF-4 (*hst*-1/K-fgf)	1.0-1.2	~23	N.R.
FGF-5	1.0	32.5-38.5	N.R.
FGF-6 (*hst*-2)	1.0	N.R.	N.R.
FGF-7 (KGF)	0.6	28	L/L
FGF-8 (AIGF)	1.1	28, 32	N.R.
FGF-9 (GAF)	0.8	30	L/L
PDGF GROWTH FACTOR FAMILY			
VEGF (VPF)	0.3, 0.9; 2.0;	34-42	S/S
PDGF	0.5	28-36	S/S
EGF-LIKE GROWTH FACTOR FAMILY			
HB-EGF	1.0-1.2	19-23	S/S
INSULIN-LIKE GROWTH FACTORS			
IGF-I	<0.5	7	S/S
IGF-II	0.5	7, 8, 16	S/S
OTHER NON-FGF HEPARIN-BINDING GROWTH FACTORS			
PLEIOTROPHIN (HBNF)	1.1-1.5	18	?/L
HGF/SF	1.3-1.4	69	L/S
GM-CSF	0.5	22	S/S
IL-3	0.5	25	S/S
IL-8	0.5	8	S/S
GROα (MGSA)	0.5	8	S/S

[a] Molar concentration. [b] L=labile; S=stable. [c] Not yet reported.

Although the same batch of heparin-Sepharose was used for all the chromatography work described here, and a reproducible FPLC system was employed for gradient elutions, direct comparisons with heparin affinities for the well characterised growth factors cannot be made. Different retention times of the same growth factors on the matrix were reported to be accounted for by batch to batch variations in HS preparations (even among those supplied by the same company) {Klagsbrun *et al.*, (1987)}. For more analytical determinations, these columns can be calibrated with purified FGFs {Gospodarowicz *et al.*, (1984)}. However, even with the use of standardised separation conditions, qualitative predictions based on heparin affinities alone, are of limited use in characterising unknown heparin-binding mitogens.

In the absence of data for the size and stability of the different heparin-binding ASAs it was difficult to predict which, if any, of the currently identified heparin-binding growth factors (FGF and non-FGF) might be responsible for the respective mitogenic activities. Comparing the affinities of the ASA in crude and fractionated HEp-2 CM, as well as in HEp-2 cell extract, it is clear that a number of growth factors could account for the high affinity ASA.

A similar problem prohibits making an informed prediction about the nature of the loosely bound ASA which was recovered at 0.25 M NaCl in this two step purification protocol). Recovery of ASA at this low salt concentration is, however, consistent with the detection of a similar low affinity ASA in HEp-2 cell extracts, following a single HS separation.

The very high affinity ASA eluting at 2.7 M NaCl (following the two-step purification), was detected in all other fractions except the 'low salt' extract. This mitogen displayed an unusually high affinity for heparin. While this could perhaps be interpreted to suggest the presence of a novel heparin-binding growth factor, it was considered that the species might also be attributed to the formation FGF multimers. Disulphide bond formation between bFGF molecules has been reported to generate heterogenious elution proflies in the separation of recombinant bFGF on a heparin-TSK HPLC column. This was evident from the appearance of multiple peaks with extended retention times, in samples which had previously single eluted as single peaks from the same column {Thompson *et al.*, (1991)}.

A number of findings in this work favour the involvement of a bFGF-like factor in the HEp-2 autocrine effect. These include the HS-affinity data, the mitogenic responsiveness of HEp-2 to bFGF, and (inasmuch as it can be relied upon) the apparent acid sensitivity of a greater portion

of the ASA in crude CM. Before any further purification was considered, the potential role of bFGF in the autocrine effect was explored using an anti-basic FGF neutralising antibody. Because aFGF exhibits similar biological activities to bFGF, but is not acid labile, and also because a portion of the ASA eluted from the HS matrix at a NaCl concentration coincident with that reported for aFGF, the potential autocrine regulation of HEp-2 by an aFGF-like factor was investigated using an anti-acidic FGF neutralising antibody.

4.2.4 Inhibition of autocrine activity with anti-FGF antibodies

Confirmation of the production of a bFGF-like ASA by HEp-2, was provided by the observation that ~50% of the ASA in crude HEp-2 CM could be effectively neutralised by co-incubation with two different lots of an anti-basic FGF antibody. A similar portion of the R30 ASA (~40%) in the R30 fraction could also be abolished with this antibody, suggesting the presence in this fraction, of a larger MW form of bFGF, or an immunologically similar species with a MW > 30 kDa. In contrast, the greater part of the 10-30 kDa ASA appeared to be a bFGF-like species. The size of the mature bFGF molecule (18 kDa), together with the lability of the R10-30 ASA, are consistent with the presence of a bFGF-like mitogen in this fraction.

Under the conditions used in these studies, the commercial anti-acidic FGF antibody was found to cross-react with bFGF. This made it impossible to assign the activities neutralised by this antibody to an acidic FGF-like species. However, as specified by the suppliers, the anti-acidic FGF antibody did not display cross-reactivity with TGFα, suggesting that the cross reactivity observed with bFGF may be accounted for by their similar sequences. If this is the case, then the results with the anti-aFGF antibody would seem to confirm the presence of an FGF-like ASA in the three HEp-2 CM fractions where bFGF-like ASA was evident.

The inability of either antibody to completely block the ASA in crude HEp-2 CM supports the hypothesis that other growth factors in HEp-2 CM combine to mediate this effect. However, in one experiment, 99% of the R10-30 ASA was neutralised by the anti-basic FGF antibody. This result was not repeated using the same concentration of a different batch of anti-basic FGF antibody, but a significant proportion of the R10-30 activity (61%) was confirmed to be neutralised by the anti-basic FGF antibody.

Batch-to-batch differences in the neutralising activity of polyclonal antisera have been cited by

other workers as factors contributing to the difficulties encountered in attempting to inhibit tumour cell growth using anti-growth factor antibodies {Herlyn *et al.*, (1990)}.

In another study, using polyclonal anti-K-fgf neutralising antibodies, two alternative explanations were proposed to explain the inability of anti-K-fgf to completely block the mitogenic response to K-fgf. The first explanation suggested that residual mitogenic activity due to K-FGF (in the presence of of the anti-K-FGF antibodies), resulted from the interaction of the growth factor with heparan sulphate extracellular cellular matrix components. This interation was proposed to prohibit recognition of K-fgf by the anti-K-fgf antibody. The second alternative, which is also applicable in the case of bFGF, is that the residual activity might be due to intracellular stimulation of the mitogenic pathway {Talarico & Basilico (1991)}.

An interesting result with the anti-FGF antibodies used in this work was the apparent increase in the mitogenic response of HEp-2 to TGFα, elicited in the presence of the antibodies. This effect, although small, was more significant with the anti-basic FGF antibody. A possible interpretation of the affects of anti-basic FGF antibodies on the response to TGFα may be explained by the ability of bFGF to modulate the number of cell surface receptors for this growth factor (i.e. the EGF receptors). Such heterologus receptor modulation by bFGF has been reported in mouse 3T3 fibroblasts, where bFGF has been shown to reduce EGF receptor binding {Hicks *et al.*, (1989)}. More recently bFGF has also been shown to mask the mitogenic effect of EGF on porcine skeletal muscle satellite cells {Doumit *et al.*, (1993)}.

The detection of immunoreactive bFGF in conditioned medium has raised the question of how such a protein as bFGF, lacking a classical signal sequence, can be secreted. Reports in the literature have detected immunoreactive bFGF in culture medium while others have not. Where there has been evidence for bFGF secretion, further questions have been posed concerning its release mechanism. Detection of a bFGF-like mitogenic activity in HEp-2 CM raises similar questions for the HEp-2 system. Moreover, retention of a bFGF-like immunoreactive species in the R30 fraction suggested that a larger molecular weight form of the growth factor might be present in HEp-2 CM, or that the classical 18 kD form was bound to a larger molecular weight species. These hypotheses are discussed in the light of evidence in the literature describing FGF binding species as well as a novel secretory mechanism utilised in the export of bFGF.

4.3 HIGHER MOLECULAR WEIGHT FORMS OF BASIC FGF

Higher molecular weight forms of bFGF were initially purified from human placenta {Sommer *et al.*, (1987)} and guinea pig brain {Moscatelli *et al.*, (1987)}. The larger MW forms arise from the alternative initiation of translation from a single mRNA species using one of three CUG codons 5′ to the classical AUG start site. Proteins arising from CUG codons result in the synthesis of N-terminal extended forms of bFGF with MWs of 22.5, 23.1 and 24.2 kDa in addition to the 18 kDa species initiated from the AUG codon {Florkiewicz *et al.*, (1989); Prats *et al.*, (1989)}. These high molecular weight forms of bFGF are also expressed in other cell line systems {Ensoli *et al.*, (1989); Rifkin & Moscatelli (1989); Renko *et al.*, (1990)}.

The 18 kDa and N-terminally extended forms of bFGF are distributed in different subcellular compartments. The former is found in the cytosol and the latter are translocated to the nucleus {Renko *et al.*, (1990); Florkiewicz *et al.*, (1991)}. The biological significance of the nuclear localisation of high MW bFGF is not clear but recent evidence suggests that bFGF may regulate transcription directly in a gene-specific manner {Nakanishi *et al.*, (1992)}.

Multimerisation of recombinant bFGF can produce dimers, trimers and tetramers of the growth factor under certain conditions {Fox *et al.*, (1988); Seno *et al.*, (1988)}. This has been shown to result from the formation of intermolecular disulphide bridges {Thompson *et al.*, (1991)}.

Pettmann *et al.*, (1991) reported that the biologically active form of natural and recombinant bFGF has an apparent MW of 27 kDa.

A 46 kDa form of bFGF has been reported in bovine adrenal glands using two polyclonal antibodies which also recognised a 24 kDa and a 30 kDa species in addition to the 17/18 kDa double band typical of bovine adrenal bFGF. The formation of multimers was ruled out by the authors because the gels were run under denaturing conditions {Grothe *et al.*, (1990)}. This 46 kDa bFGF-like species was also shown to be biologically active {Westermann *et al.*, (1990)}.

With the exception of the 46 kDa bFGF-like species detected by Grothe *et al* (1990), larger MW forms of bFGF isolated to date are smaller in size than the nominal MW cut-off of the 30 kDa ultrafiltration membrane used to prepare the R30 fraction. However, it is still theoretically possible that a species close to this limit, such as the 27 kDa form of bFGF

reported by Pettmann *et al.*, (1991) would be retained in the R30 fraction if it formed aggregates or was bound to a carrier molecule. A number of such bFGF-binding species have been described in the literature.

4.3.1 BASIC FGF BINDING SPECIES

4.3.1.1 Miscelaneous binding species for bFGF

α2-Macroglobulin has been shown to bind bFGF covalently. This proteinase inhibitor is unlikely to act as a carrier molecule for bFGF because the bound growth factor does not recognise either its high or low affinity receptors {Denis *et al.*, (1989)}.

A 17 kDa putative binding protein for acidic and basic FGF was characterised and cloned by Wu *et al.*, (1991). Growth factor binding appeared to be non-covalent and reversible, and in both cases the biological activities of the respective growth factors were inhibited. Binding was abolished in the presence of heparin, and this also removed the inhibition of biological activites.

4.3.1.2 A regulatory role for the interaction of FGFs and other growth factors which bind heparan sulphate proteoglycans

A class of proteoglycans which act as low affinity receptors for FGFs has recently been described. These heparan sulphate proteoglycans (HSPGs) bind FGFs via their heparan sulphate side chains. Yayon *et al.*, (1991) have shown that such cell surface HSPGs are required as accessory molecules for the binding of bFGF to its high affinity receptor. This observation was supported by the findings of Savona *et al.*, (1991) who suggested that heparan sulphates were necessary for the binding of bFGF to high affinity receptors in adrenocortical cells. Presta *et al.*, (1991) have demonstrated that this interaction is necessary for the proliferative response to bFGF and, as mentioned above, the study Rapraeger *et al.*, (1991) showed that bFGF-mediated fibroblast growth and the differentiation of myoblasts also required the interaction of bFGF with heparan sulphate. A number of HSPGs that interact with bFGF have have recently been cloned.

The first, a transmembrane proteoglycan called Syndecan, is expressed on mouse mammary epithelial cells and was cloned by Saunders *et al.*, (1989). Syndecan was subsequently shown to bind bFGF {Kiefer *et al.*, (1990); Bernfield and Sanderson (1990)}. Studies by Rapraeger *et al.*, (1986) had revealed that cultured mouse mammary epithelial cells shed the extracellular

domain of syndecan leaving all the glycosaminoglycan side chains intact. Syndecan shed by epithelial cells was indistinguishable from a molecule released by the same cells in response to mild trypsinisation {Jalkanen *et al.*, (1987); Weitzhandler *et al.*, (1988)}. These findings, together with the identification of a single dibasic sequence in its extracellular domain adjacent to the plasma membrane, prompted Saunders *et al.*, (1989) to propose that a trypsin-like cell surface proteinase might be involved in the release of this domain from the cell surface.

Subsequently three other members of this HSPG family of molecules have been identified namely Syndecan-2 {Marynen *et al.*, (1989)}, Syndecan-3 {Carey *et al.*, (1993)} and Syndecan-4 {Lories *et al.*, (1992)}. The biology of syndecans has been reviewed by Bernfield *et al.*, (1992).

Another HSPG with bFGF-binding capacity {Andres *et al.*,(1992)} was cloned by Lopez-Casillas *et al.*, (1991). This molecule, called betaglycan, binds bFGF via its heparan sulphate side chains, but also binds TGFβ through its core protein. Like Syndecan, betaglycan is released into the medium in a soluble form from the membrane of cells which express it {Andres *et al.*, (1989)}. A dibasic site in the predicted amino acid sequence of betaglycan was suggested by Lopez-Casillas *et al.*, (1991) as a possible target for one of the recently characterised dibasic endoproteinases {Barr (1991)}. Another potential cleavage sequence was also identified in the ectodomain of betaglycan which is identical to a sequence in the membrane-bound TGFα precursor {Lopez-Casillas *et al.*, (1991)}. The latter form of TGFα is cleaved by an elastase-like activity to release TGFα into the medium {Pandiella and Massagué (1991 a & b)}.

Endothelial cells exposed briefly to bFGF rapidly sequester the growth factor into HSPGs in their ECM, and these cells show the same mitogenic response as similar cultures continuously exposed to bFGF in their medium, suggesting that HSPGs act as a reservoir for bFGF {Flaumenhaft *et al.*, (1989)}. It has also been suggested that a pericellular proteinase might cleave the core protein of HSPGs, releasing their ectodomins with heparan sulphate-bound passenger growth factors {Sommer & Rifkin (1989)}. These and other workers have shown that bFGF-glycosaminoglycan complexes are active on sensitive cells {Saksela *et al.*, (1988)}. Although the origin of soluble HSPGs remains to be determined, studies with endothelial cells indicate that cell surface and ECM HSPGs are distinct molecules, by virtue of their different molecular weights. The cell surface HSPG has a MW of 250 kDa, compared to a MW greater

than 800 kDa for the ECM species {Saksela & Rifkin (1990)}. In addition, Flaumenhaft *et al.*, (1990) have shown that soluble HSPGs can neutralise the interaction of bFGF with fixed HSPGs, thereby functioning as carriers of bFGF which allow it to diffuse away from the producer cell. Moreover, soluble HSPG facilitates bFGF diffusion in a thermally stable {Gospodarowicz & Cheng (1986)} and proteinase resistant form {Sommer & Rifkin (1989)}.

The requirement for auxilary binding molecules in the generation of functional growth factor/receptor complexes, capable of transmitting the growth factor signal to the nuclei of target cells, appears to be a mechanism common to other growth factor signaling pathways apart from bFGF. Interleukin 6, for example, will only bind to its receptor when the auxilary glycoprotein gp130 is also present on the cell surface. This accessory molecule is required for the formation of a functional signal-transducing complex with IL-6 and its receptor {Taga & Kishimoto (1992)}.

Other growth factors interacting with HSPGs

Until recently, an *in vivo* functional role for the relatively weak binding of some growth factors to extracellular matrix and cell surface proteoglycans (compared to FGFs) was not clear. However, despite having a low affinity for heparin, the angiogenic protein, vascular endothelial growth factor (VEGF) was recently shown to be dependent on cell surface-associated heparin-like molecules for interaction with its plasma membrane receptor {Gitay-Goren *et al.*, (1992)}. In this report, the detection of VEGF receptors (by cross-linking with I^{125} VEGF) was enhanced in the presence of heparin, thereby enabling these workers to detect VEGF receptors on cell types other than endothelial cells. These included HeLa cells and NIH3T3 fibroblasts, not previously known to express VEGF receptors. The increase in sensitivity was apparently due to the induction of high-affinity cell surface binding sites in the presence of heparin at 0.1-10 μg/ml. Higher heparin concentrations (10-10000 μg/ml) were found to inhibit ligand binding.

In the context of the results with the HeLa variant HEp-2, presented in this thesis, the possibility of VEGF receptor expression suggests the involvement of one or more forms of this growth factor in an autocrine loop in HEp-2 cell regulation. In a series of studies carried out by Houck and co-workers, the four alternative transcripts of the VEGF gene were transfected into human embryonic kidney cell lines. In the first study, using the 293 kidney cell line, differences in the secretion patterns of the various VEGF transcripts was revealed {Houck *et al.*, (1991)}. Subsequently, evidence for the bioavailability of three of these VEGF transcripts

was examined in clones ('transfectants') of another human embryonic cell line, CEN4, which was transfected with constructs encoding VEGF of different length {Houck *et al.*, (1992)}.

Analysis of VEGF secretion patterns had demonstrated that the two shorter forms, $VEGF_{121}$ and $VEGF_{165}$, were efficiently secreted, and possesed simlar mitogenic activity for endothelial cells. The longer transcripts $VEGF_{189}$ and $VEGF_{205}$ were also expressed, but were not deteceted as soluble proteins in the culture medium, thereby preventing their biological activities from being determined. However, in the more recent study, biologically active $VEGF_{189}$ was recovered from a stable transfectant of the CEN4 cell line, by blocking the interaction of the $VEGF_{189}$ with its cell-surface receptors. This was achieved by using suramin, a non-specific inhibitor of growth factor/receptor interaction. Comparing the chromatographic behaviour of $VEGF_{189}$ with that in conditioned media from two other CEN4 transfectants, expressing $VEGF_{121}$ and $VEGF_{165}$ respectively, it was found that increasing length was associated with increasing affinity for VEGF on a cation-exchange matrix (S-Sepharose). The behaviour of the different VEGF forms on a heparin-Sepharose matrix was also monitered using a VEGF ELISA, and revealed that $VEGF_{121}$ failed to bind heparin whereas $VEGF_{165}$ eluted at 0.9 M NaCl. The longer $VEGF_{189}$ exhibited a binding profile similar to bFGF, with only 20-30% eluting at 0.9M NcCl and the remainder eluting with 2M NaCl. Moreover, it was found that the physiolgically relevant serine proteinase, plasmin, was capable of releasing biologically active $VEGF_{189}$ from its cell surface-bound sites.

4.3.1.3 Soluble forms of the FGF receptors

The recently characterised Fibroblast Growth Factor Receptor 1 (FGFR1) was predicted to generate multiple forms of the receptor by alternative splicing of three alternative exons {Johnson, D.E., *et al.*, (1991)}. One of these splice variants was predicted to encode a secreted form of the FGFR1. Subsequently the same group isolated a cDNA from a human placenta library encoding this protein. This secreted form of the FGFR1 was recently expressed in Chinese Hamster Ovary cells and was found to bind bFGF in preference to aFGF {Duan, D-S. R., *et al.*, (1992)}. It has also been shown that only heparin-bound bFGF binds to soluble receptors {Ornitz *et al.*, (1992)}.

The biological significance of secreted forms of the FGF receptor is not understood at present, nor is it known if such FGF-receptor complexes are mitogenic. It would be premature therefore, to infer a role for such a soluble receptor in the R30 ASA fraction. Soluble receptors for several

cytokines including IL-2, IL-4, TNF and Interferon γ (gamma), have been reported to inhibit the activities of these molecules {Larrick & Wright (1992)}. Other cytokines reported as having soluble receptors include those of IL-6 {Novick *et al.*, (1989)} and IL-7 {Goodwin *et al.*, (1990)}. Although it is not known whether the soluble forms of the IL-6 and IL-7 receptors can inhibit the activity of their respective ligands, it has been suggested that the binding of cytokines to such soluble receptors *in vivo* might increase their half-life in the circulation by protecting them from proteolytic inactivation {Fernandez-Botran (1992)}.

Natural forms of soluble receptors for other non-cytokine receptors have been reported including those for epidermal growth factor {Weber *et al.*, (1984)}, insulin-like growth factors {MacDonald *et al.*, (1989)}, colony-stimulating factor {Downing *et al.*, (1989)} and growth hormone {Leung *et al.*, (1987)}. It would appear therefore that regulation of growth factor activity by soluble receptors is a widely used mechanism among different growth factor classes.

4.3.2 SECRETORY MECHANISMS FOR THE RELEASE OF BASIC FGF FROM CELLS

Other growth factors have been found associated with HSPGs of the ECM, including IL-3 and GM-CSF {Roberts *et al.*, (1988)}, Platelet factor 4, and the non-FGF heparin binding and neurite-promoting factor, Pleiotrophin. Platelet factor 4 binds heparin-Sepharose with high affinity {Li *et al.*, (1990)} but it also has a high affinity for the chondroitin sulphate proteoglycan serglycin, with which it forms a complex in the secretory α granules of platelets {Perin *et al.*, (1988)}. Many hematopoietic cells contain serglycin in storage granules, and in macrophages at least, it is found to be largely secreted. {Kolset and Gallagher (1990)}. By comparison, heparan sulphate proteoglycans appear to be induced as macrophages mature and extravasate from the blood stream during wound healing. Syndecans are likely to act as mediators in the wound healing process since this type of heparan sulphate proteoglycan on the macrophage-like cell line P388D1 have been found to bind to bFGF-Sepharose affinity columns {Yeaman and Rapraeger (1993)}.

Heparan sulphate proteoglycans acting as carrier molecules in secretory vesicles, analogus to the role of serglycin, may explain how bFGF which lacks a typical secretory signal sequence, is released from producer cells. Westermann *et al.*, (1990) have identified immunoreactive bFGF species in the secretory granules of medullary chromaffin cells isolated from bovine adenal glands. Observations based on work with bFGF in the induction of transmitter synthesis

and storage in chromaffin cells, together with previous work demonstrating its effect on their *in vitro* survival, led Unsicker & Westermann (1992) to propose an autocrine/paracrine role for bFGF in the maintenance of these cells. Interestingly the 46 kDa form of bFGF previously identified by this group {Grothe *et al.*, (1990)} was enriched in the secretory granules with respect to the lower MW species when levels of the respective MW forms were compared in the medulla or cortex {Westermann *et al.*, (1990)}. In contrast to these findings Renko *et al.*, (1990) were unable to demonstrate the presence of bFGF in cytoplasmic vesicles isolated by cell fractionation.

If secretion of bFGF in secretory granules via exocytosis does explain the release of this growth factor from cells, the molecular mechanism by which it is recognised for translocation into secretory granules still remains to be determined. In the last few years the number of proteins secreted despite the lack of a classical signal sequence has grown. These include Interleukin-1β which was localised in the membrane-bound vesicles of activated monocytes {Rubartelli *et al.*, (1990)} and thioredoxin, a disulphide reducing enzyme secreted by normal and transformed cells. The latter molecule shares some features of the alternative pathway described for IL-1β, but was localised only in the cytosol, suggesting a direct plasma membrane translocation {Rubartelli *et al.*, (1992)}. Evidence for the excretion of a 14.5 kDa muscle lectin (L-14) by exocytosis from the cytosol to the extracellular matrix of differentiating myoblasts was reported by Cooper & Barondes, (1990). Other plasma membrane activities have been associated with the formation of exosomes (released vesicles); Johnston *et al.*, (1987) reported that selective externalisation via exocytosis is a mechanism for the shedding of transferrin receptors during reticulocyte maturation, and a similar process was described by Hale & Wuthier (1987) during the mineralisation of calcifying tissues.

Evidence for a bFGF secretory pathway independant of the endoplasmic recticulum-golgi complex was recently reported by Mignatti *et al.*, (1992). These authors had previously demonstrated that the migration of NIH 3T3 cells transfected with bFGF cDNA was modulated in an autocrine manner by bFGF. The bFGF-mediated migration was established in an isolated single cell system in which individual cell migration could only be regulated by bFGF secreted from the cell under observation (therefore excluding any possibile contribution of extracellular sources of bFGF present in mass culture experiments) {Mignatti *et al.*, (1991)}. Using this method (Phagokinetic track assay), cell migration was roughly proportional to the amount of extracellular bFGF and therefore was also proportional to bFGF release. These authors were

able to show that drugs or treatments which blocked endo- and exocytosis also inhibited cell motility, while those that inhibited protein trafficing in the Endoplasmic Recticulum-Golgi complex were ineffective in this respect. Blockers of the MDR protein pump were likewise ineffective. Of the treatments used to inhibit endo- and exocytosis in these studies, an interesting finding was that treatment with serum-free conditions appeared to inhibit bFGF release, as manifested by the inhibition of cell migration. These studies also showed that increases in motility by cells under these serum-free conditions were more sensitive to increases in the level of cell-secreted (autocrine) bFGF, irrespective of the addition of exogenous recombinant bFGF. This implies that bFGF must be concentrated near the cell surface {Mignatti *et al.*, (1992)}.

The observations of Mignatti *et al.*, (1992) raises the question of a mechanism for the release of bFGF-like activity detected in HEp-2 CM, given that HEp-2 CM was collected under serum-free conditions, and the possibility that secreted bFGF may be rapidly sequestered by producer cells. If this is the case in the HEp-2 system, then perhaps the trypsin-like activity in HEp-2 CM may be involved in a release and/or activation mechanism for the bFGF-like ASA.

Several candidates for this serine proteinase activity were detected in crude HEp-2 CM, including, trypsin, chymotrypsin, elastase and cathepsin G (see appendix D for details). The effect of a serine proteinase inhibitor (soyabean trypsin inhibitor) on the ASA in crude HEp-2 CM and its R10-30 and R30 fractions, suggests a complex regulatory role for these proteinase activities in modulating the autocrine effect. The stimulatory effect of trypsin inhibitor in the crude CM was in direct contrast to the potent inhibitory effect it displayed in the R10-30 and the R30 ultrafiltration fractions. These discrepancies may be explained in terms of the relative differences in the levels of such endogenous proteinase activities and/or the distribution of specific activities in the fractionated and unfractionated CM.

4.3.3 RELEASE OF BASIC FGF FROM EXTRACELLULAR MATRIX STORAGE SITES

The trypsin-like serine proteinase plasmin, is very abundant in many tissues. In cultures of bovine capillary endothelial cells (BCE) this proteinase has been implicated in the release of bFGF from HSPG complexes in the extracellular matrix (ECM) {Rifkin & Moscatelli (1989); Saksela *et al.*, (1990)}. Endothelial cells in culture have been shown to synthesise bFGF and this growth factor is active in effecting the biological properties of these cells in an autocrine manner {Sato & Rifkin., (1988)}. It has also been demonstrated that BCE cells secrete heparan

sulphate-bound bFGF {Saksela *et al.*, (1988)}. Given the similar role demonstrated for plasmin in the release of extracellular matrix bound-VEGF and bFGF, together with the evidence for the involvement of a trypsin-like proteinase activity in mediating both the R10-30, and the R30 autocrine stimulatory activities, it is tempting to suggest that this trypsin-like proteinase may function in a similar capacity to effect the autocrine response in HEp-2.

The implication of proteinase activity in the R10-30 ultrafiltration fraction is further supported by the work of Ho *et al.*, (1990) who reported the co-purification of serine and thiol-like lysosomal proteinases with pituitary-derived bovine bFGF on heparin-Sepharose. Two components, with apparent molecular weights of 28 and 30 kDa respectively, were separated and found to be immunoreactive against bFGF antisera. N-terminal sequence analysis revealed that these proteinases shared no sequence homology with FGFs, but that the 28 kDa component was 73% homologus with human cathepsin L and the 30 kDa component shared 57% and 47% homologies with human leukocyte elastase and cathepsin G respectively. The authors suggest that these extracellular-matrix-degrading enzymes {Sinha *et al.*, (1987), Erickson (1989)}, which are produced and secreted by transformed cells {Tryggvason *et al.*, (1987); Troen *et al.*, (1988)} may act to release matrix-bound FGFs. Another group reported indirect evidence for an endogenous trypsin-like proteinase which converted high MW bFGF species to a 'trypsin resistant' 18.8 kDa form during the purification of bFGF from bovine and rat brain on a heparin-Sepharose matirx. They found that Ca^{2+} protected the high MW heparin-bound forms from truncation by the co-purifying proteinase activity, suggesting that Ca^{2+} inhibited the activity of the proteinase responsible {Doble *et al.*, (1990)}.

A bFGF immunoreactive proteinase similar to that described by Ho *et al.*, (1990) might account for some or all of the autocrine stimulatory activity in R10-30 fraction of HEp-2 CM, but results of protease measurements in conditioned medium collected using high Ca^{2+}-containing basal medium suggest that the trypsin-like activites in HEp-2 CM are stabilised by high Ca^{2+} concentrations {O'Leary *et al.*, (1991)}. Preliminary studies in this thesis, indicated that the fold stimulation measured in high Ca^{2+}, MEM conditioned medium, was less than that detected in the low Ca^{2+} media (Section 3.2.1.4). Moreover, 'high Ca^{2+}' basal media such as MEM demonstrated higher levels of serine proteinase activities (appendix D). Therefore, although proteinase levels and ASAs were not correlated directly in the CM prepared from basal media with high and low Ca^{2+} concentrations, it is unlikely that the proteinase activities in HEp-2 are similar to the activity described by Doble *et al.*, (1990).

4.3.4 THE ROLE OF BASIC FGF *IN VIVO* AND *IN VITRO*

It is important to establish the ability of tumour cells to secrete a potent angiogenic factor such as bFGF if its role in tumourigenesis *in vivo* is to be understood. It has been reported that prolonged *in vitro* cultivation of normal endothelial cells stimulates the production of immunoreactive acidic and basic FGF growth factors above the levels found in primary cultures of these cells {Spier *et al.*, (1991)}. This poses a problem with extrapolating the findings of *in vitro* studies to the significance of these factors *in vivo*. However, studies using a transgenic mouse model system for fibrosarcoma correlated the progression from benign to a malignant phenotype in fibrosarcomas with changes in the secretory patterns of a bFGF immunoreactive species. Cell lines derived from such tumours at different stages *in vivo* demonstrated that the mildly aggressive and less vascularized fibromatosis-derived lines produced large amounts of cell associated bFGF, while those lines derived from the more highly vascularized aggressive fibromatosis and fibrosarcomas secreted bFGF into the culture medium {Kandel *et al.*, (1991)}.

Experimental evidence continues to support the idea that tumour growth is dependent on neovascularisation {Folkman (1990)} and that this process is regulated by a family of diffusible angiogenic peptides, including FGFs {Folkman *et al.*, (1987)}, as well as low molecular weight nonpeptide angiogenic molecules {Folkman *et al.*, (1992)}.

More compelling evidence for the role of bFGF as an angiogenic factor was presented by Hori *et al.*, (1991). In this mouse model system, the gene for a stablilised mutant form of bFGF retaining biological activity was fused to the signal sequence for IL-2 and the resulting construct used to transform a BALB/c3T3 cell line. A transfectant designated K1000 which secreted the bFGF mutein (bFGF CS23) formed solid tumours in BALB/c nude mice after subcutaneous implantation. Upon histological examination these tumours were intensely vascularised. The angiogenic process could be inhibited in this model system using an immunoneutralising monoclonal antibody (3H3) raised against the mutein bFGF CS23 protein, and treatment with this antibody also suppressed solid tumour growth. A direct causal relationship between the inhibition of angiogenesis and anti-tumour activity in this system was therefore established. Direct inhibition of K1000 proliferation by blocking of an autocrine loop in these cells was ruled out because the 3H3 antibody did not inhibit the *in vitro* growth of K1000 cells. The alternative possibility that tumour growth was inhibited by a non-specific immune response mediated via complement or lymphocyte binding to the tumour-bound 3H3 antibody was discounted by the lack of *in vivo* growth inhibition of the K1000 cells and the absence of any

induction of lymphocytes at the tumour periphery.

Taken together, these studies indicate that the acquirement of bFGF secretory and/or release mechanisms may be an important step in the tumourigenic process *in vivo*. This appears to be related to the paracrine stimulation of angiogenesis effected by bFGF. However, the induction of bFGF secretion *in vitro* {Spier *et al.*, (1991) suggests that this growth factor is also required for cellular adaptation to *in vitro* culture conditions.

4.3.5 ANTISENSE FGFs - evidence for an autocrine role for bFGF in HEp-2

Although the anti-basic FGF antibody used to neutralise bFGF-like ASA in HEp-2 CM did not demonstrate cross-reactivity with acidic FGF, the possibility of recognition by another member of the FGF growth factor family cannot be discounted. Attempts to corroborate the putative role of the bFGF-like factor in HEp-2 autocrine growth control using antisense oligodeoxynucleotides (unmodified) were encouraging, but not definitive. The 15 base pair sequences used were directed against the translation initiation site of basic FGF mRNA based on similar studies by Becker *et al.*, (1989) and Morrison (1991). These authors had demonstrated inhibition of proliferation in melanoma and glioma cell lines respectively with the same bFGF antisense sequence. Aside from the technical problems encountered, other parameters used in these assays may not have been optimal for detecting the effect of the arrest of bFGF protein synthesis in HEp-2. The cell density used for antisense experiments was 30-fold higher than that of the autocrine assay. Under these conditions the contribution of autocrine bFGF production to cell growth may be redundant. In addition, the fact that cultures of HEp-2 were allowed to attach overnight, before the oligos were added, may have provided time for the deposition of sufficient extracellular stores of bFGF on HSPGs of the extracellular matrix. Such a 'reservoir' of bFGF may have been ample to supplement for any deficiency in bFGF production arising from the addition of the antisense olgonucleotides. It is also important to stress that the unmodified oligonucleotides were used in these experiments, and that these were not replenished during the assay period. Unmodified oligonucleotides are sensitive to digestion by endogenous cellular nucleases, a factor which may have limited the final active intracellular concentration of oligonucleotide. Any one or a combination of these parameters may explain the failure of the basic FGF antisense sequences to affect the proliferation of HEp-2.

4.3.6 EFFECT OF HEPARIN ON HEp-2 PROLIFERATION

The negative effect of heparin on HEp-2 growth noted in the stability studies was confirmed in an experiment which measured the dose response of HEp-2 to this mucopolysaccharide at concentrations in the range 0.5-20 μg/ml. An average reduction of 40% in growth was measured over the entire concentration range with no obvious dose response relationship. This may indicate that even at 0.5 μg/ml, the threshold for the inhibitory effect of heparin is exceeded. The inhibitory effect of heparin on HEp-2 growth is consistent with the work of others.

The mitogenic activity of a heparin-binding autocrine stimulator in normal human epidermal keratinocytes, identified by Cook *et al.*, (1991a) as amphiregulin (AR), was demonstrated was found to be inhibited by heparin {Cook *et al.*, (1991b)}. These workers have subsequently shown that other sulphated polysaccharides can abrogate AR-mediated autonomous growth of keratinocytes and that sulphation of polysaccharides is necessary for this inhibition {Cook *et al.*, (1992)}. Others have also found that the antimitogenic effect of heparin appeares to depend on its size and the degree of sulphation {Wright *et al.*, (1989)}. A role for amphiregulin in the autocrine stimulation of HEp-2 is unlikely because there was no evidence for such a competitor for the EGF receptor in concentrated HEp-2 CM (Section 3.1.6.3).

4.3.6.1 Mechanism of action for heparin

Despite the functional information available, the mechanism of heparin inhibition is not fully understood. Pukac *et al.*, (1990) examined heparin inhibition in rat vascular smooth muscle cells (VSMC) and demonstrated that it could inhibit the induction of the immediate early gene expression of c-*fos* and c-*myc* by the phorbol ester, PMA (phorbol 12-myristate 13-acetate). A similar induction of c-*fos* and c-*myc* by EGF in these cells was unaffected by heparin. PMA is an activator of the growth factor signal-transducing protein kinase C (PKC) and was used in subsequent studies in VSMC by Pukac *et al.*, (1992) to show that the heparin suppression of mitogen-stimulation was operative early on in the cell cycle (i.e. during the G_o/G_1 phase). Stimulation through PKC-dependent but not PKC-independent signalling pathways was inhibited in this way. In the same study bFGF-stimulation of c-*fos* and c-*myc* was only partially sensitive to the inhibition by heparin, indicating that the bFGF acts only partially through a PKC-dependent pathway in VSMC. In another study using VSMC, heparin did not inhibit the binding of PDGF or IGF-I to VSMC {Reilly *et al.*, (1988)}.

In a more recent study, comparing the effect of heparin and other glycosaminoglycans on the growth of normal fibroblasts and a fibrosarcoma cell line, heparin was found to suppress the growth of normal cells by 25-35% in the presence of serum. In the same study however, heparin did not affect the growth of the fibrosarcoma cell line, and inhibition of cell growth was independent of serum and added growth factors. This suggested that heparin-like GAGs act directly on cell regulatory components in normal cells {Westergren-Thorsson *et al.*, (1993)}.

4.3.6.2 Possible differential effects of heparin on the mitogenic response to FGFs

The ability of heparin to bind growth factors of the FGF family is well documented but the modulatory role of heparin on the biological activity of individual FGFs is growth factor dependent. While heparin has been seen to potentiate the biological effects of aFGF in several cell systems {Thornton *et al.*, (1983); Thomas & Gimenez-Gallego (1986)}, its modulation of bFGF bioactivity seems to depend on the cell type and experimental conditions used. Some studies with heparin have reported an inhibitory effect on bFGF activity while others found that it augmented the bioactivity of this growth factor {Gospodarowicz *et al.*, (1990); Neufeld *et al.*, (1987)}. This may reflect the signalling pathways used by the particular cells used, as suggested by recent evidence with normal cells. However, the high affinity of FGFs for heparin-like GAGs, and the involvment of heparan-sulphate moieties in the formation of high affinity receptor complexes with FGFs mentioned above, suggest that heparin-like molecules may have a complex modulatory role in FGF-signalling.

In the light of evidence for heparin-induced inhibition of bFGF mitogenic activity, it is interesting to note that the percentage growth inhibtion of HEp-2 caused by added heparin was in the same order (~40%) as the percentage reduction in ASA effected by the incubation of anti-basic FGF antibody with the crude HEp-2 CM fraction (~50%). This would seem to support the involvment of a bFGF-like species in the autocrine effect but the negative effect of heparin on HEp-2 growth is difficult to reconcile with a role for aFGF in the HEp-2 autocrine effect. Factor-specific inhibition of FGF growth factors by heparin has been reported in other cell line systems too.

In the SC-3 cell line mentioned earlier (Section 1.3.5.7) Kasayama *et al.*, (1991) found that heparin enhanced the bioactivity of low concentrations of acidic and basic FGF, but was antagonistic to the mitogenic effects of a distinct FGF-like autocrine activity (AIGF), secreted by these cells in response to androgen induction. A 50% reduction in SC-3 growth was reported

in the presence of 0.1 μg/ml of heparin with SC-3 cells. This is quantitatively similar to the percentage reduction observed at 0.5 μg/ml heparin in the HEp-2 system.

AIGF was later shown to be the sole FGF-like growth factor secreted by SC-3 cells {Nonomura *et al.*, (1989)}. The same group found that the SC-3 autocrine activity could be inhibited by anti-basic FGF antibodies {Yamanishi *et al.*, (1991)} suggesting a cross-reactivity with bFGF. This cross-reactivity between the anti-basic FGF polyclonal antibody and AIGF was confirmed by Western blotting (unpublised data) in a publication reporting the cloning and characterisation of the SC-3 autocrine growth factor (AIGF) as the eight member of the murine FGF family {Tanaka *et al.*, (1992)}.

The possibility that an AIGF-like heparin-binding autocrine growth factor is produced by HEp-2 cells in culture cannot be ruled out. This is supported by the recovery of an ASA from HEp-2 CM which eluted from a HS matrix between 1.0 and 1.3 M-NaCl (Section 3.3.5) — an affinity which is coincidental to that reported for AIGF (eluting at 1.1 M-NaCl in a buffer containing 0.1% CHAPS) {Nonomura *et al.*, (1988)}.

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

The original aim of this project was to improve the exisiting methodology and culture conditions for the establishment of primary non-small cell lung carcinomas (particularly the squamous cell subtype), as permanent *in vitro* cultures. Experience with many NSCLCs made it clear that successful cultures could only be derived from large tumour samples, such as those obtained at lobectomy etc. Moreover, deficiencies in the growth medium used were found to favour growth arrest and terminal differentiation. In a minority of cases, cultures could be subcultured beyond the primary stage, but only one continuous cell line was established, and at that, the source was a secondary lung metastatsis. Despite their poor performance *in vitro,* the propensity of primary NSCLC tumours to metastasise *in vivo* is well documented. This suggests that a subpopulation of cells in these tumours should have similar potential for continuous proliferation *in vitro* — given the correct growth conditions.

In this project, the lack of proliferative potential of NSCLC growth *in vitro* was considered in terms of insufficient or absent growth factors. In the search for novel growth supplments for NSCLCs effusions from cancer patients were considered. The labour-intensive nature of the primary culture work mitigated against the use of these cultures as indicators of growth-promoting activity for NSCLCs. This was important if sufficient progress was to be made in the duration of the project. Therefore a change in the direction of this work was taken.

To this end, an attempt was made to use the established squamous cell lung carcinoma cell lines DLRP, SK-MES-1, and DLKP as 'indicator cell lines' to screen malignant effusions for growth stimulators relevant to NSCLCs. Preliminary investigation of autocrine growth factor production in DLRP and DLKP was also attempted. No evidence of autocrine activity was apparent in CM from either of these cell lines in the assay systems used. This may reflect the nature of the monolayer-based detection systems employed because when seeded in semi-solid agar medium, both cell lines demonstrated definite 'cut-off' points, below which no cells formed colonies. This density-dependent growth in the more stringent agar system is indicative of autocrine growth stimulation, and may provide the basis for developing agar-based assays for detecting novel NSCLC growth factors using these cell lines.

The use of DLRP, SK-MES-1 and DLKP as 'indicator cell lines' for effusion-derived growth-promoting activities also proved impractical. This was due to the slow growing nature of these cell lines, and the difficulty in obtaining sufficient numbers of clinical samples. However, when the relatively small number of such samples available were assayed in the HEp-2 proliferation assay, analysis of the effects of various MEs associated with benign and malignant diseases did not indicate that growth-promoting activity was specific to cancer-related diseases. Moreover, in two cases where autologous plasma and/or serum was also available, there was evidence that the effusion-derived activities were also systemic. A variety of previously characterised growth factors have since been identified by others in malignant effussions. The apparent complexity of MEs, as well as reports in ovarian effusions of the NSCLC inhibitor IL-6, suggest that it would prove difficult to isolate novel growth factors for NSCLC from these biological fluids, contrary to our initial expectations.

Recent developments in the literature suggest that some of the commercially available growth factors such as HGF/SF, IGF-I, PDGF, and bFGF might prove useful in fresh attempts to

develop selective serum free media for NSCLC. The same may be true for KGF and the peptide VIP when these become avaliable as highly purified preparations.

Use of the HEp-2 cell line led to the discovery of an apparently complex autocrine growth control network in this cell system, which in itself was a useful model for the study of autocrine growth control. HEp-2 cells produce both autocrine growth inhibitors, and autocrine growth stimulators.

The low MW (1-5 kDa) autocrine inhibitory species identified was not investigated further because this work was focused on the identification of epithelial growth stimulatory species. However, the size of this inhibitor makes it interesting, in that less is known about low MW growth inhibitors than larger protein inhibitors such as TGF-β. It would be of interest also to determine the molecular size of the inhibitory activity separated by cation-exchange chromatography, given that a low MW inhibitory activity was also recovered by gel-filtration. These separation techniques might form the basis of a purification strategy for this inhibitory activity if further physicochemical analysis suggested that it might be a novel factor.

Autocrine stimulatory activity (ASA) in HEp-2 can be fractionated into two molecular weight classes, a 10-30 kDa MW fraction, and a >30 kDa MW fraction. A significant portion of the ASA in both fractions can be accounted for by a bFGF-like growth factor, but the greater portion of the 10-30 kDa MW ASA was attributed to this species. The lability of this fraction to heat, acid treatment, and storage, is also consistent with the properties of bFGF. An unexpected finding was the evidence for a serine proteinase-like enzyme activity in the 10-30 MW kDa fraction. This proteinase appears to mediate the autocrine activity of this fraction, but its role in the overall autocrine regulation of HEp-2 growth remains to be validated.

The presence in the larger >30 kDa MW fraction of a similar enzyme activity, albeit at lower levels, supports the hypothesis that a serine proteinase activity may function to release and/or activate a growth factor bound to a carrier molecule, or in a larger MW form, analagous to the role of plasmin in the release of bFGF from heparan sulphate proteoglycans. Another candidate which might be involved in this 'activation' mechanism is VEGF. The detection of VEGF receptors on the HEp-2-related HeLa cells suggests this possibility.

On the basis of the different stability of the >30 kDa MW fraction, it is more difficult to ascribe the bFGF-like ASA in the >30 kDa fraction to bFGF. Further characterisation of this fraction is needed to distinguish between the possibility that the larger bFGF-like activity represents an aggregate of the 18 kDa form of bFGF, or that it may be a complex of the 18 kDa form and a larger carrier molecule. Treatment with of the >30 kDa fraction with the reducing agent dithiothreitol should help to eliminate the possibile contribution of bFGF multimers, and exposure to heparanase and/or other extracellular-matrix-degrading enzymes should help to address the second possibility.

The affinities of the ASAs in both MW fractions has yet to be determined and compared with those in crude HEp-2 conditioned medium. Before any further purification is considered, more physicochemical stability analysis of the MW fractions are needed. The acid stability property in particular would be informative, as many of the growth factors characterised to date are acid stable. However to do this, the negative effect of HCl treatments on the control medium needs to be circumvented. A useful experiment might be to determine the effect of chlorate ions in the HEp-2 system, or to acid treat a CM sample collected in a different basal medium.

A greater understanding of the role of heparin may also be informative, particularly its effect on bFGF-induced stimulation of HEp-2. If heparin does not inhibit the bFGF mitogenic activity, then it may be interacting with another growth factor activity such as amphiregulin.

Several mitogens with different affinites for immobilised heparin are recovered from separations of HEp-2 CM. The possibility that one or more of the FGF family are active in the HEp-2 system cannot be eliminated because of the potential for cross-reactivity with these, and perhaps previously unidentified FGFs. In an effort to characterise the heparin-binding ASAs further, qualitiative analysis of heparin-Sepharose-fractionated CM and or its MW fractions might be considered using currently the available ELISAs for acidic and basic FGF, as well as several other members of this family such as FGF-4 and FGF-6. Further development of the assay parameters is required to overcome the technical difficulties associated with the use of the more specific antisense oligonucleotides.

Finally, not all of the HEp-2 ASA can be attributed to heparin-binding mitogens. From the evidence presented here, demonstrating that IL-1 can stimulate HEp-2 proliferation, and a publication in the literature reporting the secretion an IL-1-like activity by HEp-2 cells, the potential exists for an autocrine loop involving IL-1. The more recent discovery that functional IL-6 cytokine, and its receptor components are expressed in HEp-2, indicates the possibility of yet another autocrine growth regulatory mechanism in the HEp-2 system. These mechanisms remain to be verified. It would also be ineresting to determine the interactions between IL-1 and IL-6, and the putative bFGF autocrine loop, given that both cytokines are known to modulate bFGF secretion in other cell types utilising bFGF autocrine mechanisms.

6.0 ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor Martin Clynes for his advice and support, but above all else, his patience with 'deadlines' throughout my postgraduate years at Dublin City University.

Mr. Vincent Lynch provided not only the primary tissue material, but also an enthuastic dedication to this work, which was an inspiration to me in the face of adversity. I also acknowledge Dr. Michael Moriarty, and Dr. Maeve Pomeroy, for the supply of malignant effusion samples.

I am indebted to the generosity of Thelma Pentony for the extended loan of her PC, and to Dr. Mohammad Saleh, who took the time to load the software and configure it for the printer at the National Cell and Tissue Culture Centre, DCU. Thanks are also due to Maireád Callan, and Yvonne Reilly, for helping me get started with WordPerfect, and for putting up with many intrusions into their routine. To my user-friendly 'computer graphics consultant', Joanne Keenan, and to Dr. Liz Moran, for sharing her workspace with me during the final hectic weeks of writing up, a big thank you.

I thank also, Dr. Patricia Mulcahy and Dr. Brendan O'Connor for sharing their technical expertise, and for their help during the purification work. Una Gilvarry's help is acknowledged for the histopathology work, and for her advice with the photography.

Several colleagues blessed me with their friendship, humour, and emotional support; so to Susan Neenan, Ann Conway and Ursula Pattison, I say — "it was very much appreciated"! For all of these qualities, and for their loyalty, a big thank you to my 'Large-scale lab comrades' - Dr. Breda Carey and Angela O'Toole, who also contributed their time by reference gathering and proof-reading.

Two 'classy ladies' deserve my special thanks — Clodagh for being right there at the right time with her calls, and Dr. Angela Martin, who, during the worst days of the write-up, 're-membered' me with the conviction that I had it in me to finish this thesis. For all that, and for your practical contribution, thank you Ang!

Thanks also to Dorothy, the tin man, and all our friends — especially Marty and John, whose friendship during the 'difficult days of this dissertation', shed light on this 'road through yellow woods'. There were many others — who I count among angels and the Unseen Hands that guided these words. To you on all planes, and our Mother, my heart-felt thanks.

Finally, to my parents, for the years of financial sacrifice and support during my education - go raibh míle míle maith agaibh.

Bernard Gregory, March 1994.

7.0 REFERENCES

7.0 REFERENCES

Aaronson, S.A. 1991. Growth factors and cancer. **Science, 254:1146-1153.**

Acland, P., Dixon, M., Peters, G. and Dickson, C. 1990. Fate of the INT-2 oncoprotein is determined by choice of initiation codon. **Nature, 343:662-665.**

Adams, J.C., Furlong, R.A. and Watt, F.M. 1991. Production of scatter factor by ndk, a strain of epithelial cells and inhibition of scatter factor activity by suramin. **J. Cell Sci., 98:385-394.**

Adreef, A.M. and Welte, K. 1979. Hematopoietic colony-stimulating factors. **Semin. Oncol., 16:211-229.**

Algate, P.A. and McCubrey, J.A. 1993. Autocrine transformation of hemopoietic cells resulting from cytokine message stabilization after intracisternal A particle transposition. **Oncogene, 8:1221-1232.**

Andres, J.L., Defalcis, D., Noda, M. and Massagué, J. 1992. Binding of two growth factor families to separate domains of the proteoglycan betaglycan. **J. Biol. Chem., 265:5927-5930.**

Andres, J.L., Stanley, K., Chiefetz, S. and Massagué, J. 1989. Membrane anchorage and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor β. **J. Cell Biol., 109:3137-3145.**

Ankrapp, D.P. and Bevan, D.R. 1993. Insulin-like growth factor-I and human lung fibroblast-derived insulin-like growth factor-I stimulate the proliferation of human lung carcinoma cells *in vitro*. **Cancer Res., 53:3399-3404.**

Antoniades, H.N. 1991. PDGF: a multifunctional growth factor. **Bailleres Clin. Endocrinol. Metab., 5:595-613.**

Antoniades, H.N., Bravo, M.A., Avila, R.E., Galanopoulos, T., Neville-Golden, J., Maxwell, M. & Selman, M. 1990. Platelet-derived growth factor in idiopathic pulmonary fibrosis. **J. Clin. Invest., 86:1055-1064.**

Antoniades, H.N., Galanopoulos, T., Neville-Golden, J., Kiristy, C.P. & Lynch S.E. 1991. Injury induces *in vivo* expression of platelet-derived growth factor (PDGF) and PDGF-receptor messenger-RNAs in skin epithelial cells and messenger RNA in connective tissue fibroblasts. **Proc. Natl. Acad. Sci. USA, 88:565-569.**

Antoniades, H.N., Galanopoulos, T., Neville-Golden, J. and O'Hara, C.J. 1992. Malignant epithelial cells in primary human lung carcinomas coexpress *in vivo* platlet-derived growth factor (PDGF) and PDGF receptor mRNAs and their protein products. **Proc. Natl. Acad. Sci. USA, 89:3942-3946.**

Araujo, P.M. and Cotman, C.W. 1992. Basic FGF in astroglial, microglial, and neuronal cultures: characterization of binding sites and modulation of release by lymphokines and trophic factors. **J. Neurosci., 12:1668-1678.**

Arteaga, C.L., Hanauske, A.R., Clark, G.M., Osborne, C.K., Hazarika, P., Pardue, R.L., Tio, F. and Von Hoff, D.D. 1988. Immunoreactive α transforming growth factor activity in effusions from cancer patients as a marker of tumor burden and patient prognosis. **Cancer Res., 48:5025-5028.**

Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M. and Sporn, M.B. 1983. Transforming growth factor-beta in human platelets. **J. Biol. Chem., 258:7155-7160.**

Avivi, A., Yayon, A. and Givol, D. 1993. A novel form of FGF receptor-3 using an alternative exon

in the immunoglobulin domain III. **FEBS Lett., 330:249-252.**

Bach, L.A., *et al.*, 1993. **Acta. Paediatr. Scand., 372:107.**

Baghidiguian, S., Verrier, B., Gerard, C. & Fantini, J. 1992. Insulin-like growth factor-I is an autocrine regulator of human colon cancer cell differentiation and growth. **Cancer Letts., 62:23-33.**

Bailie-Johnson, H., Twentyman, P.R., Fox, N.E., Walls, G.A., Workman, P., Watson. J.V., Johnson, N., Reeve, J.G. & Bleehen, N.M. 1985. Establishment of cell lines from patients with lung cancer (predominantly small cell carcinoma). **Br. J. Cancer, 52:495-504.**

Baird, A., Esch, F., Mormède, P., Ueno,N., Ling, N., Böhlen, P., Ying, S.-Y., Wehrenberg, W.B. and Guillemin, R. 1986. molecular characterisation of fibrobalst growth factor distribution and biological activities in various tissues. **Recent Prog. Horm. Res., 42:143-205.**

Baird, A., Mormède, P. and Böhlen, P. 1985. Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests its identity with macrophage-derived growth factor. **Biochem. Biophys. Res. Commun., 126:358-364.**

Barnard, J.A., Lyons, R.M. and Moses, H. 1990. The cell biology of transforming growth factor β. **Biochim. Biophys. Acta, 1032:79-87.**

Barr, P.J. 1991 Mammalian subtilisins: the long-sought dibasic processing endoproteases. **Cell, 66:1-3.**

Barrondon. Y. and Green, H. 1987. Cell migration is essential for sustained growth of keratinocyte colonies: the roles of transforming growth factor-α and epidermal growth factor. **Cell, 50:1131-1137.**

Basilico, C. and Moscatelli, D. 1992. The FGF family of growth factors and oncogenes. **Adv. Cancer Res., 59:115-165.**

Bates, B., Hardin, J., Zhan, X., Drickamer, K. and Goldfarb, M. 1991. Biosynthesis of human fibroblast growth factor-5. **Mol. Cell. Biol., 11:1840-1845.**

Bauman, H., Jahreis, G.P., Sauder, D.N. and Koj, A. 1984. Human keratinocytes and monocytes release factors which regulate the synthesis of major acute phase plasma-proteins in hepatic cells from man, rat, and mouse. **J. Biol. Chem., 259:7331-7342.**

Baumann, M., Baumann, H. and Fey, D.H. 1990. Molecular cloning, characterisation and functional expression of the rat liver interleukin-6 receptor. **J. Biol. Chem., 265:9853-9862.**

Baxter, R.C. and Martin, J.L. 1989. Structure of the MW 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity labelling. **Proc. Natl. Acad. Sci. USA, 86:6898-6902.**

Bazan, J.F. 1991. Neuropoietic cytokines in the hematopoietic fold. **Neuron, 7:197-208.**

Bazil, G.W., Haynes, M., Garland, J. and Dexter, T.M. 1983. Characterization and partial purification of a haemopoietic cell growth factor in WEHI-3 cell conditioned medium. **Biochem. J., 210:747-759.**

Becker, D., Meier, C.B. & Herlyn, M. 1989. Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides targeted against basic fibroblast growth factor. **EMBO J., 8:3685-3691.**

Berdel, W.E., Danhauser-Riedl, S., Steinhausser, G. and Winton, E.F. 1989. Various human hematopoietic growth factors (interleukin-3, GM-CSF, G-CSF) stimulate clonal growth of nonhematopoietic tumor cells. **Blood, 73:80-83.**

Berger, M.S., Guillick, W.J., Greenfield, C., Evans, S., Addis, B.J. and Waterfield, M.D. 1987. Epidermal growth factor receptor in lung tumours. **J. Pathol., 152:297-307.**

Bergh, J., Nilsson, K., Zech, L. and Giovanella, B. 1981. Establishment and characterization of continuous lung squamous cell carcinoma cell line U-1752. **AntiCancer Res., 1:317-322.**

Bernfield, M. and Sanderson, R.D. 1990. Syndecan, a developmentally regulated cell surface proteoglycan that binds extracellular matrix and growth factors. **Phil. Trans. R. Soc. Lond.B., 374:171-186.**

Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M.T., Spring, J., Gallo, R.L. and Lose, E.J. 1992. Biology of the syndecans. **Annu. Rev. Cell Biol., 8:365-393.**

Berridge, M.J. and Irvine, R.F. 1984. **Nature, 308:315-321.**

Besner, G.E., Higashiyama, S. and Klagsbrun, M. 1990. Isolation and characterization of a macrophage-derived heparin-binding growth factor. **Cell Regul., 1:811-819.**

Besner, G.E. and Klagsbrun, M. 1988. Characterization and purification of a heparin-binding growth factor from mononuclear cell conditioned medium. **J. Cell. Biol., 107:A2706.**

Bhatt, H., Brunet, L.S. and Stewart, C.L 1991. Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. **Proc. Natl. Acad. Sci. USA, 88:1408-1412.**

Bishayee, S., Majumdar, S., Khire, J. and Das, M. 1989. Ligand-induced dimeriszation of the platelet-derived growth factor receptor. Monomer-dimer interconversion occurs independent of receptor phosphorylation. **J. Biol. Chem., 264:11699-11705.**

Blundell, T.L., Bedarkar, S., Rinderknecht, E. and Humbel, R.E. 1978. **Proc. Natl Acad. Sci. USA, 75:180-184.**

Böhlen, P. and Kovesdi, I. 1991. HBNF and MK, members of a novel gene family of heparin-binding proteins with potential roles in embryogenesis and brain function. **Prog. Growth Factor Res., 3:143-157.**

Borset, M., Helseth, E., Naume, B. and Waage, A. 1993. Lack of IL-1 secretion from human myeloma cells highly purified by immunomagnetic separation. **Br. J. Haematol., 85:446-451.**

Bottaro,, D.P., Rubin, J.S., Faletto, D.L., Chan, A.M.L., Kmiecik, T.E., vanade Woude, G.F. and Aaronson, S.A. 1991. Identification of the hepatocyte growth factor receptor as the c-*met* proto-oncogene product. **Science, 251:802-804.**

Brachmann, R., Lindquist, P.B., Nagashima, M., Kohr, W., Lipari, T., Napier, M. and Derynck, R. 1989. Transmembrane TGFα precursors activate EGF/TGFα receptors. **Cell, 56:691-700.**

Brambilla, E., Gazzeri, S., Gouyer, V. and Brambilla, C. 1993. Mechanisms of lung oncogenesis. **Rev. Prat., 43:807-814.**

Brem, H. and Klagsbrun, M. 1993. The role of fibroblast growth factors and related oncogenes in tumor growth. In: **Oncogenes and Tumor Suppressor Genes in Human Malignacies. (eds) Benz, C.C. and Liu, E.T. Kluwer Academic Publishers, Boston, pp211-231.**

Browder, T.M., Dunbar, C.E. and Nienhuis, A.W. 1989. Private and public autocrine loops in neoplastic cells. **Cancer Cells, 1:9-17.**

Brower, M., Carney, D.N., Oie, H.K., Gazdar, A.F. and Minna, J.D. 1986. Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. **Cancer Res., 46:798-806.**

Brown, T.J Lioubin, M.N. and Marquard, H. 1987. Purification and characterisation of cytostatic lymphokines-T-synergistic antiproliferative activity of transforming growth factor beta-1, interferon gamma, and oncostatin-M for human melanoma cells. **J. Immunol., 139:2977-2983.**

Bruce, A.G., Linsley, P.S. and Rose, T.M. 1992. Oncostatin M. **Prog. Growth Factor Res., 4:157-170.**

Burgess, A.W. *et al.*, 1990. In: **Peptide growth factors and their receptors In: Sporn M.B. and Roberts, A.B. eds. Springer-Verlag, New York, pp 190.**

Burgess, A.W., Metcalf, D., Kozka, I.J., Simpson, R.J., Vairo, G., Hamilton, J.A. and Nice, E.C. 1985. Purification of two forms of colony-stimulating factor from mouse L-cell conditioned medium. **J. Biol. Chem., 260: 16004-16011.**

Burton, P.B. and Knight, D.E. 1992. Parathyroid hormone-related peptide can regulate the growth of human lung cancer cells, and may form part of an autocrine TGFα loop. **FEBS Lett., 305:228-232.**

Carney, D.N. and deLeij, L. 1988. Lung Cancer Biology. **Seminars in Oncology, 15:199-214.**

Carey, D.J., Evans, D.M., Stahl, R.C., Asundi, V.K., Conner, K.J., Garbes, P. and Cizmeci-Smith, G. 1992. Molecular cloning and characterization of N-syndecan, a novel transmembrane heparan sulfate proteoglycan. **J. Cell Biol., 117:191-201.**

Carney, D.N., Gazdar, A.F., Bepler, G., Guccion, J.G., Marangos, P.J., Moody, T.W., Zweig, M.H. and Minna, J.D. 1985. Establishment and identification of small cell lung cancer cell lines having classic and variant features. **Cancer Res., 45:2913-2923.**

Carpenter, G. and Cohen, S. 1979. Epidermal growth factor. **Ann. Rev. Biochem., 48:193-216.**

Carpeneter, G. and Wahl, I. 1990. The epidermal growth factor family. In: **Peptide Growth Factors and their Receptors. Sporn, M.B. and Roberts. A.B. (eds), Springer-Verlag, New York, pp69.**

Cate,R.L., Mattaliano, R.J., Hession, C., Tizard, R., Farber, N.M., Cheung, A., Ninfa, E.G., Frey, A.Z., Gash, D.J., Chow, E.P., Fisher, A., Bertonis, J.B., Torres, G., Wallner, B.P., Ramachandran, K.L., Ragin, R.C., Managanaro, T.F., MacLaughlin, D.T. and Donahoe, P.K. 1986. Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. **Cell, 45:685-698.**

Causin, C., Weheed, A., Braulke, T., Junghans, U., Maly, P., Humbel, R.E. and von Figura, K. 1988. Mannose 6-phosphate/insulin-like growth factor II-binding proteins in human serum and urine. Their relation to the mannose 6-phosphate/insulin-like growth factor II receptor. **Biochem. J., 252:795-799.**

Chang, W., Upton, C., Hu, S.-L., Purichio, A.F. and MacFadden, G. 1987. The genome of Shope fibroma virus, a tumorigenic poxvirus, contains a growth factor gene with sequence similarity to those encoding epidermal growth factor and trnasforming growth factor α. **Mol. Cell. Biol., 7:535-540.**

Charnock-Jones, D.S., Sharkey, A,M., Rayputwi, J., Burch, D., Scholfield, J.P., Fountain, S.A., Boocock, C.A. and Smith, S.K. 1993. Identification and localisation of alternatively spliced messenger RNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. **Biol. Reprod., 48:1120-1128.**

Chauhan, A.K., Li, Y.-S. and Deuel, T.F. 1993. Pleiotrophin transforms NIH3T3 cells and induces tumours in nude mice. **Proc. Natl. Acad. Sci. USA, 90:679-682.**

Chen, T.R. 1977. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. **Exp Cell Res., 104:255-262.**

Cheifetz, S., Andres, J.L. and Massagué, J. 1988. The transforming growth factor-β receptor type III is a membrane proteoglycan. **J. Biol. Chem., 263:16984-16991.**

Cheifetz, S., Like, B. and Massagué, J. 1986. Cellular distribution of type I and type II receptors for transforming growth factor-β. **J. Biol. Chem., 261:9972-9978.**

Cheifetz, S., Weatherbee, J.A., Tsang, M.L.S., Anderson, J.K., Mole, J.E., Mole, J.A., Lucas, R. and Massagué, J. 1987. The transforming growth factor-β system, a complex pattern of cross-reactive lignands and receptors. **Cell, 48:409-415.**

Chodak, G.W., Hospelhorn, V., Judge, S.M., Mayforth, R., Koeppen, H. and Sasse, J. 1988. Increased levels of fibroblast growth factor-like activity in urine from patients with bladder or kidney cancer. **Cancer Res., 48:2083-2088.**

Ciccodicola, A., Donno, R., Oblici, S., Simeone, A., Zollo, M. and Persico, M.G. 1989. Molecular characterization of a gene of the 'EGF family' expressed in undifferentiated human NTERA2 teratocarcinoma cells. **EMBO. J., 8:1987-1991.**

Claesson-Welch, L., Eriksson, A., Morén, A., Severinsson, L., Ek, B., Östman, A., Betsholtz, C., and Heldin C.-H. 1988. cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. **Mol. Cell Biol., 8:3476-3486.**

Claesson-Welch, L., Eriksson, A., Westermark, B. and Heldin, C.-H. 1989. cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. **Proc. Natl. Acad. Sci. USA, 86:4917-4921.**

Clemmons, D.R., Jones, J.I., Busby, W.H. and Wright, G. 1993. Role of insulin-like growth factor binding proteins in modifying IGF actions. **Ann. New York Acad. Sci., 692:10-21.**

Coffey, R.J., Bascom, C.C., Sipes, N.J., Graves-Deal, R., Weissmann, B.E. and Moses, H.L. 1988. Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor-beta. **Mol. Cell. Biol., 8:3088-3093.**

Coffey, R.J.Jr, Derynck, R., Wilcox, J.N., Bringman, T.S., Goustin, A.S., Moses, H.L. and Pittelkow, M.R. 1987. Production and auto-induction of transforming growth factor-α in human keratinocytes. **Nature, 328:817-820.**

Coffey, R.J.Jr., Leof, E.B., Shipley, G.D. and Moses, H.L. 1987. Suramin inhibition of growth factor receptor binding and mitogenic activity in AKR-2B cells. **J. Cell Physiol., 132:143-148.**

Colouscou, J-M., Garrouste, F., Remacle-Bonnet, M., Bettetini, D., Marvaldi, J. and Pommier, G. 1988. Autocrine secretion of a colorectum-derived growth factor by HT-29 human colon carcinoma cell line. **Int. J. Cancer, 42:895-901.**

Colouscou, J-M., Remacle-Bonnet, M., Carlton, G.W., Plowman, G.D. and Shoyab, M. 1992. Colorectum cell-derived growth factor (CRDGF) is homologus to amphiregulin, a member of the epidermal growth factor family. **Growth Factors, 7:195-205.**

Conn, G., Soderman, D.D., Schaeffer, M.-T., Wile, M., Hatcher, V.B. and Thomas, K.A. 1990. Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. **Proc. Natl. Acad. Sci. USA, 87:1323-1327.**

Connolly, D.T., Knight, M.B., Harakas, N.K., Wittwer, A.J. and Feder, J. 1986. Determination of

number of endothelial cells in culture using an acid phosphatase assay. **Anal. Biochem., 152:136-140.**

Connolly, D.T., Stoddard, B.L., Harakas, N.K. and Feder, J. 1987. Human fibroblast-derived growth factor is a mitogen and chemoattractant for endothelial cells. **Biochem. Biophys. Res. Commun., 144:705-712.**

Cook, P.W., Mattox, P.A., Keeble, W.W. and Shipley, G.D. 1992. Inhibition of autonomous human keratinocyte proliferation and amphiregulin mitogenic activity by sulfated polysaccharides. **In Vitro Cell. Dev. Biol., 28A:218-222.**

Cook, P.W., Mattox, P.A. & Keeble, W.W. Pittelkow, M.R., Plowman, G.D., Shoyab, M., Adelman, J.P. and Shipley, G.D. 1991a. A heparan sulfate-regulated human keratinocyte autocrine factor is similar or identical to amphiregulin. **Mol. Cell Biol., 11:2547-2557.**

Cook, P.W., Pittelkow, M.R. and Shipley, G.D. 1991b. Growth factor-independent proliferation of normal human neonatal keratinocytes and production of autocrine- and paracrine acting mitogenic factors. **J. Cell Physiol., 146:277-289.**

Cook, R.M., Miller, Y.E. and Bunn, D.A.Jr. 1993. Small cell lung cancer: etiology, biology, clinical features, staging, and treatment. **Curr. Probl. Cancer, 17:69-141.**

Cooper, D.N.W. and Barondes, S.H. 1990. Evidence for export of a muscle lectin from cytsol to extracellular matrix and a novel secretory mechanism. **J. Cell Biol., 100:1681-1691.**

Cornil, I., Theodorescu, S., Man, M., Herlyn, M., Jambrosic, J. and Kerbel, R.S. 1991. Fibroblast cell interactions with melanoma cells affect tumor cell growth as a function of tumor progression. **Proc. Natl. Acad. Sci. USA, 88:6028-6032.**

Courty, J., Dauchel, M.C., Caruelle, D., Perderiset, M. and Barritault, D. 1991. Mitogenic properties of a new endothelial cell growth factor related to pleiotropin. **Biochem. Biophys. Res. Commun., 180:145-151.**

Cuttitta, F., Carney, D.N., Mulshine, J., Moody, J.W., Fedorko, J., Fischler, A. & Minna, J.D. 1985. Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. **Nature, 316:823-826.**

Dalloul, A.H., Arock, M., Fourcade, C., Hatzfeld, A., Bertho, J.M., Debre, P. and Mossaley, M.D. 1991. Human thymic epithelial cells produce interleukin-3. **Blood, 77:69-74.**

Davidson, N.E., Gelman, E.P., Lippman, M.E. and Dickson, R.B. 1987. Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. **Mol Endocrinol, 1:216-233.**

Davis, S., Aldrich, T.M., Stahl, N., Dan, L., Taga, T., Kishimoto, T., Ip, N.Y. and Yancopou, G.D. 1993 LIF-beta and gp-130 as heterodimerizing signal transducers of the tripartite CNTF receptor. **Science, 260:1805-1808.**

De Jong, P.M., Van Sterkenburg, M.A.J.A., Kempenaar, J.A., Dijkman, J.H. and Ponec, M. 1993. Serial culturing of human bronchial epithelial cells derived from biopsies. **In Vitro Cell. Dev. Biol., 29A:379-387.**

DeLapriere, O., Rosnet, O., Benharroch, D., Raybaud, F., Marchetto, S., Planche, J., Galland, F., Mattei, M.-G., Copeland, N.G., Jenkins, N.A., Coulier, F. and Birnbaum, D. 1990. Structure, chromosome mapping and expression of the murine FGF-6 gene. **Oncogene, 5:823-831.**

Delli-Bovi, P., Curatola, A.M., Kern, F.G., Greco, A., Ittman, M. and Basilico, C. 1987. An oncogene

isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. **Cell, 50:729-737.**

Delli-Bovi, P., Curatola, A.M., Newman, K.M., Sato, Y., Moscatelli, D., Kewick, R.M., Rifkin, D.B. and Basilico, C. 1988. Processing, secretion, and biological properties of a novel growth factor of the fibroblast growth factor family with oncogenic potential. **Mol. Cell. Biol., 8:2933-2941.**

Denis, P.A., Saksela, O., Harpel, P. and Rifkin, D.B. 1989. α_2-Macroglobulin is a binding protein for basic fibroblast growth factor. **J. Biol. Chem., 264:7210-7216.**

Derynck, R. 1992. The physiology of transforming growth factor-α. **Adv. Cancer Res., 58:27-52.**

Derynck, R., Goeddel, D.V., Ullrich, A., Gutterman, J.U., Williams, R.D., Bringman, T.S. and Berger, W.H. 1987. Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. **Cancer Res., 47:707-712.**

Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B. and Goeddel, D.V. 1985. Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. **Nature, 316:701-705.**

Derynck, R., Roberts, A.B., Winkler, M.E., Chen, E.Y. and Goeddel, D.V. 1984. Human transforming growth factor-α: precursor structure and expression in *E. coli*. **Cell, 38:287-297.**

Devare, S.G., Reddy, E.P., Law, J.D., Robbins, K.C. and Aaronson, S.A. 1983. Nucleotide sequence of the simian sarcoma-virus genome — demonstration that its acquired cellular sequences encode the transforming gene product p28sis. **Proc. Natl. Acad. Sci, USA, 80:731-735.**

de Vries, C., Escobebo, J.A., Ueno, H., Houck, K., Ferrara, N. and Williams, L.T. 1992. The fms-like receptor for vascular endothelial growth factor. **Science, 255:989-991.**

Dickson, C. and Peters, G. 1987. Potential oncogene product related to growth factors. **Nature, 326:833.**

Dickson, C., Smith, R., Brookes, S. and Peters, G. 1984. Tumourigenesis by mouse mammary tumour virus: proviral activation of a cellular gene in the common integration region *int-2*. **Cell, 37:529-536.**

Dickson, R.B., Bates, S.E., McManaway, M.E. and Lippman, M.E. 1986. Characterization of estrogen responsive transforming activity in human breast cancer cells. **Cancer Res., 46:1707-1713.**

Dietal, M., Arps, H., Gerding, D., Trapp, M. and Niendorf, A. 1987. Establishment of primary cell cultures: experiences with 155 cell strains. **Klin. Wochenscher, 65:507-512.**

Dinarello, C.A. 1991. Interleukin-1 and interleukin 1 antagonism **Blood, 77:1627-1652.**

Dinarello, C.A. & Thompson, R.C. 1991. Blocking IL-1-Interleukin 1-receptor antagonism *in vivo* and *in vitro*. **Immunol. Today, 12:404-410.**

Dinarello, C.A. & Wolff, S.M. 1993. Mechanisms of disease — the role of interleukin-1 in disease. **N. Engl. J. Med. 328:106-113.**

Dixon, M., Deed, R., Acland, P., Moore, R., Whyte, A., Peters, G. and Dickson, C. 1989. Detection and characterization of the fibroblast growth factor-related oncoprotein INT-2. **Mol. Cell. Biol., 9:4896-4902.**

Doble, B.W., Fandrich, R.R., Liu, L., Padua, R.R. and Kardami, E. 1990. Calcium protects pituitary basic fibroblast growth factors from limited proteolysis by co-purifying proteases. **Biochem. Biophys. Res. Comm., 173:1116-1122.**

Doolittle, R.F., Hunkrapil, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.N. 1983. Simian sarcoma virus oncogene, v-*sis*, is derived from the gene (or genes) encoding a platelet-derived growth factor. **Science, 221:275-277.**

Doumit, M.E., Cook, D.R. and Merkel, R.A. 1993. Fibroblast growth factor, Epidermal growth factor, Insulin-like growth factor, and Platelet-derived growth factor-BB stimulate proliferation of clonally derived porcine myogenic satellite cells. **J. Cell Physiol., 157:326-332.**

Downing, J.R., Roussel, M.F. and Sherr, C.J. 1989. Protein kinase C 'transmodulates' the colony stimulating factor 1 receptor by activating a membrane associated protease that specifically releases the receptor ligand-binding domain from the cell. **Clin. Res., 37:544A.**

Downward, J., Yarden, Y., Mayers, E., Scrace, G., Totty, N., Stockwel, P., Ullrich, A., Schlessi, J. and Waterlie, M.D. 1984. Dose similarity of epidermal growth factor receptor and v-*erb-B* oncogene protein sequences. **Nature, 307:521-527.**

Duan, D-S.R., Werner, S. and Williams, T. 1992. A naturally occuring secreted form of fibroblast growth factor (FGF) receptor 1 binds basic FGF in preference over acidic FGF. **J. Biol. Chem., 267:16076-16080.**

Dvorak, H.F., Sioussat, T.M., Brown, L.F., Berse, B., Nagy, J.A., Sotpel, A., Manesaw, E.J., Vandewat, L. and Senger, D.R. 1991. Distribution of vascular-permeability factor (vascular endothelial growth factor) in tumours — concentration in tumour blood vessels. **J, Exp. Med., 174:1275-1278.**

Dvorak, H.F. 1986. Tumors: Wounds that do not heal. **N. Engl. J. Med., 315:1650-1659.**

Ennis, B.W., Valverius, E.M., Bates, S.E., Lippman, M.E., Bellot, F., Kris, R., Schlessinger, J., Masui, H., Golderberg, A., Mendelsohn, J. and Dickson, B. 1989. Anti-epidermal growth factor receptor antibodies inhibit the autocrine-stimulated growth of MDA-468 human breast cancer cells. **Mol. Endocrinol., 3:1830-1838.**

Ensoli, B., Nakamura, S., Salahuddin, S.Z., Biberfeld, P., Larson, L., Beaver, B., Wong-Staal, F. and Gallo, R.C. 1989. AIDS — kaposis sarcoma-derived cells express cytokines with autocrine and paracrine growth effects. **Science, 243:223-226.**

Erickson, A.H. 1989. Biosynthesis of lysosomal endopeptidases. **J. Cell Biochem., 40:31-41.**

Eriksson, A., Rorsman, C., Ernlund, A., Claesson-Welch, L. and Heldin, C.-H. 1992. Ligand-induced homo- and hetero-dimerization of platelet-derived growth factor α- and β- receptors in intact cells. **Growth Factors**, 6:1-4.

Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Böhlen, P. and Guillemin, R. 1985. Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. **Proc. Natl. Acad. Sci. USA, 82:65077-6511.**

Eschenfeldt, W.H. and Berger, S.L. 1986. The human prothymosin α gene is polymorphic and induced upon growth stimulation: evidence using a cloned cDNA. **Proc. Natl. Acad. Sci. USA, 83:9403-9407.**

Fang, W.J, Hartmann, N., Chow, D.T., Riegal, A.T. and Wellstei, A. 1992. Pleiotrophin stimulates fibroblasts and endothelial and epithelial cells and is expressed in human cancer. **J. Biol. Chem., 267:5889-5897.**

Fernandez-Botran, R. 1991. Soluble cytokine receptors: their role in immunoregulation. **FASEB J., 5:2567-2574.**

Finch, P.W., Rubin, J.S., Miki, T., Ron, D. and Aaronson, S.A. 1989. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. **Science, 245:752-755.**

Flaumenhaft, R., Moscatelli, D. and Rifkin, D.B. 1990. Heparin and heparan-sulphate increase the radius of diffusion and action of basic fibroblast growth factor. **J. Cell Biol., 111:1651-1659.**

Fleming, T.P., Matsui, T., Heidaran, M.A., Molloy, C.J., Artrip, J. and Aaronson, S.A. 1992. Demonstration of an activated platelet-derived growth factor autocrine pathway and its role in human tumor cell proliferation *in vitro*. **Oncogene, 7:1355-1359.**

Florkiewicz, R.Z., Baird, A. and Gonzalez, A.-M. 1991. Multiple forms of bFGF: differential nuclear and cell surface localization. **Growth Factors, 4:265-275.**

Florkiewicz, R.Z. and Sommer, A. 1989. Human basic fibroblast growth factor gene encodes four polypeptides: Three initiate translation from non-AUG codons. **Proc. Natl. Acad. Sci. USA, 86:3978-3981.**

Folkman, J. 1990. What is the evidence that tumours are angiogenesis dependent? **J. Natl. Cancer Inst., 82:4-6.**

Folkman, J. and Klagsbrun, M. 1987. Vascular physiology. A family of angiogenic peptides. **Nature, 329:671-672.**

Folkman, J. and Shing, Y. 1992. Angiogenesis. **J. Biol. Chem., 267:10931-10934.**

Foulke, R.S., Marshall, M.H., Trotta, P.P. and Von Hoff, D.D. 1990. *In vitro* assessment of the effects of granulocyte-macrophage colony-stimulating factor on primary human tumors and derived cell lines. **Cancer Res., 50:6264-6267.**

Forberg, K., Bergh, J. and Westermark, B. 1993. Expression of functional PDGFβ receptors in a human large-cell lung carcinoma cell line. **Int. J. Cancer, 53:556-560.**

Fox, G.M., Schiffer, S.G., Rhode, M.F., Tsal, L.B., Banks, A.R. and Arakawa, T. 1988. Production, biological activity, and structure of recombinant basic fibroblast growth factor and an analog with cysteine replaced by serine. **J. Biol. Chem., 263:18452-18458.**

Freed, K.A. and Herington, A.C. 1989. Insulin-like growth factor-I and its autocrine role in growth by MCF-7 human breast cancer cells in culture. **J. Mol. Endocrinol., 3:183-190.**

Freshney, R.I. 1987. Culture of specific cell types. *In:* **Culture of animal cells. (2nd eds), Liss, A.R., Inc. New York. pp257-288.**

Frolik, C.A., Wakefield, L.M., Smith, D.M and Sporn, M.B. 1984. Characterization of a membrane receptor for transforming growth factor-β in normal rat kidney fibroblasts. **J. Biol. Chem., 259:10995-11000.**

Fujimoto, K., Ichimori, Y., Kakizoe, T., Okajima, E., Sakamoto, H., Sugimura, T. and Terada, M. 1991. Increased serum levels of basic fibroblast growth factor in patients with renal cell carcinoma. **Biochem. Biophys. Res. Commun., 180:386-392.**

Furlong, R.A. 1992. The biology of hepatocyte growth factor/scatter factor. **BioEssays, 14:613-617.**

Garver, R.I. Jr., Chan, C.S. and Milner, P.G. 1993. Reciprocal expression of pleiotrophin and midkine in normal versus malignant lung tissues. **Am. J. Respir. Cell Mol. Biol., 9:463-466.**

Gastl, G.A., Abrahms, J.S., Nanus, D.M., Oosterba, R., Silver, J., Liu, F., Chen, M., Albino, A.P.

and Bander, N.H. 1993. Interleukin-10 production by human carcinoma cell lines and its relationship to interleukin-6 expression. **Int. J. Cancer, 55:96-101.**

Gazdar, A.F., Carney, D.N., Nau, M.M. and Minna, J.D. 1985. Characterization of variant subclasses of cell lines derived from small cell lung cancer having distinctive biochemical, morphological growth properties. **Cancer Res., 45:2924-2930.**

Gazdar, A.F. and Oie, H.K. 1986a. Cell culture methods for human lung cancer. **Cancer Genet. Cytogenet., 19:5-10.**

Gazdar, A.F. and Oie, H.K. 1986b. Letter to the editor re: Martin Brower *et al.* 'Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium' in Cancer Res, 46:798-806. **Cancer Res., 46:6011-6012.**

Gearing, D.P., Thut, C.J., Vandenbo, T., Gimpel, S.D., Delaney, P.B., King, J., Price, V., Cosman, D. and Beckmann, M.P. 1991. Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. **EMBO. J., 10:2839-2848.**

Gentry, L.E. and Nash, B.W. 1990. The pro domain of pre-pro-transforming growth factor beta 1 when independently expressed is a functional binding protein for the mature growth factor. **Biochem, 29:6851-6857.**

Gilbert, D.A., Reid, Y.A., Gail, M.H., Pee, D., White, C., Hay, R.J. and O'Brien, S.J. 1990. Application of DNA fingerprints for cell-line individualization. **Am. J. Hum. Genet., 47:499-514.**

Gilvarry, U., Farrell, D., Lynch, V., Moriarty, M., Dooley, M., Law, E. and Clynes, M. 1990. Cytological differences between normal and malignant human cell populations in culture. **Cancer Res., 50:3390-3393.**

Giordano, S., Ponzetto, C., Di Renzo, M.F., Cooper, C.S. and Comoglio, P.M. 1989. Tyrosine kinase receptor indistinguishable from the c-met protein. **Nature, 339:155-156.**

Giri, J.G., Lomendico, P.T. and Mizel, S.B. 1985. Studies on the synthesis and secretion of interleukin-1.1 A 330000 molecular weight precursors for Interleukin-1. **J. Immunol., 134:343-349.**

Gitay-Goren, H., Soker, S., Vlodavsky, I. and Neufeld, G. 1992. The binding of vascular endothelial growth factor to its receptor is dependent on cell surface-associated heparin-like molecules. **J. Biol. Chem., 267:6093-6098.**

Givol, D. and Yayon, A. 1992. Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. **FASEB J., 6:3362-3369.**

Goldstein, A.L., Low, T.L.K., McAdoo, M., McClure, J., Thurman, G.B., Rossio, J., Lai, C.Y., Chang, D., Wang, S.S., Harvey, C., Ramer, A.H. and Meienhofer, J. 1977. Thymosin α1: isolation and sequence analysis of an immunologically active thymic polypeptide. **Proc. Natl Acad. Sci. USA, 74:725-729.**

Goodall, G.J., Bagley, C.J., Vadas, M.A. and Lopez, A.F. 1993. A model for the interaction of the GM-CSF, IL-3 and IL-5 receptors with their ligands. **Growth Factors, 8:87-97.**

Goodwin, R.G., Friend, D., Ziegler, S.F., Jerzy, R., Falk, B.A., Gimpel, S., Cosman, D., Dower, S.K., March, C.J., Namen, A.E. and Park, L.S. 1990. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. **Cell, 60:941-951.**

Gospodarowicz, D. 1987. Purification of brain and pituitary FGF. **Methods. Enzymol., 147:106-119.**

Gospodarowicz, D. 1989. Fibroblast growth factor. In: Pimental, E. and Perucho, M. (eds) **Critical Reviews in Oncogenesis. CRC Press: Boca Raton, Fl. pp1-26.**

Gospodarowicz, D., Plouet, J. and Fujii, D.K. 1989. Ovarian germinal epithelial cells respond to basic fibroblast growth factor and express its gene: implications for early folliculogenesis. **Int. J. Dev. Neuro., 7:401-409.**

Gospodarowicz, D. 1990. Fibroblast growth factor and its involvment in developmental processes. **Current Topics Dev. Biol., 24:57-93.**

Gospodarowicz, D., Abraham., J.A. and Schillin, J. 1989. Isolation and characterisation of a vascular endothelial cell mitogen produced by the pituitary derived folliculo stellate cells. **Proc. Natl. Acad. Sci. USA, 86:7311-7315.**

Gospodarowicz, D. and Cheng, J. 1986. Heparin protects basic and acidic FGF from inactivation. **J. Cell Physiol., 128:475-484.**

Gospodarowicz, D., Cheng, J., Lui, G.-M., Baird, A. and Böhlen, P. 1984. Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: Identity with pituitary fibroblast growth factor. **Proc. Natl. Acad. Sci, USA., 81:6963-6967.**

Gospodarowicz, D., Massoglia, S., Cheng. J., Lui, G.-M. and Böhlen, P. 1985. Isolation of pituitary fibroblast growth factor by fast protein liquid chromatography (FPLC): partial chemical and biological characterization. **J. Cell. Physiol., 122:323-332.**

Gospodarowicz, D., Plouet, J. and Malerstein, B. 1990. Comparison of the ability of basic and acidic fibroblast growth factor to stimulate the proliferation of an established keratinocyte cell line: Modulation of their biological effect by heparin, transforming growth factor β (TGFβ), and epidermal growth factor. **J. Cell. Physiol., 142:325-333.**

Grabstein, K., Eisemannm J., Mochizuk, D., Shanbec, K., Conlon, P., Hopp, T, March, C. and Gillis, S. 1986. Purification to homogeneity of B-cell stimulatory factor-A molecule that stimulates proliferation of multiple lymphokine dependent cell lines. **J. Exp. Med., 163:1405-1414.**

Grangou-Laxaridis, M., Clinton, M., Goodall, G.T. and Horecker, B.L. 1988. Prothymosin α and parathymosin: amino acid sequence deduced from the cloned rat spleen cDNAs. **Arch. Biochem. Biophys., 263:305-310.**

Gray, A., Dull, T.J. and Ullrich, A. 1983. Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000-molecular weight protein precursor. **Nature, 303:722-725.**

Grey, A -M., Schor, A.M., Rushton, G., Ellis, I. and Schor, S. 1989. Purification of the migration stimulating factor produced by fetal and breast cancer patients. **Proc. Natl. Acad. Sci. USA., 86:2438-2442.**

Grimaldi, J.C. and Meeker, T.C. 1989. The t(5-14) chromosomal translocation in a case of acute lymphocytic-leukemia joins the interleukin-3 gene to the immunoglobulin heavy-chain gene. **Blood, 73:2081-2085.**

Grothe, C., Zachmann, K., Unsicker, K. and Westermann, R. 1990. High molecular weight forms of basic fibroblast growth factor recognised by a new anti-bFGF antibody. **FEBS Lett., 260:35-38.**

Gruss, H.J., Brach, M.A., Mertelsmann, R.H. and Herrmann, F. 1991. Interferon-gamma interrupts autocrine growth mediated by endogenous interleukin-6 in renal-cell carcinoma. **Int. J. Cancer, 49:770-773.**

Guillaume, T., Sekhavat, M., Rubinstein, D.B., Hamdan, O. & Symann, M.L. 1993. Transcription of genes encoding granulocyte-macrophage colony stimulating factor, interleukin 3 and interleukin 6 receptors and lack of proliferative response to exogenous cytokines in nonhematopoietic human malignant cell lines. **Cancer Res., 53:3139-3144.**

Hale, J.E. and Wuthier, R.E. 1987. The mechanisms of matrix vesicle formation. Studies on the composition of the chondrocyte microvilli and on the effects of microfilament perturbing agents on cellular vesiculation. **J.Cell. Biol., 262:1916-1925.**

Halaban, R., Kwon, B.S., Ghosh, S., Delli-Bovi, P. and Baird, A. 1988. bFGF as an autocrine growth factor for human melanomas. **Oncogene Res., 3:177-186.**

Haegeman, G., Content, J., Volckaer, G., Derynck, R. Tavernie, J. and Fiers, W. 1986. Structural analysis of the sequence coding for an inducible 26 kDa protein in human fibroblasts. **Eur. J. Biochem., 159:625-632.**

Ham, R.G. and McKeehan, W.L. 1979. Media and growth requirements. **Meth. Enzymol., 53:44-93.**

Hamburger, A.W., Reid, Y.A., Pelle, B., Milo, G.E., Noyes, I., Krakauer, H. and Fuhrer, J.P. 1985. Isolation and characterization of a monoclonal antibody specific for epithelial cells. **Cancer Res., 45:783-790.**

Hamburger, A.W., Salmon, S.E., Kim, M.B., Trent, J.M., Soehnlen, B., Alberts, D.S. and Schmidt, H.J. 1978. Direct cloning of human ovarian carcinoma cells in agar. **Cancer Res., 38:3438-3444.**

Hanauske, A.-R., Degen, D., Marshall, M.H., Hilsenbeck, S.G., Banks, P., Stuckey, J., Leahy, M. and Von Hoff, D.D. 1992. Effects of recombinant human interleukin-1α on clonogenic growth of primary human tumors *in vitro*. **J. Immunol., 11:155-158.**

Hannink, M. and Donoghue, D.J. 1988. Autocrine stimulation by the v-*sis* gene product requires a ligand-receptor interaction at the cell surface. **J. Biol. Chem., 107:287-298.**

Harris, A.L. 1990. EGF receptor (EGFR) expression in human primary cancers. **Proc. Am. Assoc. Cancer Res., 31:458-460.**

Harvey, T.S., Wilkinson, A.J., Tappin, M.J., Cooke, R.M. and Campbell, I.D. 1991. The solution structure of human transforming growth factor α. **Eur. J. Biochem, 198:555-562.**

Hazudu, D.J., Lee, J.C. and Young, P.R. 1988. The kinetics of interleukin 1 secretion from activated monocytes — differences between interleukin-1-alpha and interleukin 1-beta. **J. Biol. Chem., 263:8473-8479.**

Heldin, C.-H., Ernlund, A., Rorsman, C. and Rönnstrand, L. 1989. Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. **J. Biol. Chem., 264:8905-8912.**

Heldin, C.-H. and Westermark, B. 1984. Growth factors: mechanisms of action and relation to oncogenes. **Cell, 37:9-20.**

Hérbert, J.M., Basilico, C., Goldfarb, M., Haub, O. and Martin, G.R. 1990. Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression during embryogenesis. **Dev. Biol., 138:454-463.**

Herlyn, M., Clark, W.H., Rodeck, U., Mancianti, M.L., Jambrosic, J. and Koprowski, H. 1987. Biology of tumor progression in human melanocytes. **Proc. Natl. Acad. Sci. USA, 56:461-474.**

Herlyn, M., Kath, R., Williams, N., Valyi-Nagy, I. and Rodeck, U. 1990. Growth-regulatory factors for normal, premalignant, and malignant human cells *in vitro*. **Adv. Cancer Res., 54:213-234.**

Hermann, F., *et al.*, 1988. **J. Clin. Invest., 81:1415.**

Hermansson, M., Funa, K., Hartman, M., Claesson-Welch, L., Heldin, C.-H., Westermark, B. and Nistér, M. 1992. Platelet-derived growth factor (PDGF) and its receptors in human glioma tissue; expression of mRNA and protein suggests the presence of autocrine and paracrine loops. **Cancer Res., 52:3213-3219.**

Herschman, H.R. 1991. Primary response genes induced by growth factors and tumor promotors. **Annual. Rev. Biochem., 60:281-319.**

Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T. and Kishimoto, T. 1990. Molecular cloning and expression of an IL-6 signal transducer, gp130. **Cell, 63:1149-1157.**

Hicks, K., Friedman, B. and Rosner, M.R. 1989. Basic and acidic fibroblast growth factors modulate the epidermal growth factor receptor by a protein kinase C-independent pathway. **Biochem. Biophys. Res. Commun., 164:796-803.**

Higashio, K., Nobuyuki, S., Goto, M., Itagaki, Y., Nagao, M., Yasuda, H. and Morinaga, T. 1990. Identity of a tumor cytotoxic factor from human fibrobalsts and hepatocyte growth factor. **Biochem. Biophys. Res. Commun., 170:397-404.**

Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C. and Klagsbrun, M. 1991. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. **Science, 251:936-939.**

Higashiyama, S., Lau, K., Besner, G.E., Abraham, J.A. and Klagsbrun, M. 1992. Structure of heparin-binding EGF-like growth factor; multiple forms, primary structure and glycosylation of the mature protein. **J. Biol. Chem., 267:6205-6212.**

Higashiyama, S., Abraham, J.A. and Klagsbrun, M. 1993. Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparin sulfate. **J. Cell. Biol., 122:933-940.**

Hirano, T., Yasukawa, K., Harada, H., Taga, T., Watanabe, Y., Matsuda, T., Kashiwam, S., Nakajima, K., Koyama, K. and Iwamatsu, A., 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces lymphocytes-B to produce immunoglobulin. **Nature, 324:73-76.**

Ho, P.L., Carpenter, M.R., Smillie, L.B. and Gambarini, A.G. 1990. Co-purification of proteases with basic fibroblast growth factor (FGF). **Biochem. Biophys. Res. Commun., 170:769-774.**

Hodges, G.M., Livingston, D.C. & Franks, L.M. 1973. Localization of trypsin in cultured mammalian cells. **J. Cell Sci., 12:887-902.**

Hollstein, M., Sidransky, B., Vogelstein, B. and Harris, C.C. 1991. p53 mutations in human cancers. **Science, 253:49-53.**

Hori, A., Sasada, R., Matsuttani, E., Naito, K., Sakura, Y., Fujita, T. and Kozai, Y. 1991. Suppression of solid tumour growth by immunoneutralizing monoclonal antibody against basic fibroblast growth factor. **Cancer Res., 51:6180-6184.**

Horn, D., Fitzpatrick, W.C., Compper, P.T., Ochs, V., Bolton-Hanson, M., Zarling, J., Malik, N., Todaro, G.J., and Linsley, P.S. 1990. Regulation of cell growth by recombinant oncostatin M. **Growth Factors, 2:157-165.**

Hosang, M. 1985. Suramin binds to platelet-derived growth factor and inhibits its biological activity. **J. Cell Biochem., 29:265-273.**

Houck, K.A., Ferrara, N., Winer, J., Cachianes, G., Li, B. and Leung, D.W. 1991. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. **Mol. Endocrinol., 5:1806-1814.**

Houck, K.A., Leung, D.W., Rowland, A.M., Winer, J. and Ferrara, N. 1992. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. **J. Biol. Chem., 267:26031-26037.**

Huang, J.S., Huang, S.S. and Deuel, T.F. 1984. Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF cell-surface receptors. **Cell, 39:79-87.**

Huff, K.K., Kaufman, D., Gabbay, K.H., Martin-Spencer, E., Lippman, M.E. and Dickson, R.B. 1986. Secretion of an insulin-like growth factor-I-related protein by human breast cancer cells. **Cancer Res., 46:4613-4619.**

Hughes, S.E. and Hall, P.A. 1993. Immunolocalization of fibroblast growth factor receptor and its ligands in human tissues. **Lab. Invest., 69:173-182.**

Humbel, R.E. 1990. Insulin-like growth factors I and II. **Eur. J. Biochem., 190:445-462.**

Hunts, J., Ueda, M., Ozawa, S., Abe, O., Pastan, I. and Shimizu, N. 1985. Hyperproduction and gene amplification of the epidermal growth factor receptor in squamous cell carcinomas. **Jpn. J. Cancer Res., 76:663-666.**

Idzerda, R.L., March, C.J., Mosley, B., Lynam, S.D., Vandenbo, T., Gimpel, S.D., Din, W.S., Grabstel, K.H., Widmer, M.B. and Park, L.S. 1990. Human interleukin-4 receptor confers biological resposiveness and defines a novel receptor superfamily. **J. Exp. Med., 171:861-873.**

Ihle, J. N., Pepersac, L. and Rebar, L. 1981. Regulation of T-cell differentiation: *in vitro* induction of of 20 alpha hydroxysteroid dehydrogenase in splenic lymphocytes from athymic mice by a unique lymphokine. **J. Immunol., 126:2184-2189.**

Ii, M., Yoshida, H., Aramaki, Y., Masuya, H., Hada, T., Terada, M., Hatanaka, M. and Ichimori, Y. 1993. Improved enzyme immunoassay for human basic fibroblast growth factor using a new enhanced chemiluminescence system. **Biochem. Biophys. Res. Commun., 193:540-545.**

Imanishi, K., Yamaguchi, K. and Honda, S. 1988. Transforming growth factor-α as a possible autocrine growth factor for human adenocarcinoma of the lung. *In:* **Imura, H., Shizume, K., and Yoshida, S. (eds.), Progress in Endocrinology, Amsterdam, Elsevier, pp 1363-1368.**

Imanishi, K., Yamaguchi, K., Kuranami, M., Kyo, E., Hoxumi, T. and Abe, K. 1989. Inhibition of growth of human lung adenomacarcinoma cell lines by anti-transforming growth factor-α monoclonal antibody. **J. Natl. Cancer Inst., 81:220-223.**

Ito, F., Takii, Y., Suzuki, J. *et al.*, 1982. Reversiblbe inhibition by human serum lipoproteins of cell proliferation. **J. Cell Physiol., 113:1-13.**

Jakobovits, A., Shackleford, G.M., Varmus, H.E. and Martin, G.R. 1986. Two proto-oncogenes implicated in mammary carcinogenesis, *int*-1 and *int*-2, are independently regulated during mouse development. **Proc. Natl. Acad. Sci. USA, 83:7806-7810.**

Jakowlew, S.B., Dillard, P.J., Kondaiah, P., Sporn, M.B. and Roberts, A.B. 1988a. Complementary

deoxyribonucleic acid cloning of a novel transforming growth factor-β messenger ribonucleic acid from chick embryo chondrocytes. **Mol. Endocrinol., 2:747-755.**

Jakowlew, S.B., Dillard, P.J., Sporn, M.B. and Roberts, A.B. 1988b. Complementary deoxyribonucleic acid cloning of a messenger ribonucleic acid encoding transforming growth factor beta 4 from chicken embryo chondrocytes. **Mol. Endocrinol., 2:1186-1195.**

Jalkanen, M., Rapraeger, A., Saunders, S. and Bernfield, M. 1987. Cell surface proteoglycan of mouse mammary epithelial cells is shed by cleavage of its matrix-bound ectodomain from its membrane-associated domain. **J.Cell Biol., 105:3087-3096.**

Jaques, G., Rotsch, M., Wegman, C., Worsch, U., Maasberg, M. and Haveman, K. 1988. Production of immunoreactive insulin-like growth factor-I and response to exogenous IGF-I in small cell lung cancer cell lines. **Exp. Cell Res., 176:336-343.**

Jaye, M., Schlessinger, J. and Dionne, C. 1992. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. **Biochim. Biophys. Acta, 1135:185-199.**

Jeffreys, A.J., Wilson, V. and Thien, S.L. 1985a. Hypervariable minisatellite regions in human DNA. **Nature, 314:67-73.**

Jeffreys, A.J., Wilson, V. and Thien, S.L. 1985b Individual specific fingerprints of human DNA. **Nature, 316:76-79.**

Johnson, D.E., Lu, J., Chen, H., Werner, S. and Williams, L.T. 1991. The human fibroblast growth factor receptor genes: a commom structural arrangement underlies the mechanisms for generating forms that differ in their third immunoglobulin domain. **Mol. Cell. Biol., 11:4627-4634.**

Johnsson, A., Betsholtz, C., Heldin, C.-H. and Westermark, B. 1985. Antibodies against platelet-derived growth factor inhibit acute transformation by simian sarcoma virus. **Nature, 317:438-440.**

Johnston, R.M., Adam, M., Hammond, J.R., Orr, L. and Turbide, C. 1987. Vesicle formation during rectiulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). **J. Biol. Chem., 262:9412-9420.**

Jorissen, M., Van der Schueren, B., Van den Berghe, H. and Cassimen, J,J. 1991. Contribution of *in vitro* culture methods for respiratory epithelial cells to the study of the physiology of the respiratory tract. **Eur. Respir. J., 4:210-217.**

Joseph-Silverstein, J. and Rifkin, D.B. 1987. Endothelial cell growth factors and the vessel wall. **Seminars in Thrombsis and Hemostasis, 13:504-513.**

Kadomatsu,K., Tonomura, M. and Muramatsu, T. 1988. cDNA cloning and sequencing of a new gene intensely expressed in early differentiation stages of embryonal carcinoma cells and in mid-gestation period of mouse embryogenesis. **Biochem. Biophys. Res. Commun., 151:1312-1318.**

Kaiser, U., Schardt, C., Brandscheidt, D., Wollmer, E. and Havemann, K. 1993. Expression of insulin-like growth factor receptors I and II in normal human lung and in lung cancer. **J. Cancer Res. Clin. Oncol., 119:665-668.**

Kajimoto, Y. and Rotwein, P. 1991. Structure of the chicken IGF-I gene reveals conserved promotor elements. **J. Biol. Chem., 266:9724-9731.**

Kan, M., Shi, E.-G. and McKeehan, W.L. 1991. Identification and assay of fibroblast growth factor receptors. **Methods. Enzymol., 198:158-171.**

Kanakaraj, P., Raj, S., Khan, S.A. and Bishayee, S. 1991. Ligand-induced interaction between alpha- and beta-type platelet-derived growth factor (PDGF) receptors: Role of receptor heterodimers in kinase activation. **Biochemistry, 30:1761-1767.**

Kandel, J., Bossy-Wetzel, E., Randvanyl, F., Klagsbrun, M., Folkman, J. and Hanahan, D. 1991. Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. **Cell, 66:1095-1104.**

Kasayama, S., Sumitani, S., Tanaka, A., Yamanishi, H., Nakamura, N., Matsumoto, K. and Sato, B. 1991. Heparin inhibits autocrine stimulation but not fibroblast growth factor stimulation of cell proliferation of androgen-responsive shionogi carcinoma 115. **J. Cell Physiol., 148:260-266.**

Kasuga, M., Van Obberghen, E., Nissley, S.P. and Rechler, M.M. 1981. Demonstration of two subtypes of insulin-like growth factor receptors by affinity cross-linking. **J. Biol. Chem., 256:5305-5308.**

Katayama, M. and Kan, M. 1991. Heparin-binding (fibroblast) growth factors are potential autocrine regulators of esophageal cell proliferation. **In Vitro Cell. Dev. Biol., 27A:533-541.**

Kawano, M., Hirano, T., Matsuda, T., Taga, T., Horii, Y., Iwato, K., Asaoku, H., Tang, B., Tanabe, O., Tanaka, H., Kuramoto, A. and Kishimoto, T. 1988. Autocrine regulation and essential requirement of BSF-2/IL-6 for human multiple myeloma. **Nature, 332:83-85.**

Keating, M.T. and Williams, L.T. 1988. Autocrine stimulation of intracellular PDGF receptors in v-*sis* transformed cells. **Science, 239:914-916.**

Kent Osborne, C., Coronado, E.B., Kitten, L.J., Artega, C.I., Fuqua, S.A.W., Ramasharma, K., Marshall, M. and Li, C.H. 1989. Insulin-like growth factor-II (IGF-II): a potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor.

Kiefer, M.C., Stephans, J.C., Crawford, K., Okino, K. and Barr, P.J. 1990. Ligand-affinity cloning and structure of a cell surface heparan sulfate proteoglycan that binds basic fibroblast growth factor. **Proc. Natl. Acad. Sci. USA, 87:6985-6989.**

Kirk, D., Brober, T., Irwin, J.C., Kenney, W.C. and Jones, L.W. 1993. Fibroblast inhibition of tumour cells may be mediated by TGF-β1. **In Vitro Cell Dev. Biol., 29A:4-8.**

Kishimoto, T., Hibi, M., Murakami, M., Narazaki, M., Saito, M., Taga, T. 1992. The molecular biology of interleukin-6 — and its receptor. **Ciba Found. Symp., 167:5-23.**

Klagsbrun, M. 1989. The fibroblast growth factor family: structural and biological properties. **Prog. Growth Factor. Res., 1:207-235.**

Klagsbrun, M. & Baird, A. 1991. A dual receptor system is required for basic fibroblast growth factor activity. **Cell, 67:229-231.**

Klagsbrun, M. & Shing, Y. 1985. Heparin affinity of anionic and cationic capillary endothelial cell growth factors: analysis of hypothalamus-derived growth factors and fibroblast growth factors. **Proc. Natl. Acad. Sci., USA, 82:805-809.**

Klagsbrun, M., Sullivan, R., Smith, S., Rybka, R. and Shing, Y. 1987. Purification of endothelial cell growth factors by heparin affinity chromatography. **Meths. Enzymol., 147:95-105.**

Kobayashi, S., Teramura, M., Sugawara, I., Oshimi, K. and Mizogichi, H. 1993. Interleukin-11 acts as an autocrine growth factor for human megakaryoblastic cell lines. **Blood, 81:889-893.**

Kobrin, M.S., Yamanaka, Y., Friess, H., Lopez, M.E. and Korc, M. 1993. Aberrant expression of

type I fibroblast growth factor receptor in human pancreatic adenocarcinomas. **Cancer Res., 53:4741-4744.**

Koch, C.A., Anderson, D., Moran, D.F., Ellis, C. and Dawson, T. 1991. SH2 and SH3 domains — elements that control interactions of cytoplasmic signaling proteins. **Science, 252:668-674.**

Koda, T., Sasada, A., Matsushima, S. and Kakinuma, M. 1987. A transforming gene, *hst*, found in NIH3T3 cells transformed with DNA from three stomach cancers and a colon cancer. **Jpn. J. Cancer Res., 78:325-328.**

Kohase, M., Henriksen-DeStefano, D., May, L.T., Vilcek, J. and Sehgal, P.B. 1986. Induction of beta 2-interferon by tumor necrosis factor: a homeostatic mechanism in the control of cell proliferation. **Cell., 45:659-666.**

Kolset, S.O. and Gallagher, J.T. 1990. Proteoglycans in haemopoietic cells. **Biochim. Biophys. Acta., 1032:191-211.**

Kondaiah, P., Sands, M.J., Smith, J.M., Fields, A., Roberts, A.B., Sporn, M.B. and Melton, D.A. 1990. Identification of a novel transforming growth factor-β (TGF-β5) mRNA in *Xenopus laevis*. **J. Biol. Chem., 265:1089-1093.**

Korc, M., Chandrasekar, B., Yamanaka, Y., Friess, H., Buchler, M. and Berger, H.G. 1992. Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increase in levels of epidermal growth factor and transforming growth factor alpha. **J. Clin. Invest., 90:1352-1360.**

Kurzrock, R., Estrov, Z., Wetzler, M., Cutterma, S.U. and Talpaz, M. 1991. LIF — not just a leukemia inhibitory factor. **Endocrine Rev., 12:208-217.**

Larrick, J.W. and Wright, S.C. 1992. Native cytokine antagonists. **Baillieres Clin. Haematol., 5:681-702.**

Law, E., Gilvarry, U., Lynch, V., Gregory, B., Grant, G. and Clynes, M. 1992. Cytogenetic comparison of two poorly differentiated human lung squamous cell carcinoma lines. **Cancer Genet. Cytogenet., 59:111-118.**

Layton, J.E., Scanlon, D.B., Soveny, C. and Morstyn, G. 1988. Effects of bombesin antagonists on the growth of small cell lung cancer cells *in vitro*. **Cancer Res., 48:4783-4789.**

Lechner, J.F. and LaVeck, M.A. 1985. A serum-free method for culturing normal bronchial epithelial cells at clonal density. **J. Tiss. Cult. Methods., 9:43-48.**

Lee, D.C., Rose, T.M., Webb, N.R. and Todaro, G.J. 1985. Cloning and sequence analysis of a cDNA for rat transforming growth factor-α. **Nature, 313:489-491.**

Lee, J., Horuk, R., Rice, G.C., Bennett, G.I., Camerato, T. and Wood, W.I. 1992. Characterisation of 2 high affinity human interleukin-8 receptors **J. Biol. Chem., 267:6283-6287.**

Lee, M., Draoui, M., Zia, F., Gazdar, A.F., Oie, H., Tarr, C., Bellot, F., Kris, R. and Moody, T.W. 1992. Epidermal growth factor receptor monoclonal antibodies inhibit the growth of lung cancer cell lines. **J. Natl. Cancer Inst., 13:117-123.**

Leibovitz, A. 1986. Development of tumor cell lines. **Cancer Genet. Cytogenet., 19:11-19.**

Levett, D., *et al.*, 1986. **J. Immunol., 136:-340.**

Leung, D.W., Cachianes, G., Kuang, W.-J., Goeddel, D.V., and Ferrara, N. 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. **Science, 246:1306-1309.**

Leung, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W.J., Barnard, R., Waters, M.J. and Wood, W.I. 1987. Growth hormone receptor and serum binding protein; purification, cloning and expression. **Nature, 330:537-543.**

Leveen, P., Claesson-Welch, L., Heldin, C.-H., Westermark, B. and Betsholtz, C. 1990. Expression of messenger RNAs for platelet-derived growth factor and its receptors in human sarcoma cell lines. **Int. J. Cancer, 46:1066-1070.**

Levine, A.J., Momand, J. and Finlay, C.A. 1991. The p53 tumour suppressor gene. **Nature, 351:453-456**

Li, Q., Blacher, R., Esch, F., and Cognate, F. 1990. Heparin-binding erythroid cell stimulating factor from fetal bovine serum has the N-terminal sequence of insulin-like growth factor II. **Biochem. Biophys. Res. Commun., 166:557-561.**

Li, Y-S., Hoffman, R.M., Lebeau, M.M., Espinosa, R., Jenkins, N.A., Gilbert, D.J., Copeland, N.G. and Deul, T.F. 1992. Characterisation of the human pleiotrophin gene-promoter region and chromosomal localisation. **J. Biol. Chem., 267:6011-6016.**

Li, Y.-S., Milner, P.G., Chauhan, A.K., Watson, M.A., Hoffman, R.M., Kodner, C.M., Milbrandt, J. & Deuel, T.F. 1990. Cloning and expression of a developmentally regulated protein that induces mitogenic and neutrite outgrowth activity. **Science, 250:1690-1694.**

Linsley, P.S., Kallesta, J., Ochs, V. and Neubawer, M. 1990. Clevage of a hydrophobic C-terminal domain increases growth inhibitory activity of oncostatin M. **Mol. Cell. Biol., 10:1882-1890.**

Liotta, L.A., Mandler, R., Murano, G., Katz, D.A., Gordon, R.K., Ching, P.K. and Schiffmann, E. 1986. Tumor cell autocrine motility factor. **Proc. Natl. Acad. Sci. USA, 83:3302-3306.**

Liu, C. and Tsao, M.S. 1993. Proto-oncogene and growth factor/receptor expression in the establishment of primary non-small cell lung carcinoma cell lines. **Am. J. Pathol., 142:413-423.**

Liu, J., Modrell, B., Aruffo, A., Marken, J.S., Taga, T., Yasukawa, K., Murakami, M., Kishimoto, T. and Shoyab, M. 1992. Interleukin-6 signal transducer gp130 mediates oncostatin M signaling. **J. Biol. Chem., 267:16763-16766.**

Liu, X.H., Wiley, H.S. and Meikle, A.W. 1993. Androgens regulate proliferation of human prostate cancer cells in culture by increasing transforming growth factor α (TGFα) and epidermal growth factor (EGF)TGFα receptor. **J. Clin. Endocrinol. Metabol., 77:1472-1478.**

Lobel, P., Dahms, N.M. and Kornfeld, S. 1988. Cloning and sequence analysis of the cation-independent mannose 6-phosphate receptor. **J. Biol. Chem., 264:2563-2570.**

Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J.L., Lane, W.S. and Massagué, J. 1991. Structure and expression of the membrane proteoglycan Betaglycan, a component of the TGFβ receptor system. **Cell, 67:785-795.**

Lories, V., Cassiman, J.-J., Van den Berghe, H. and David, G. 1992. Differential expression of cell surface heparan sulfate proteoglycans in human mammray epithelial cells and lung fibroblasts. **J. Biol. Chem., 267:1116-1122.**

Lowenthal, J.W., Castle, B.E., Christia, J., Schreurs, J., Remick, D., Arai, N., Hoy, P., Takebey, Y. and Howard, M. 1988. Expression of high affinity receptors for murine interleukin-4 (BSF-1) of

hematopoietic and nonhematopoietic cells. **J. Immunol., 140:456-464.**

Lu, C. and Kerbel, R.S. 1993. Interleukin-6 undergoes transition from paracrine growth inhibitor to autocrine stimulator during human melanoma progression. **J. Cell Biol., 120:1281-1288.**

Luster, W., Gropp, C., Kern, H.F. and Havemann, K. 1985. Lung tumour cell lines synthesizing peptide hormones established from tumours of four histological types: Characterization of the cell lines and analysis of their peptide hormone production. **Br. J. Cancer, 51:865-875.**

Lyons, R.M., Gentry, L.E., Purchio, A.F. and Moses, H.L. 1990. Mechanism of activation of latent recombinant transforming growth factor-beta-1 by plasmin. **J. Cell Biol., 110:1361-1367.**

Lyons, K., Graycar, J.L., Lee, A., Hashami, S., Lindquist, P.B., Chen, E.Y., Hogan, B.L.M. and Derynck, R. 1989. Bgr-1, a mammalian gene related to *xenopus Vg-1*, a member of the transforming growth factor gene superfamily. **Proc. Natl. Acad. Sci. USA, 86:4554-4558.**

MacDonald, R.G., Tepper, M.A., Clairmon, K.B., Perregau, S.B. and Czech, M.P. 1989. Serum form of the rat insulin-like growth factor-II/mannose 6-phosphate receptor is truncated in the carboxyl-terminal domain. **J. Biol. Chem., 264:3256-3261.**

Madtes, D.K., Raines, E.W., Sakariassen, K.S., Assoian, R.K., Sporn, M.B., Bell, G.I. and Ross, R. 1988. Induction of transforming growth factor-alpha in activated human alveolar macrophages. **Cell, 53:285-293.**

Mailleaux, P., Vanderwi, J.M., and Vanderha, J.J. 1992. The new growth factor pleiotrophin (HB-GAM) messenger RNA is selectively present in the mengiothelial cells of human mengingiomas. **Neurosci. Lett., 142:31-35.**

Marics, I., Adelaide, J., Rayboud, F., Mattei, M.-G., Coullier, F., Planche, J., De Lapeyriere, O. and Birnbaum, D. 1989. Characterization of the *HST*-related FGF 6 gene, a new member of the fibroblast growth factor gene family. **Oncogene, 4:335-340.**

Marquardt, H., Hunkapiller, M.W., Hood, L.E., Twardzik, D.R., DeLarco, J.E., Stephenson, J.R. and Todaro, G.J. 1983. Transforming growth factors produced by retrovirus transformed rodent fibroblasts and human melanoma cells, amino acid sequence homology with epidermal growth factor. **Proc. Natl. Acad. Sci. USA, 80:4684-4688.**

Martin, A. 1992. Cytotoxicity testing *in vitro:* investigation of 5 miniaturised, colorimetric assays. **Ph.D. thesis, Dublin City University, Glasnevin, Dublin 9, Republic of Ireland.**

Martin, A. and Clynes, M. 1991. Acid phosphatase: endpoint for *in vitro* toxicity tests. **In Vitro Cell. Dev. Biol., 27A:183-184.**

Martin, A. and Clynes, M. 1993. Comparison of 5 microplate colorimetric assays for *in vitro* cytotoxicity testing and cell proliferation assays. **Cytotechnology, 11:49-58.**

Marynen, P., Zhang, J., Cassiman. J.-J., Van den Berghe, H. and David, G. 1989. Partial and primary structure of the 48- and 90-kilodalton core proteins of cell surface-associated heparan sulfate proteoglycans of lung fibroblasts. **J. Cell Biol., 264:7017-7024.**

Mason, A.J., Hayflick, J.S., Ling, N., Esch, F., Ueno, N., Ying, Y., Guillemin, R., Niall, H. and Seeberg, P.H. 1985. Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. **Nature, 318:659-663.**

Massagué, J. 1990a. The transforming growth factor-β family. **Annu. Rev. Cell Biol., 6:597-641.**

Massagué, J., Cheifetz, S., Boyd, F.T. and Andres, J.L. 1990b. TGF-Beta receptors and TGF-Beta binding binding proteoglycans — recent progress in identifying their functional properties. **Ann. N.Y. Acad. Sci., 593-59-72.**

Massagué, J. and Czech, M.P. 1982. The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. **J. Biol. Chem., 257:5038-5045.**

Masui, T., Wakefield, L.M., Lechner, J.F., LaVeck, M.A., Sporn, M.B. and Harris, C.C. 1986. Type β transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. **Proc. Natl. Acad. Sci. USA, 83, 2438-2442**

Masui, T., Lechner, J.F., Mark III, G.E., Pfeifer, A.M.A., Miyashita, D.L. and Harris, C.C. 1988. Growth and differentiation programs of normal and transformed human bronchial epithelial cells. In: **Growth Factors, Tumour Promotors, and Cancer Genes. (eds) Colburn, N.H., Moses, H.L., and Stanbridge, E.J. Alan R. Liss Inc. New York. pp, 191-201.**

Matsui, T., Heidaran, M., Miki, T., Popescu, W., La Rochell, W., Kraus, M., Pierce, J. and Aaronson, S. 1989. Isolation of a novel receptor cDNA establishes the presence of two PDGF receptor genes. **Science, 243:800-804.**

Matsumoto, K., Tajima, H., Okazaki, H. and Nakamura, T. 1992. Negative regulation of hepatocyte growth factor gene expression in human lung fibroblasts and leukemic cells by transforming growth factor β_1 and glucocorticoids. **J. Biol. Chem., 267:24917-24920.**

Matuo, Y., Nishi, N., Muguruma, Y., Yoshitake, Y., Masuda, Y., Nishikawa, K. and Wada, F. 1988. Stabilization of fibroblast growth factors by a non-cytotoxic zwitterionic detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS). **In Vitro Cell. Dev. Biol., 24:477-480.**

Maurer, H.R. 1992. Towards serum-free, chemically defined media for mammalian cell culture. *In:* **Rickwood, D., & Hames, B.D. (eds.), Animal Cell Culture, A Practical Approach. Freshney, R.I. IRL Press, Oxford University Press, New York. pp 15-43.**

May, L.T., Helfgott, D.C. and Sehgal, P.B. 1986. Anti-beta interferon antibodies inhibit the increased expression of HLA-B7 messenger-RNA in tumour necrosis factor treated human fibroblasts — structural studies of the beta-2 interferon involved. **Proc. Natl. Acad. Sci. USA, 83:8957-8961.**

Melsert, R., Hoogerbrugge, J.W. and Rommerts, F.F.G. 1989. Albumin, a biologically active protein acting on leydig cells. **Mol. Cell. Endocrinol., 64:35-44.**

Merritt, W.D., Weissler, M.C., Turk, B.F. and Gilmer, T.M. 1990. Oncogenes amplification in squamous cell carcinoma of the head and neck. **Arch. Otoaryngol Head Neck Surg., 116:1394-1398.**

Metcalf, D. 1992. Leukemia inhibitory factor — a puzzling polyfunctional regulator. **Growth Factors, 2:7:169-173.**

McCormick, F. 1989. ras GTPase activating protein: signal transmitter and signal terminator. **Cell, 56:5-8.**

McDonnell, S. Investigation on growth of human epithelial cells in culture. 1987. **Ph.D thesis, National Institute for Higher Education, Dublin [NIHED].**

McMahon, S.B. and Monroe, J.G. 1992. Role of primary response genes in generating cellular responses to growth factors. **FASEB. J. 6:2707-2715.**

Mignatti, P., Morimoto, T. and Rifkin, D.B. 1992. Basic fibroblast growth factor a protein devoid of

a secretory signal sequence is released by cells via a pathway independent of the endoplasmic recticulum-golgi complex. **J. Cell Physiol., 151:81-93.**

Mignatti, P., Morimoto, T. and Rifkin, D.B. 1991. Basic fibroblast growth factor released by single, isolated cells stimulates their migration in an autocrine a manner. **Proc. Natl. Acad. Sci. USA, 88:11007-11011.**

Mignatti, P., Tsuboi, R., Robbins, E. and Rifkin, D.B. 1989. *In vitro* angiogenesis on the human amniotic membrane: requirement for basic fibroblast growth factor-induced proteinases. **J. Biol. Chem., 108:671-682.**

Miki, S., Iwano, M., Miki, Y., Yamamoto, M., Tang, B., Yokokawa, K., Sonoda, T., Hirano, T. and Kishimoto, T. 1989. Interleukin-6 (IL-6) functions as an *in vitro* autocrine growth factor in renal cell carcinomas. **FEBS Lett., 250:607-610.**

Miles, S.A., Martinez, O., Rezai, A., Magpenta, I., Kishimoto, T., Nakamura, S., Radka, S.F., Linsley, P.S. 1992. Oncostatin-M as a potent mitogen for AIDS — Kaposi's sarcoma derived cells. **Science, 255:1432-1434.**

Mills, G.B., May, C., McGill, M., Roifman, C.M. & Mellors, A. 1988. A putative new growth factor in ascitic fluid from ovarian cancer patients: identification, characterization, and mechanism of action. **Cancer Res., 48:1066-1071.**

Millauer, B., Wizigman, S., Schnurch, H., Martinez, R., Moller, N.P.H, Risau, W. and Ullrich, A. 1993. High-affinity VEGF-binding and developmental expression suggest FLK-1 as a major regulator of vasculogenesis and angiogenesis. **Cell, 72:835-846.**

Minuto, F., Del Monte, P., Barreca, A., Alama, A., Cariola, G. and Giordano, G. 1988. Evidence for autocrine mitogenic stimulation by Somatomedin-C/Insulin-like growth factor I on an established human lung cancer cell line. **Cancer Res., 48:3716-3719.**

Minuto, F., Del Monte, P., Barreca, A., Fortini, P., Cariola, G., Catrambone, G. and Giordano, G. 1986. Evidence for an increased somatomedian-C/insulin-like growth factor I content in primary human lung tumours. **Cancer Res., 46:985-988.**

Miyamoto, M., Naruo, k., Seko, C., Matsumoto, S., Kondo, T. and Kurokawa, T. 1993. Molecular cloning of a novel cytokine encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. **Mol. Cell Biol., 13:4251-4259.**

Miyauchi, S., Moroyama, T., Kyoizumi, S., Asakawa, J.-I., Okamoto, T. and Takada, K. 1988. Malignant tumor cell lines produce interleukin-1-like factor. **In Vitro Cell Dev. Biol., 24:753-758.**

Miyazaki, K., Masui, H. and Sato, G.H. 1984. Growth and differentiation of human bronchogenic carcinoma cells in serum-free media. In: **Barnes, D.W., Sirbasku, D.A., and Sato, G.H. (eds). Cell Culture Methods For Molecular and Cell Biology. Alan R. Liss, Inc, New York, Volume 3, pp 83-94.**

Miyazono, K., Hellman, U., Wernstedt, C., Heldin, C-H. 1988. Latent high molecular weight complex of transforming growth factor β1. **J. Biol. Chem., 263:6407-6415.**

Mizel, S.B. 1988. Interleukin 1. Biology and molecular biology. In: **Poste, G., and Crooke, S.T. (eds). Cellular and Molecular aspects of inflammation. pp 75-96.**

Moats-Staats, B.M., Retsch-Bogart, G.Z., Price, W.A., Jarvis, H.W., D'Ercole, A.J. and Stiles, A.D. 1993. Insulin-like growth factor (IGF-1) antisense oligodeoxynucleotide mediated inhibition of DNA synthesis by WI-38: evidence for autocrine actions of IGF-I. **Mol. Endocrinol., 7:171-180.**

Moody, T.W., Fagarasan, M., Zia, F., Cesnjaj, M. and Goldstein, A.L. 1993b. Thymosin α1 down-regulates the growth of human non-small cell lung cancer cells *in vitro* and *in vivo*. **Cancer Res., 53:5214-5218.**

Moody, T.W., Zia, F., Goldstein, A., Naylor, P., Sarin, E., Brenneman, D., Koros, A.M.C., Reubi, J.C. and Gozes, I. 1993a. VIP analogues inhibit small cell lung cancer growth. **Biomed. Res., 13:131-136.**

Moore, A.E., Sabachewsky, L. and Toolan, H.W. 1955. **Cancer Res., 15:598.**

Morrison, D.K., Kaplan, D.R., Rapp, U. and Roberts, T.M. Signal transduction from membrane to cytoplasm. **Proc. Natl. Acad. Sci. USA, 85:8855-8859.**

Morrison, R.S., Gross, J.L., Herblin, W.F., Reilly T.M., LaSala, P.A., Alterman, R.L., Moskal, J.R., Kornblith, P.L. and Dexter, D.L. 1990. Basic fibroblast growth factor-like activity and receptors are expressed in a human glioma cell line. **Cancer Res., 50:2524-2529.**

Morrison, R.S. 1991. Suppression of basic fibroblast growth factor expression by antisense oligodeoxynucleotides inhibits the growth of transformed human astrocytes. **J. Biol. Chem., 266:728-734.**

Moscatelli, D., Joseph-Silverstein, J., Manejias, R. and Rifkin, D.B. 1987. M_r 25,000 heparin-binding protein from guinea pig brain is a high molecular weight form of basic fibroblast growth factor. **Proc. Natl. Acad. Sci. USA, 84:5778-5782.**

Moscatelli, D., Presta, M., Joseph-Silverstein, J. and Rifkin, D.B. 1986. Both normal and tumor cells produce basic fibroblast growth factor. **J. Cell. Physiol., 129:273-276.**

Mosley, B., Beckmann, M.P., March, C.J., Idzerda, R.L., Gimpel, S.D., Vandenbo, T., Friend, D., Alpert, A., Anderson, D. and Jackson, J. 1989. The murine interleukin-4 receptor-molecular-cloning and characterisation of secreted and membrane-bound forms. **Cell, 59:335-348.**

Mulshine, J.L., Cuttitta, F., Bibro, M., Fedorko, J., Fargion, S., Little, C., Carney, D.N., Gazdar, A.F. and Minna, J.D. 1983. Monoclonal antibodies that distinguish non-small cell from small cell lung cancer. **J. Immunol., 131:497-502.**

Murphy, P.R., Sato, Y. and Knee, R.S. 1992. Phosphorothioate antisense oligonucleotides against basic fibroblast growth factor inhibit anchorage-dependent and anchorage-independent growth of a malignant glioblastoma cell line. **Mol. Endocrinol., 6:877-884.**

Myoken, Y., Kayada, Y., Okamoto, T., Kan, M., Sato, G.H. and Sato, J.D. 1991. Vascular endothelial cell growth factor (VEGF) produced by A-431 human epidermoid carcinoma cells and identification of membrane binding sites. **Proc. Natl. Acad. Sci. USA, 88:5819-5823.**

Nair, B.C., Devico, A.L., Nakamura, S., Copeland, T.D., Chen, Y., Patel. A., O'Neill, T., Oroszlan, S., Gallo, R.C. and Sarngadh, M.G. 1992. Identification of a major growth factor for AIDS Kaposi's-sarcoma cells as oncostatin-M. **Science, 255:1430-1432.**

Naglich, J.G., Metherall, J.E., Russell, D.W. and Eidels, L. 1992. Expression cloning of a Diptheria toxin receptor: identity with heparin-binding EGF-like growth factor precursor. **Cell, 69:1051-1061.**

Nakane, T., Szentendrei, T., Stern, L., Virmani, M., Seely, J., Kunos, G. 1990. Effects of IL-1 and cortisol on β-adrenergic receptors in cultured A549 lung tumor cells. **J. Immunol., 145:260-266.**

Nakanishi, Y., Mulshine, J.L., Kasprzyk, P.G., Natale, R.B., Maneckjee, R., Avis, I., Treston, A.M., Gazdar, A.F., Minna, J.D. and Cuttitta, F. 1988. Insulin-like growth factor-I can mediate autocrine

proliferation of human small cell lung cancer cell lines *in vitro*. **J. Clin. Invest., 82:354-359.**

Nakanishi, Y., Kihara, K., Mizuno, K., Masamune, Y., Yoshitake, Y. and Nishikawa, K. 1992. Direct effect of basic fibroblast growth factor on gene transcription in a cell-free system. **Proc. Natl. Acad. Sci. USA, 89:5216-5220.**

Naruo, K.-I., Seko, C., Kuroshima, K.-I., Matsutani, E., Sasada, R., Kondo, T. and Kurokawa, T. 1993. Novel secretory heparin-binding factors from human glioma cells (glia-activating factors) involved in glial cell growth. **J. Biol. Chem., 268:2857-2864.**

Navarro, S. Delbili, N., Bernaudi, J.F., Vainchen, W. and Doly, J. 1989. Regulation of the expression of IL-6 in human monocytes. **J. Immunol., 142:4339-4345.**

Neame, P.J., Young, C.N., Brock, C.W., Treep, J.T., Garvey, T.M., Sasse, J. and Rosenberg, L.C. 1993. Pleiotrophin is an abundant protein in dissociative extracts of bovine foetal epiphysical cartilage and nasal newborns. **J. Orthopaed. Res., 11:479-491.**

Neote, K., Darbonne, W., Ogez, J., Horuk, R. and Schaii, T.J. 1993. Identification of a promiscuous inflammatory peptide receptor on the surface of red blood cells. **J. Biol. Chem., 268:2247-2249.**

Nettesheim, P., Fitzgerald, D.J., Kitamura, H., Walker, C.L., Gilmer, T.M., Barrett, J.C. and Gray, T.E. 1987. *In vitro* analysis of multistage carcinogenesis. **Environ. Health Perspect., 75:71079.**

Neufeld, G., Gospodarowicz, D., Dodge, L. and Fujii, D.K. 1987. Heparin modulation of neurotrophic effects of acidic and basic fibroblast growth factors and nerve growth factor on PC12 cells. **J. Cell. Physiol., 131:131-140.**

New, B.A. and Yeoman, L. 1992. Identification of basic fibroblast growth factor sensitivity and receptor and ligand expression in human colon tumor cell lines. **J. Cell Physiol., 150:320-326.**

Nguyen, M., Watanabe, H., Budson, A., Richie, J.P., Hayes, D.F. and Folkman, J. 1992. Basic fibroblast growth factor is elevated in urine of patients with a wide variety of neoplasms. **Mol. Biol. Cell, (Suppl.) 3:234a.**

Nicola, N.A. & Metcalf, D. 1991. Subunit promiscuity among hemopoietic growth factor receptors. **Cell, 67:1-4.**

Nishizuka, Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. **Nature, 334:661-665.**

Nissley, P. and Lopaczynski, W. 1991. Insulin-like growth factor receptors. **Growth Factors, 5:29-43.**

Nistér, M., Libermann, T.A., Betsholtz, C., Pettersson, M., Claesson-Welch, L., Heldin, C.-H., schlessinger, J. and Westermark, B. 1988. Expression of mRNAs for platelet-derived growth factor and transforming growth factor-alpha and their receptors in human malignant glioma cell lines. **Cancer Res, 48:3910-3918.**

Nonomura, N., Nakamura, M., Sudo, K., Sato, B. and Matsumoto, K. 1989. Proliferation of shionogi carcinoma cells caused by androgen-induced fibroblast growth factor (FGF)-like peptide in an autocrine mechanism. In: **Serino, M. (eds). Perspectives in Andrology. Raven Press, New York, pp 431-438.**

Nonomura, N., Nakamura, N., Uchida, N., Noguchi, S., Sato, B., Sonoda, T. and Matsumoto, K. 1988. Growth-stimulating effect of androgen-induced autocrine growth factor(s) secreted from Shionogi carcinoma 115 cells on androgen-unresponsive cancer cells in a paracrine mechanism. **Cancer Res., 48:4904-4908.**

Novick, D., Engelmann, H., Wallach, D. and Rubinstein, M. 1989. Soluble cytokine receptors are present in normal human urine. **J. Exp. Med., 170:1409-1414.**

O'Brien, W., Stenman, G. and Sager, R. 1986. Suppression of tumour growth by senescence in virally transformed human fibroblasts. **Proc. Natl. Acad. Sci. USA., 83:8659-8663.**

Occleston, N.L. and Walker, C. 1993. Production of multiple growth factors by a human non-small cell lung carcinoma cell line. **Cancer Lett., 71:203-210.**

Ohmura, E., Okada, M., Onoda, N., Kamiya, Y., Murakami, H., Tsushima, T. and Shizume, K. 1990. Insulin-like growth factor I and transforming growth factor α as autocrine growth factors in human pancreatic cancer cell growth. **Cancer Res., 50:103-107.**

Okumura, N., Takimiti, K., Okada, M. and Nakagawa, H. 1989. C6 glioma cells produce basic fibroblast growth factor that can stimulate their own proliferation. **J. Biochem., 106:904-909.**

O'Leary, R., Gregory, B., Carey, B., McGlinchey, E., O'Toole, A., Clynes, M. and O'Connor, B. 1991. Factors influencing protease levels in animal cells *in vitro*. **Biochem. Soc. Trans., 19:245-246.**

Oppenheim, J.J., Zacharia, C.O., Mukaida, N. and Matsushi, K. 1991. Properties of the novel proinflammatory supergene intercrine cytokine family. **Annu. Rev. Immunol., 9:617-648.**

Oppenheim, J.J., Kovacs, E.J., Matsushi, K. and Duram, S.K. 1986. There is more than one interleukin-1. **Immunol. Today, 7:45-56.**

Ornitz, D.M., Yayon, A., Flanagan, J.G., Svahn, C.M., Levi, E. and Leder, P. 1992 Heparin is required for cell-free binding of basic fibroblast growth-factor to a soluble receptor and for mitogenesis in whole cells. **Mol. Cell. Biol., 12:240-247.**

Oshima, A., Nolan, C.M., Kyle, J.W., Grubb, J.H. and Sly, W.S. 1988. The human cation-independent mannose 6-phosphate receptor. **J. Biol. Chem., 263:2553-2562.**

Ozanne, B., Richards, C.S., Hendler, F., Burns, D. and Gusterson, B. 1986. Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. **J. Pathol., 149:9-14.**

Padgett, R.W., St Johnston, R.D. and Gelbart, W.M. 1987. A transcript from a *Drosophila* pattern gene predicts a protein homologus to the transforming growth factor-β family. **Nature, 325:81-84.**

Pandiella, A. and Massagué, J. 1991a. Clevage of the membrane precursor for TGFα is a regulated process. **Proc. Natl. Acad. Sci. USA, 88:1726-1730.**

Pandiella, A. and Massagué, J. 1991b. Multiple signals activate cleavage of the membrane transforming growth factor α precursor. **J. Biol. Chem., 266:5769-5733.**

Pai, S.B., Steele, V.E. and Nettesheim, P. 1983. Neoplastic transformation of primary tracheal epithelial cell cultures. **Carcinogenesis, 4:369-374.**

Panos, R.J., Ribin, J.S., Aaronson, S.A. and Mason, R.J. 1993. Keratinocyte growth factor and hepatocyte growth factor/scatter factor are heparin-binding growth factors for alveolar type II cells in fibroblast-conditioned medium. **J. Clin. Invest., 92:969-977.**

Partanen, J., Mäkelä, T., Eerola, E., Koehonen, J., Hirvonen, H., Claesson-Welsh, L. and Alitalo, K. 1991. FGFr-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. **EMBO. J., 10:1347-1354.**

Paterno, G.D., Gillespie, L.L., Dixon, M.S., Slack, J.M. and Heath, J.K. 1989. Mesoderm-inducing

properties of INT-2 and kFGF: Two oncogene-encoded growth factors related to FGF. **Development, 106:79-83.**

Periera-Smith, O. and Smith, J. 1988. Genetic analysis of indefinite division in human cells: Identification of four complementation groups. **Proc. Natl. Acad. Sci. USA, 85:6042-6046.**

Perin, J.-P., Bonnel, F., Maillet, P. and Jolles, P. 1988. Characterisation of an N-terminal sequence of human-platelet proteoglycan. **Biochem. J., 255:1007-1013.**

Perroteau, I., Salomon, D., DeBortali, M., Kidwell, W., Hazarika, P., Pardue, R., Dedman, J. and Tam, J. 1986. Immunological detection and quantification of alpha-transforming growth-factors in human breast carcinoma cells. **Breast Cancer, 7:201-210.**

Peterseim, U.M., Sarkar, S.N. and Kupper, T.S. 1993. Production of IL-3 by non-transformed primary neonatal murine keratinocytes: evidence for constitutive IL-3 gene expression in neonatal epidermis. **Cytokine, 5:240-249.**

Pettmann, B., Janet, T., Labourdette, G., Sensenbrenner, M., Manthorpe, M. and Varon, S. 1991. Biologically active basic fibroblast growth factor migrates at 27 kD in 'non-denaturing' SDS-Polyacrylamide Gel Electrophoresis. **Growth Factors, 5:209-220.**

Pfeffer, S. and Ullrich, A 1985. Epidermal growth factor. Is the precursor a receptor? **Nature, 313:184.**

Pfeifer, A.M.A., Lechner, J.F., Masui, T., Reddel, R.R., Mark, G.E. & Harris, C.C. 1989. Control of growth and squamous differentiation in normal human bronchial epithelial cells by chemical and biological modifiers and transferred genes. **Environ. Health Perspect., 80:209-220.**

Pittelkow, M.R., Lindquist, P.B., Abraham, R., Graves-Deal, R., Derynck, R. and Coffey, R.J. 1989. Induction of transforming growth factor-alpha expression in human keratinocytes by phorbol esters. **J. Biol. Chem., 264:5164-5171.**

Ploüet, J., Schilling, J. and Gospodarowicz, D. 1989. Characterisation of a newly identified endothelial cell mitogen produced by ATT-20 cells. **EMBO. J., 8:3807-3806.**

Plowman, G.D, Green, J.M., McDonald, V.L., Neubauer, M.G., Disteche, C.M., Todaro, G.J and Shoyab, M. 1990. The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. **Mol. Cell Biol., 10:1969-1981.**

Podolsky, D.K., Pleskow, D.K. and Jafari, H. 1988. Latent transformed growth-inhibiting factor in human malignant effusions. **Cancer Res., 48:418-424.**

Pollak, M. and Richard, M. 1990. Suramin blockade of insulin-like growth factor I-stimulated proliferation of human osteosarcoma cells. **J. Natl. Cancer Inst., 82:1349-1352.**

Prat, M., Narsimhan, R.P., Crepaldi, T., Nicotra, M.R., Natali, P.G. and Comoglio, P.M. 1991. The receptor encoded by the human c-*met* oncogene is expressed in hepatocytes, epithelial cells and solid tumors. **Int. J. Cancer., 49:323-328.**

Prats, H., Kaghad, M., Prats, A.C., Klagsbrun, M., Lelias, J.M., Liauzun, P., Chalon, P., Yauber, J.P., Amairic, F., Smith, J. and Caput, D. 1989. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. **Proc. Natl. Acad. Sci. USA, 86:1836-1840.**

Presta, M., Tiberio, L., Rusnati, M., Dell'Era, P. and Ragnotti, G. 1991. Basic fibroblast growth factor requires a long-lasting activation of protein kinase C to induce cell proliferation in transformed fetal bovine aortic endothelial cells. **Cell Reguln., 2:719-726.**

Pukac, L.A., Castellot, J.J., Wright, T.C., Caleb, B.L. and Karnovsky, M.J. 1990. Heparin inhibits c-*fos* and c-*myc* messenger RNA expression in vascular smooth muscle cells. **Cell Regul., 1:435-443.**

Pukac, L.A., Ottlinger, M.E. and Karnovsky, M.J. 1992. Heparin suppresses specific second messenger pathways for protooncogene expression in rat vascular smooth muscle cells. **Proc. Natl. Acad. Sci. USA, 267:3707-3711.**

Ralston, R. and Bishop, M. 1985. The product of the protooncogene c-*src* is modified during the cellular response to platelet-derived growth factor. **Proc. Natl. Acad. Sci. USA, 82:7845-7849.**

Rapraeger, A., Jalkanen, M. and Bernfield, M. 1986. Cell surface proteoglycan associates with the cytoskeleton ay the basolateral cell surface of mouse mammary epithelial cells. **J. Cell Biol., 103:2683-2696.**

Rapraeger, A.C., Krufka. A. and Olwin, B.B. 1991. Requirement of heparan-sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. **Science, 252:1705-1708.**

Rathjen, P.D., Toth, S., Willis, A., Heath, J.K. and Smith, A.G. 1990. Differentiaion inhibitory activity is produced in matrix-associated and diffusible forms that are generated by alternate promoter usage. **Cell, 62:1105-1114.**

Reeve, J.G., Payne, J.A. and Bleehen, N.M. 1990. Production of immunoreactive insulin-like growth factor-I (IGF-I) and IGF-I binding proteins by human lung tumors. **Br. J. Cancer, 61:727-731.**

Reilly, C.F., Fritze, L.M. and Rosenberg, R.D. 1988, Heparin-like molecules regulate the number of epidermal growth factor receptors on vascular smooth muscle cells. **J. Cell. Physiol., 136:23-32.**

Renko, M., Quarto, N., Morimoto, T. and Rifkin, D.B. 1990. Nuclear and cytoplasmic localisation of different basic fibroblast growth factor species. **J. Cell Physiol., 144:108-114.**

Richmond, A. and Thomas, H.G. 1986. Purification of melanoma growth stimulatory activity. **J. Cell. Physiol., 129:375-384.**

Rifkin, D.B. and Moscatelli, D. 1989. Recent developments in the cell biology of basic fibroblast growth factor. **J. Cell Biol., 109:1-6.**

Rizzino, A. 1984. Behaviour of transforming growth factors in serum-free medium, an improved assay for transforming growth factors. **In Vitro Cell. Dev. Biol., 20:815-822.**

Roberts, R., Gallagher, J., Spooncer, E., Allen, T.D., Bloomfield, F. and Dexter, T.M. 1988. Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. **Nature, 332:376-378.**

Roberts, A.B. and Sporn, M.B. 1993. Physiological actions and clinical applications of transforming growth factor beta (TGF-beta). **Growth Factors, 8:1-9.**

Rodan, S.B., Wesolowski, G. Thomas, K. and Rodan, G.A. 1987. Growth stimulation of rat calcaria osteoblastic cells by acidic fibroblast growth factor. **Endocrinology, 121:1917-1923.**

Rodeck, U., Becker, D. and Herlyn, M. 1991. Basic fibroblast growth factor in melanoma. **Cancer Cells, 3:308-311.**

Rollins, B.J. and Stiles, C.D. 1988. Regulation of c-*myc* and c-*fos* proto-oncogene expression by animal cell growth factors. **In Vitro Cell. Dev. Biol., 24:81-84.**

Rose, T.M. and Bruce. A.G. 1991. Oncostatin-M is a member of a cytokine family that includes

leukemia inhibitory factor, granulocyte colony-stimulating factor and interleukin-6. **Proc. Natl. Acad. Sci. USA,88:8641-8645.**

Rosen, E.M., Goldberg, I.D., Kacinski, B.M., Buckholz, T. and Vinter, D.W. 1989. Smooth muscle releases an epithelial cell scatter factor which binds to heparin. **In Vitro Cell. Dev. Biol., 25:163-173.**

Ross, R., Raines, E.W. and Bowen-Pope, D.F. 1986. The biology of platelet-derived growth factor. **Cell, 46:155-169.**

Rotwein, P. 1991. Structure, evolution, expression, and regulation of insulin-like growth factors. **Growth Factors, 5:3-18.**

Rotwein, P. and Hall, L.J. 1990. Evolution of insulin-like growth factor II, characterisation of mouse IGF II gene and identification of two pseudo exons. **DNA and Cell Biol., 9:725-735.**

Rozengurt, E. 1981. Measure of the proliferative effects of cytokines. In: **Cytokines. A Practical Approach, pp253-259.**

Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. and Sitia, R. 1992. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. **J. Biol. Chem., 267:24161-24164.**

Rubartelli, A., Cozzolino, F., Talio, M. and Sitia, R. 1990. A novel secretory pathway for interleukin-1-beta, a protein lacking a signal sequence. **EMBO. J., 9:1503-1510.**

Rubin, J.S., Chan, A.M.-L., Bottaro, D.P., Burgess, W.H., Taylor, W.G., Cech, A.C., Hirschfield, D.W., Wong, J., Miki, T., Finch, P.W. and Aaronson,S.A. 1991. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. **Proc. Natl. Acad. Sci. USA, 88:415-419.**

Rubin, J.S., Osada, H., Finch, P.W., Taylor, W.G., Rudikoff, S. and Aaronson, S.A. 1989. Purification and characterization of a newly identified growth factor specific for epithelial cells. **Proc. Natl. Acad. Sci. USA, 86:802-806.**

Rusch, V., Baselga, J., Cordon-Cardo, C., Orazem, J., Zaman, M., Hoda, S., McIntosh, J., Kurie, J. and Dmitrovsky, E. 1993. Differential expression of the epidermal growth factor receptor and its ligands in primary non-small cell lung cancers and adjacent benign lung. **Cancer Res., 53 (Suppl. 10):2379-2385.**

Rygaard, K., Klausedn, B., Nakamura, T. and Spargtho, M. 1993. Growth inhibition and change in morphology and mobility of SCLC cell-lines by hepatocyte growth factor scatter factor. **Int. J. Oncol., 2:991-996.**

Sager, R., *et al* 1991. In: **Chemotactic Cytokines. Westwick *et al.*, (eds). Plenum Press. New York, pp73.**

Safi, A., Sadmi, M., Martinet, N., Menard, O., Valliant, P., Gallati, H., Hosang, M. & Martinet, Y. 1992. Presence of elevated levels of platelet-derived growth factor (PDGF) in lung adenocarcinoma pleural effusions. **Chest, 102:204-207.**

Sakaguchi, K. 1992. Acidic fibroblast growth factor autocrine system as a mediator of calcium-regulated parathyroid cell growth. **J. Biol. Chem., 267:24554-24562.**

Sakaguchi, K., Yanagishita, M., Takeuchi, Y. and Aurbach, G.D. 1991. Identification of heparan-sulphate proteoglycan as a high affinity receptor for acidic fibroblast growth factor (aFGF) in a parathyroid cell line. **J. Biol. Chem., 266:7270-7278.**

Sakamoto, H., Mori, M., Taira, M., Yoshida, T., Matsukawa, S., Shimizu, K., Sekiguchi, M., Terada, M. and Sugimura, T. 1986. Transforming gene for from human stomach cancers and a noncancerous portion of stomach mucosa. **Proc. Natl. Acad. Sci. USA, 83:3997-4001.**

Saksela, O., Moscatelli, D., Sommer, A. and Rifkin, D.B. 1988. Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. **J. Cell Biol., 107:743-571.**

Saksela, O. and Rifkin, D.B. 1990. Release of basic fibroblast growth factor-heparan sulphate complexes from endothelial cells by plasminogen activator mediated proteolytic activity. **J. Cell Biol., 110:767-775.**

Saksela, D. and Rifkin, D.B. 1988. Cell associated plasminogen: regulation and physiological functions. **Annu. Rev. Cell. Biol., 4:93-126.**

Saunders, S., Jalkenen, M., O'Farrell, S. and Bernfield, M. 1989. Molecular cloning of syndecan, an integral membrane proteoglycan. **J. Cell Biol., 108:1547-1556.**

Savage, C.R., Hash, J.H. and Cohen, S. 1973. Epidermal growth factor location of disulphide bonds. **J. Biol. Chem., 248:7669-7672.**

Savage, C.R., Inagami, T. and Cohen, S. 1972. Primary structure of EGF. **J. Biol. Chem., 247:5928-5934.**

Savona, C., Chambaz, E.M. and Feige, J.J. 1991. Proteoglycan sulfates contribute to the binding of basic FGF to its high affinity receptors on bovine adrenocortical cells. **Growth Factors, 5:273-282.**

Scholfield, P.N. 1991. **Acta. Paediatr. Scand., 372:83.**

Schweigerer, L., Neufeld, G., Friedman, J., Abraham, J.A., Fiddes, J.C. and Gospodarowicz, D. 1987. Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. **Nature, 325:257-259.**

Segarini, P.R. 1990. Cell type specificity of TGF-beta binding. **Ann, N.Y. Acad. Sci., 593:73-90.**

Segarini, P.R. 1991. A system of transforming growth factor-beta receptors. **Am. J. Respir. Cell Mol. Biol., 4:395-396.**

Sehgal, P.B., May, L.T., Tamm, I. and Vilcek, J. 1987. Human β_2 interferon and B-cell differentiation factor BSF-2 are identical. **Science, 235:731-732.**

Seifert, R.A., Hart, C.E., Phillips, P.E., Forstrom, J.W., Ross, R., Murray, M.J. and Bowen-Pope, D.F. 1989. Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. **J. Biol. Chem., 264:8771-8778.**

Seitz, R., Rappe, N., Kraus, M., Immel, A., Wolf, M., Maasberg, M., Egbring, R., Pfab, R. and Havemann, K. 1993. Activation of coagulation and fibrinolysis in patients with lung cancer: relation to tumor stage and prognosis. **Blood Coagul. Fibrinolysis, 4:249-254.**

Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzi, C.A., Harvey, V.S. and Dvorak, H.F. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. **Science, 219:983-985.**

Seno, M., Sasada, R., Iwane, M., Sudo, K., Kurokawa, T., Ito, K. and Igarashi, K. 1988. Stabilising basic fibroblast growth factor using protein engineering. **Biochim. Biophys. Res. Commun., 151:701-708.**

Seo, M.K., Lynch, K.E. and Podolsky, D.K. 1988. Multiplicity of transforming growth factors in human malignant effusions. **Cancer Res., 48:1792-1797.**

Serve, H., Steinhauser, G., Oberberg, D., Flegel, W.A., Northoff, H. and Berdel, W.E. 1991. Studies on the interaction between interleukin-6 and human malignant nonhaematopoeitic cell lines. **Cancer Res., 51:3862-3866.**

Shaw, G.L., Gazdar, A.F., Phelps. R., Linnoila, R.I., Ihde, D.C., Johnson, B.E., Oie, H.K., Pass, H.I., Steinberg, S.M., Ghosh, B., Walsh, T.E., Nesbitt, J.C., Cotelingam, J.D., Minna, J.D. and Mulshine, J.L. 1993. Individualized chemotherapy for patients with non-small cell lung cancer determined by prospective identification of neuroendocrine markers and *in vitro* drug sensitivity testing. **Cancer Res., 53:5181-5187.**

Sherwin, R.P. and Richters, A. 1975. Human lung cancer in tissue culture. In: **J. Fogh (ed.), Human Tumor cells *in vitro*. Plenum Publishing Corp., New York, pp 267-297.**

Shima, N., Nagao, M., Ogaki, F., Tsuda, E., Murakami, A. and Higashio, K. 1991. Tumor cytotoxic factor/hepatocyte growth factor from human fibroblasts: cloning of its cDNA, purification and characterization of recombinant protein. **Biochem. Biophys. Res. Commun., 180:1151-1158.**

Shimizu, S., Hirano, T., Yoshioka, R., Sugai, S., Matsuda, T., Taga, T., Kishimoto, T. and Kondo, S. 1988. Interleukin-6 (B-Cell stimulatory factor 2)-dependent growth of Lennert's lymphoma-derived T-Cell line (KT-3). **Blood, 72:1826-1828.**

Shing, Y., Folkman, J., Murphy, M. and Klagsbrun, M. 1983. Purification by affinity chromatography on heparin-Sepharose of a growth factor that stimulates capillary endothelial cell proliferation. **J. Cell Biol., 97:395a.**

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murphy, J. and Klagsbrun, M. 1984. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. **Science, 223:1296-1299.**

Shipley, G.D., Keeble, W.W., Hendrickson, J.E., Coffey, R.J. Jr. and Pittelkow, M.E. 1989, Growth of normal human kerationcytes and fibroblasts in serum-free medium is stimulated by acidic and basic fibroblast growth factor. **J. Cell. Physiol., 138:511-518.**

Shoyab, M., McDonald, V.M., Bradley, J.G. and Todaro, G.J. 1988. Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7. **Proc. Natl. Acad. Sci. USA, 85:6528-6532.**

Shoyab, M., Plowman, G.D., McDonald, V.L., Bradley, J.G. and Todaro, G.J. 1989. Structure and function of human amphiregulin: a member of the epidermal growth factor family. **Science, 243:1074-1076.**

Shweiki, D., Itin, A., Neufeld, G., Gitaygor, H. and Keshet, E. 1993. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. **J. Clin. Invest., 91:2235-2243.**

Silletti, S. & Raz, A. 1993. Autocrine motility factor is a growth factor. **Biochem. Biophys. Res Commun., 194:446-457.**

Sims, J.E., March, C.J., Cosman, D., Widmer, M.B., MacDonal, H.R., McMahon, C.J., Grubin, C.E., Wignall, J.M., Jackson, J.L. and Call, S.M. 1988. c-DNA expression cloning of the IL-1 receptor. A member of the immunoglobulin superfamily. **Science, 241:585-589.**

Sinha, S., Watorek, W., Karr, S., Giles, J., Bode, W. and Travis, J. 1987. Primary structure of human neutrophil elastase. **Proc. Natl. Acad. Sci. USA, 84:2228-2232.**

Smits, A., Funa, K., Vassbotn, F.S., Beausang-Linder, M., af Ekenstam, F., Heldin, C-H., Westermark, B. and Nistér, M. 1992. Expression of platelet-derived growth factor and its receptors in proliferative disorders of fibroblastic origin. **Am. J. Pathol., 140:639-648.**

Soderdahl, G., Betsholtz, C., Johansson, A., Nilsson, K. and Bergh, J. 1988. Differential expression of platelet-derived growth factor and transforming growth factor genes in small- and non-small cell human lung carcinoma cell lines. **Int. J. Cancer, 41:636-641.**

Sommer, A., Brewer, M.T., Thompson, R.C., Moscatelli, D., Presta, M. and Rifkin, D.B. 1987. A form of basic fibroblast growth factor with an extended amino terminus. **Biochem. Biophys. Res. Commun., 144:543-550.**

Sommer, A. and Rifkin, D.B. 1989. Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. **J. Cell Physiol., 138:215-220.**

Soutter, A.D., Nguyen, M., Watanabe, H. and Folkman, J. 1993. Basic fibroblast growth factor secreted by an animal tumor is detectable in urine. **Cancer Res., 53:5297-5299.**

Spiegel, A.M. 1992. G proteins in cellular control. **Curr. Opin. Cell Biol., 4:203-211.**

Spier, E., Sasse, J., Shrivastav, S. and Casscells, W. 1991. Culture-induced increase in acidic and basic fibroblast growth factor activities and their association with the nuclei of vascular endothelial and smooth muscle cells. **J. Cell. Physiol., 147:362-373.**

Sporn, M.B. and Roberts, A.B. 1988. Peptide growth factors are multifunctional. **Nature, 332:217-219.**

Sporn, M.B. and Roberts, A.B. 1985. Autocrine growth factors and cancer. **Nature, 313:745-747.**

Sporn, M.B. & Todaro, G.J. 1980. Autocrine secretion and malignant transformation of cells. **N. Engl. J. Med., 303:878-80.**

Steele-Perkins, G., Turner, J., Edmond, C., Hari, T., Pierce, S.B., Stover, C., Rutter, W.J. and Roth, R.A. 1988. Expression and characterisation of a functional human insulin-like growth factor I receptor. **J. Biol. Chem., 263:11486-11492.**

Stiles. A.D. and Moats-Staats, B.M. 1989. Production and action of insulin-like growth factor-I/somatomedin-C in primary cultures of fetal lung fibroblasts. **Am. J. Respir. Cell Mol. Biol., 1:21-26.**

Stokes, C.L., Rupnick, M.A., Williams, S.K. and Lauffenburger, D.A. 1990. Chemotaxis of human microvessel endothelial cells in response to acidic fibroblast growth factor. **Lab. Invest., 63:657-668.**

Stoker, M. 1989. Effect of scatter factor on motility of epithelial cells. **J. Cell. Physiol., 139:565-569.**

Stromberg, K., Collins, T.J., Gordon, A.W., Jackson, C.L. and Johnson, G.R. 1992. Transforming growth factor-α acts as an autocrine growth factor in ovarian carcinoma cell lines. **Cancer Res., 52:341-347.**

Stroobant, P., Rice, A.P., Guillick, W.J., Cheng, D.J., Kerr, I.M. and Waterfield, M.D. 1985. Purification and characterization of vaccinia virus growth factor. **Cell, 42:383-393.**

Taga, T. and Kishimoto, T. 1992. Cytokine receptors and signal transduction. **FASEB J., 6:3387-3396.**

Takahashi, J.A., Fukumoto, M., Igarashi, K., Oda, Y., Kikuchi, H. and Hatanaka, M. 1992. Correlation of basic fibroblast growth factor expression levels with the degree of malignancy and vascularity in human gliomas. **J. Neurosurg., 76;792-798.**

Takahashi, T., Okuno, Y. and Ohsugi, Y. 1993. Growth characteristics of a human myeloma cell line transfected with IL-6 cDNA. **Rinsho Ketsueki., 34:423-426.**

Takizawa, H., Ohtoshi, T., Ohta, K., Yamashita, N., Hirohata, S., Hirai, K., Hiramatsu, K. and Ito, K. 1993. Growth inhibition of human lung cancer cell lines by interleukin 6 *in vitro*: a possible role in tumor growth via an autocrine mechanism. **Cancer Res., 53:4175-4181.**

Takizawa, H., Ohtoshi, T., Ohta, K., Hirohata, S., Yamaguchi, M., Suzuki, N., Ueda, T., Ishii, A., Shindoh, G., Oka, T., Hiramatsu, K. and Ito, K. 1992. Interleukin 6/B cell stimulatory factor-II is expressed and released by normal and transformed human bronchial epithelial cells. **Biochem. Biophys. Res. Commun., 187:596-602.**

Talarico, D. and Basilico, C. 1991. The K-fgf/hst oncogene induces transformation through an autocrine mechanism that requires extracellular stimulation of the mitogenic pathway. **Mol. Cell Biol., 11:1138-1145.**

Tanaka, A., Miyamoto, K., Minamino, N., Matsuo, H. and Matsumoto, K. 1992. Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. **Proc. Natl. Acad. Sci. USA, 89:8928-8932.**

Tanaka, Y., Adams, D.M. and Shaw, S. 1993. Proteoglycans on endothelial cells present adhesion inducing cytokines to leukocytes. **Immunol. Today, 14:111-114.**

Ten Dijke, P., Hansen, P., Iwata, K.K., Pieler, C. and Foulkes, J.G. 1988. Indetification of another member of the transforming growth factor type beta gene family. **Proc. Natl. Acad. Sci. USA, 85:4715-4719.**

Terman B.I., Dougherew, M., Carrion, M.E., Dimitrov, D., Armellin, D.C., Gospodarowicz, D. and Böhlen, P. 1992. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. **Biochem. Biophys. Res. Commun.,187:1579-1586.**

Terman, B.I., Carrion, M.E., Kovacs, E., Rasmusse, B.A., Eddy, R.L. and Shows, T.B. 1991. Identification of a new endothelial cell growth factor receptor tyrosine kinase. **Oncogene, 6:1677-1683.**

Terzaghi-Howe, M. 1989. Changes in response to, and production of transforming growth factor type β during neoplastic progression in cultured rat tracheal epithelial cells.

Texidó, J. and Massagué, J. 1988. Structural properties of a soluble bioactive precursor for transforming growth factor-α. **J. Biol. Chem., 263:3924-3929.**

Thacker, J., Webb, M.B.T. and Debenham, P.G. 1988. Fingerprinting cell lines: use of hypervariable DNA probes to characterize mammalian cell cultures. **Somatic Cell and Molecular Genetics, 14:519-525.**

Theillet, C., Adelaide, J., Louason, G., Nonnet-Dorion, F., Jacquemier, J., Adnane, J., Longy, M., Katsaros, D., Sismondi, P., Gaudray, P., *et al.* 1993. FGFR1 and PLAT genes and DNA amplification at 8p12 in breast and ovarian cancers. **Genes Chromosom. Cacner, 7:219-226.**

Thelliet, C., Le Roy, X., De Lapeyriere, O., Grosgeorges, J., Adnane, J., Raynaud, S.D., Simoy-Lafontaine, J., Goldfarb, M., Escot, C., Birnbaum, D. and Gaudray, P. 1989. Amplification of FGF-related genes in human tumors: Possible involvment of *HST* in breast carcinomas. **Oncogene, 4:915-922.**

Thomas, K.A., Gimenez-Gallego, G. 1986. Fibroblast growth factors: broad spectrum mitogens with potent angiogenic activity. **Trends. Biochem. Sci., 11:81-94.**

Thomas, K.A., Rios-Candelore, M. and Fitzpatrick, S. 1984. Purification and characterization of acidic

fibroblast growth factor from bovine brain. **Proc. Natl. Acad. Sci. USA, 81:357-361.**

Thomassen, D.G. 1993. Neoplastic progression of rat tracheal epithelial cells is associated with a reduction in the number of growth factors required for clonal proliferation in culture.

Thompson, S.A., Protter, A.A., Bitting, L., Fiddes, J.C. and Abraham, J.A. 1991. Cloning, recombinant expression and characterization of basic fibroblast growth factor. **Methods. Enzymol., 198:96-116.**

Thornton, S.C., Mueller, S.N. and Levine, E.M. 1983. Human endothelial cells: use of heparin in cloning and long term serial cultivation. **Science, 222:623-625.**

Thornton, S.C., Robbins, J.M., Penny, R. and Breit, S.N. 1992. Fibroblast growth factors in connective tissue disease associated interstitial lung disease. **Clin. Exp. Immunol., 90:447-452.**

Tiesman, J., Meyer, A., Hines, R.N. and Rizzino, A. 1988. Production of growth factors related to fibroblast growth factor and platelet-derived growth factor by human embryonal carcinoma cells. **In Vitro Cell Dev. Biol., 24:1209-1216.**

Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J.C. and Abraham, J.A. 1991. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. **J. Biol. Chem., 266:1947-1954.**

Todaro, G.J. and deLarco, J.E. 1978. Growth factors produced by sarcoma virus-transformed cells. **Cancer Res., 38:4147-4154.**

Toi, M., Bicknell, R. and Harris, A.L. 1992. Inhibition of colon and breast carcinoma cell growth by IL-4. **Cancer Res., 52:4147-4154.**

Topp, M.S., Koenigsmann, M., Mire-Sluis, A., Oberberg, D., Eitelbach, F., von Marschall, Z., Notter, M., Reufi, B., Stein, H., Thiel, E., *et al.* 1993. Recombinant human interleukin-4 inhibits growth of some human lung tumor cell lines *in vitro* and *in vivo*. **Blood, 82:2837-2844.**

Tricoli, J.V., Rall, L.B., Scott, J., Bell, G.I. and Shows, T.B. 1984. Localisation of insulin-like growth factor genes to human chromosomes 11 and 12. **Nature, 310:784-786.**

Troen, B.R., Ascherman, D., Atlas, D. and Gottesman, M.M. 1988. Cloning and expression of the gene for the major excreted protein of transformed mouse fibroblasts. A secreted lysosomal protease regulated by transformation. **J. Biol. Chem., 263:254-261.**

Tryggvason, K., Hoyhtya, M. and Salo, T. 1987. Proteolytic degradation of extracellular-matirx in tumour invasion. **Biochem. Biophys. Acta., 907:191-217.**

Tsao, M.S., Zhu, H., Giaid, A., Viallet, J., Nakamura, T. and Park, M. 1993. Hepatocyte growth factor/scatter factor is an autocrine factor for human normal bronchial epithelial and lung carcinoma cells. **Cell Growth Differ., 4:571-579.**

Tsuda, T., Nakatani, H., Matsumura, T., Yoshida, K., Tahara, E., Nishihira, T., Sakamoto, H., Yoshida, T., Terada, M. and Sugimura, T. 1988. Amplification of the *hst*-1 gene in human esophageal carcinomas. **Jpn. J. Cancer Res., 79:584-588.**

Tucker, R.F., Branum, E.L., Shipley, G.D., Ryan, R.J. and Moses, H.L. 1984. Specific binding to cultured cells of I^{125} labelled type beta-transforming growth factor from human platelets. **Proc. Natl. Acad. Sci. USA, 81:6757-6761.**

Tungekar, M.F., Turley, H., Dunnill, M.S., Gatter, K.C., Ritter, M.A. and Harris, A.L. 1991.

Interleukin 4 receptor expression on human lung tumors and normal lung. **Cancer Res., 51:261-264.**

Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Liebermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D. and Seeburg, P.H. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. **Nature, 309:418-425.**

Ullrich, A. and Schlessinger, J. 1990. Signal transduction by receptors with tyrosine kinase activity. **Cell, 61:203-212.**

Ueno, N., Baird, A., Esch, F., Ling, N. and Guillemin, R. 1986. Isolation of an amino-terminal extended form of basic fibroblast growth factor. **BBRC, 138:580-588.**

Unsicker, K. and Westermann, R. 1992. Basic fibroblast growth factor promotes transmitter storage and synthesis in cultured chromaffin cells. **Brain Res. Dev Brain Res., 65:211-216.**

Upton, C. Macen, J.L. and MacFadden, G. 1987. Mapping and sequencing of a gene from myxoma virus that is related to those encoding epidermal growth factor and transforming growth factor alpha. **J. Virol., 61:1271-1275.**

Urdal, D.L., Call, S.M., Jackson, D.L. and Dower, S.K. 1988. Affinity purification and chemical analysis of the interleukin 1 receptor. **J. Biol. Chem., 263:2870-2877.**

Vainikka, S., Partanen, J., Bellosta, P., Coulier, F., Basilico, C., Jaye, M. and Alitalo, K. 1992. Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction. **EMBO. J., 11:4273-4280.**

Varadi, D.P., Cifonelli, J.A. and Dorfman, A. 1967. The acid mucopolysaccharides in normal urine. **Biochim. Biophys. Acta., 141:103-117.**

Vassbotn, F.S., Östman, A., Langeland, N., Holmsen, H., Westermark, B., Heldin, C.-H. and Nistér, M. 1994. Activated platelet-derived growth factor autocrine pathway drives the transformed phenotype of a human glioblastoma cell line. **J. Cell. Physiol., 158:381-389.**

Veale, D., Kerr, N., Gibson, G.J. and Harris, A.L. 1989. Characterization of epidermal growth factor receptor in primary human non-small cell lung cancer. **Cancer Res., 49:1313-1317.**

Venkatesan, S., Gershowitz, A. and Moses, B. 1982. **J. Virol., 61:637-646.**

Versnel, M.A., Claesson-Welch, L., Hammacher, A., Bouts, M.J., van der Kwast, T.H., Eriksson, A., Willelmsen, R., Weima, S.M., Hoogsteden, H.C., Hagemeijer, A. and Heldin, C-H. 1991. Human malignant mesothelioma cell lines express PDGF beta-receptors whereas cultured normal mesothelial cells express predominantly PDGF alpha-receptors. **Oncogene, 6:2005-2011.**

Vlasselaer, P.V., *et al.*, 1992. **Prog. Growth Factor Res., 4:337.**

Vlodavsky, I., Bar-Shivat, R., Ishai-Michaeli, R., Bashkin, P. and Fuks, Z. 1991. Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism. **TIBS, 16:268-271.**

Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J. and Klagsbrun, M. 1987. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. **Proc. Natl. Acad. Sci. USA, 84:2292-2296.**

Wakefield, L.M., Smith, D.M., Flanders, K.C. and Sporn, M.B. 1988. Latent transforming growth factor-β from human platelets. **J. Biol. Chem., 263:7646-7654.**

Watanabe, H., Carmi, P., Hogan, V., Raz, T., Silletti, S., Nabi, I.R. and Raz, A. 1991. Purification of human tumor cell autocrine motility factor and molecular cloning of its receptor. **J. Biol Chem., 266:13442-13448.**

Watanabe, H., Chigira, M. and Raz, A. 1993. The role of autocrine motility factor receptor in metastasis. **Gan To Kagaku Ryoho, 20:405-409.**

Watanabe, H., Nguyen, M., Schizer, M., Li, V.A., Hayes, D.F., Sallan, S. and Folkman, J. 1992. Basic fibroblast growth factor in human serum — a prognostic test for breast cancer. **Mol. Biol. Cell, (Suppl.) 3:234a.**

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroban, P., Johnsson, A., Wasteson, A., Westerman, B., Heldin, C.-H., Huang, J.S. and Deuel, T.F. 1983. Platelet-derived growth factor is structurally related to the putative transforming protein — p28SIS of simian sarcoma virus. **Nature, 304:35-39.**

Watson, J.M., Sensintaffar, J.L., Berek, J.S. and Martinez-Maza, O. 1990. Constitutive production of interleukin 6 by ovarian cancer cell lines and primary ovarian tumor cultures. **Cancer Res., 50:6959-6965.**

Watson, J.M., Berger, J.S. and Martinez-Maza, O. 1993. Growth inhibition of ovarian cancer cells induced by antisense IL-6 oligonucleotides. **Gynecol. Oncol., 49:8-15.**

Weber, W., Gill, G. and Spiess, J. 1984. Production of an epidermal growth factor receptor-related protein. **Science, 224:294-298.**

Weeks, D.C. and Melton, D.A. 1987. A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF-β. **Cell, 51:861-867.**

Weich, H.A., Iberg, N., Klagsbrun, M. and Folkman, J. 1990. Expression of acidic and basic fibroblast growth factors in human and bovine vascular smooth muscle cells. **Growth Factors, 2:313-320.**

Wellstein, A., Fang, W., Khatri, A., Lu, Y., Swain, S.S., Dickson, R.B., Sasse, J., Riegel, A.T. and Lipman, M.E. 1992. A heparin-binding growth factor secreted from breast cancer cells homologous to a developmentally regulated cytokine. **J. Biol. Chem., 267:2582-2587.**

Werner, S., Peters, K.G., Longaker, M.T., Fuller-Pace, F., Banda, M.J. and Williams, L.T. 1992. Large induction of keratinocyte growth factor expression in the dermis during wound healing. **Proc. Natl. Acad. Sci. USA, 89:6896-6900.**

Werner, S., Kurt Roth, W., Bates, B., Goldfarb, M. and Hofschneider, P.H. 1991. Fibroblast growth factor 5 proto-oncogene is expressed in normal human fibroblasts and induced by serum growth factors. **Oncogene, 6:2137-2144.**

Westendorf, J.J., Ahmann, G.J., Armitage, R.J., Spriggs, M.K., Lust, J.A., Greipp, P.R., Katzmann, D.F. and Jelinek, D.F. 1994. CD40 expression in malignant plasma cells — role in stimulation of autocrine IL-6 secretion by a human myeloma cell line. **J. Immunol., 152:117-128.**

Westegren-Thorsson, G., Persson, S., Isaksson, A., Onnervik, P.O., Malmstrom, A. and Fransson, L.A. 1993. L-iduronate-rich glycosaminoglycans inhibit growth of normal fibroblasts independently of serum or added growth factors. **Exp. Cell Res., 206:93-99.**

Westermann, R., Johannsen, M., Unsicker, K. and Grothe, C. 1990. Basic fibroblast growth factor (bFGF) immunoreactivity is present in chromaffin granules. **J. Neurochem., 55:285-292.**

Wilbur, D.W., Camacho, E.S., Hilliard, D.A., Dill, P.L. & Weisenthal, L.M. 1992. Chemotherapy of non-small cell lung carcinoma guided by an *in vitro* drug resistance assay measuring total tumour cell kill. **Br. J. Cancer, 65:27-32.**

Wilson, A.P., Fox, H., Scott, I.V., Lee, H., Dent, M. and Golding, P.R. 1991. A comparison of the growth promoting properties of ascitic fluids, cyst fluids and peritoneal fluids from patients with ovarian tumours. **Br. J. Cancer, 63:102-108.**

Wong, S.T., Winchell, L.F., McCune, B.K., Earp, H.S., Texidó, J., Massagué, J., Herman, B. and Lee, D.C. 1989. The TGFα precursor expressed on the cell surface binds to the EGF receptor on adjacent cells, leading to signal transduction. **Cell, 56:495-506.**

Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Herwick, R.M. and Wang, E.A. 1988. Novel regulators of bone formation: molecular clones and activities. **Science, 242:1528-1534.**

Wright, T.C. Jr., Castellot, J.J. Jr., Petitou, M., Lormeau, J.C., Choay, J. and Karnovsky, M.J. 1989. Structural determinants of heparins growth inhibitory activity. **J. Biol. Chem., 264:1534-1542.**

Wu, D., Kan, M., Sato, G.H., Okamoto, T. and Sato, J.D. 1991. Characterization and molecular cloning of a putative binding protein for heparin-binding growth factors. **J. Biol Chem., 266:16778-16765.**

Yamada, Y. and Serrero, G. 1988. Autocrine growth induced by the insulin-related factor in the insulin-independent teratoma cell line 1246-3A. **Proc. Natl. Acad. Sci. USA, 85:5936-5940.**

Yamaguchi, T., Kawamoto, K., Uchida, N., Uchida, K. and Watanabe, S. 1992. Three cell lines showing androgen-dependent, -independent, and suppressed phenotypes, established from a single tumor of androgen-dependent shionogi carcinoma 116. **In Vitro Cell. Dev. Biol., 245-254.**

Yamasaki, K., Taga, T., Hirata, Y., Yawata, H., Kawanish, Y., Seed, B., Taniguch, T., Mirano, T. and Kishimoto, T. 1988. Cloning and expression of the human interleukin-6 (BSF-2/IFN beta-2) receptor. **Science, 241:825-828.**

Yan, G., Fukabori, Y., McBride, G., Nikolaropolous, S. and McKeehan, W.L. 1993. Exon switching and activation of stromal and embryonic fibroblast growth factor (FGF)-FGF receptor genes in prostate epithelial cells accompany stromal independence and malignancy. **Mol. Cell Biol., 13:4513-4522.**

Yarden, Y., Escobedo, J.A., Kuang, W-J., Yang-Feng, T.L., O'Daniel, T.O., Tremble, P.M., Chen, E.Y., Ando, M.E., Harkins, R.N., Francke, U., Fried, V.A., Ullrich, A. and Williams, L.T. 1986. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. **Nature, 323:226-232.**

Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P. and Ornitz, D.M. 1991. Cell surface heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. **Cell, 64:841-848.**

Yeaman, C. and Rapraeger, A.C. 1993. Membrane-anchored proteoglycans of mouse macrophages: P388D1 cells express a syndecan-4-like heparan sulfate proteoglycan and a distinct chondroitin sulfate form. **J. Cell Physiol., 157:413-425.**

Yee, C., Biondi, A., Iscove, N.N., deSousa, G.G., Aarden, l.A., Wong, G.G., Clark, S.C., Messener, H.A. and Minden, M.D. 1989. A possible autocrine role for interleukin-6 in two lymphoma cell lines. **Blood, 74:798-804.**

Yee, D., Morales, F.R., Hamilton, T.C. and Von Hoff, D. 1991. Expression of insulin-like growth factor I, its binding proteins, and its receptor in ovarian cancer. **Cancer Res, 51:5107-5112.**

Yen, Y.P., Cox, T.C. and Goodman, G.E. 1986. Malignant effusion stimulates the cloning of fresh human tumours in soft agar. **Proc. Am. Assoc. Cancer Res., 27:32.**

Yin, T.G, Taga, T., Tsang, M.L.S., Yasukawa, K., Kishimoto, T. and Yang, Y.C. 1993. Involvement of IL-6 signal transducer GP-130 in IL-11 mediated signal transduction. **J. Immunol., 151:2555-2561.**

Yoshida, T., Miyagawa, K., Odagiri, H., Sakamoto, H., Little, P.F.R., Terada, M. and Sugimura, T. 1987. Genomic sequence of *hst*, a transforming gene encoding a protein homologus to fibroblast growth factors and the *int*-2-encoded protein. **Proc. Natl. Acad. Sci. USA, 84:7305-7309.**

Yoshinga, Y., Fujita, S., Gotoh, M., Nakamura, T., Kikuchi, M. and Hirohashi, S. 1992. Human lung cancer cell line producing hepatocyte growth factor/scatter factor. **Jpn. J. Cancer Res., 83:1257-1261.**

Yoshitake, Y. and Nishikawa, K. 1991. Distribution of fibroblast growth factors in cultured tumor cells and their transplants. **In Vitro Cell. Dev. Biol., 28A:419-428.**

Zapf, J., Waldvoge, M. and Froech, E.R. 1975. Binding of non-suppressible insulin-like activity to human serum — evidence for a carrier protein. **Arch. Biochem. Biophys., 168:638-645.**

Zarling, J.M., Shoyab, M., Marquardt, H., Hanson, M.B., Lioubin, M.N. and Todaro, G.J. 1986. Oncostatin M: a growth regulator produced by differentiated histiocytic lymphoma cells. **Proc. Natl. Acad. Sci. USA, 83:9739-9743.**

Zhan, X., Bates, B, Hu, X. and Goldfarb, M. 1988. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. **Mol. Cell. Biol., 8:3487-3495.**

Zhou, D.J., Casey, G. and Cline, M.J. 1988. Amplification of human *int*-2 in breast cancers and squamous carcinomas. **Oncogene, 2:279-282.**

Zilberstein, A., Ruggieri, R., Korn, J.M. and Revel, M. 1986. Structure and expression of cDNA and genes for human interferon beta-2, a distinct species inducible by growth stimulatory cytokines. **EMBO. J., 5:2529-5237.**

Zimmer, Y., Givol, D. and Yayon, A. 1993. Multiple structural elements determine ligand binding of fibroblast growth factor receptors. Evidence that both Ig domain 2 and 3 define receptor specificity. **J. Biol. Chem., 268:7899-7903.**

8.0 APPENDICES

APPENDIX A

Calculation of settings to give a "g" value of 25,000 using the SM-24 rotor on the Sorval

The following equation is used to calculate the settings on the sorvall for a particular "g" value when the SM-24 rotor is used.

$$g = 0.0000284 \times r \times N^2$$

Where, r = radius of the rotor; N = revelutions/minute ($\times$ 1000 on the Sorval).

SM-24 rotor dimensions

r = 6.48 cm (inner row in rotor)
r = 8.99 cm (outer row in rotor)

The tubes for centrifugation were placed in the outer row of this rotor and the "g" value at this distance from the rotor shaft was calculated as;

$$\therefore \text{9895 rev/min} = \frac{25{,}000}{8.99 \times 0.0000284}$$

The centrifuge was therefore set at 10,000 rev/min to ahieve a "g" force of 25,000.

APPENDIX B

Sequences of Oligodeoxynucleotides Sense and Antisense to Acidic FGF mRNA

ANTISENSE aFGF: (ASA)

5′ TTC CCC TTC AGC CAT 3′

SENSE aFGF: (SA)

5′ ATG GCT GAA GGG GAA 3′

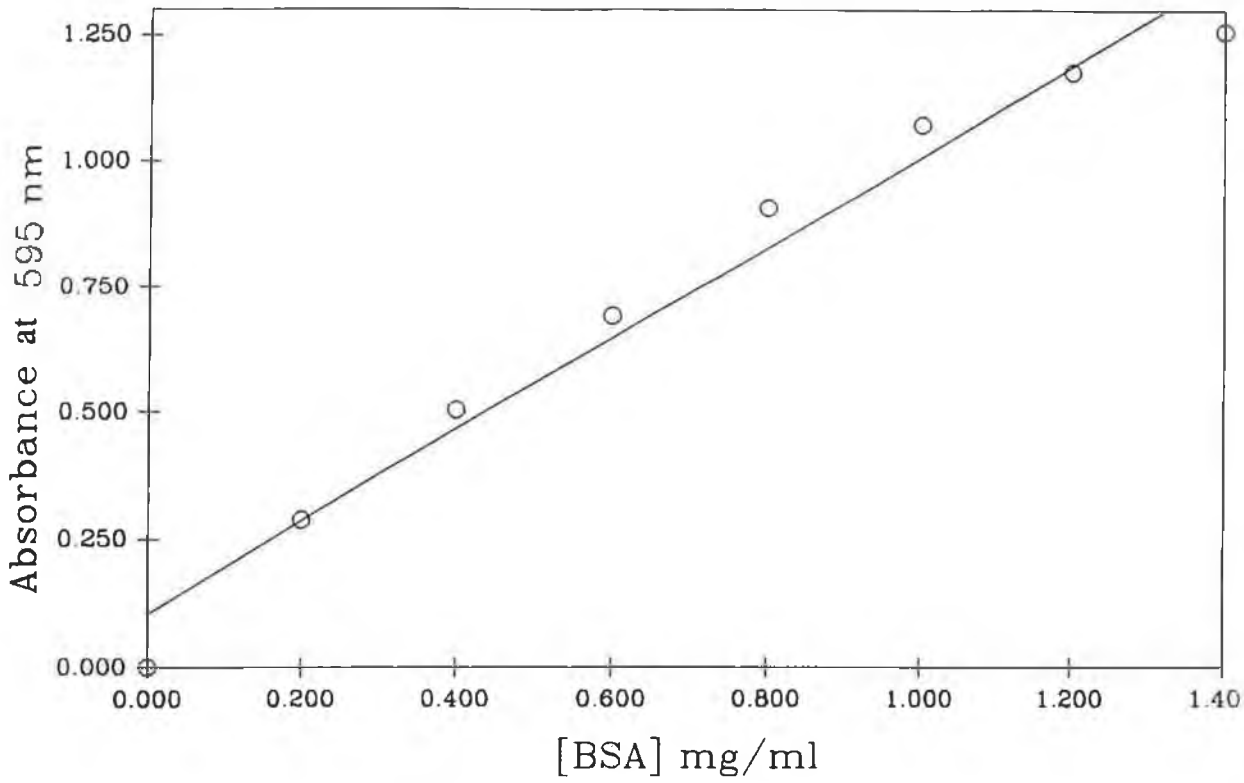
ANTISENSE bFGF: (ASB)

5′ GGC TGC CAT GGT CCC 3′

SENSE bFGF: (SB)

5′ GGG ACC ATG GCA GCC 3′

APPENDIX C



Standard curve for protein concentration versus absorbance @ 595 nm — determined using the BioRad protein assay.

APPENDIX D

The following table of serine proteinase activities is taken from O'Leary *et al.*, (1991).

Serine proteinase levels in HEp-2 CM grown in five different basal culture media

BASAL MEDIUM	Proteinase activity (μmol PNA[a]released/min/ml)			
	Trypsin	Chymotrypsin	Elastase	Cathepsin G
RPMI-2650	<0.10	<0.10	<0.10	<0.10
DMEM	0.81	0.77	0.75	0.85
Ham's F12	<0.10	<0.10	<0.10	<0.10
MEM	1.42	1.34	0.98	1.36
DMEM/Ham's F12[b]	0.53	0.43	0.40	0.56

[a] The substrate used was p-nitroanalide. [b] A 1:1(v/v) DMEM/Ham's F12 mixture.