"Growth factor production by the human carcinoma cell line RPMI-2650"

by

Margaret Dooley

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The experimental work described here was carried out under the supervision of Dr Martin Clynes

School of Biological Sciences,
National Institute for Higher Education,
Glasnevin,
Dublin 9

ABSTRACT

Proliferation of a human carcinoma cell line (RPMI-2650) was found to be density dependent in culture In a double-layer agar clonal assay, no growth was observed below a critical "cut-off" density unless autologous feeder cells were present Medium conditioned by RPMI-2650 cells could substitute for the physical presence of feeder cells, thus opening the way to biochemical analysis of the nature of the feeder and density dependence effects RPMI-2650 conditioned medium (CM) was shown, using specific cell culture bioassays, to contain TGF-α (transforming growth factor-α)-like, TGF-β-like and autostimulatory activities, as well as EGF (Epidermal growth factor) receptor binding activity Following large-scale production of CM in roller and suspension culture, these activities were fractionated using ultrafiltration, gel filtration in acetic acid and HPLC HPLC separated TGF-α and TGF-β activities, but autostimulatory activity was lost RPMI-2650 cells had lower EGF receptor levels than 3 other human carcinoma cell lines examined (A431, T-47D, SCC-9) EGF receptor levels in some of these lines were shown to be regulated by steroid hormones The results presented here indicate that the autostimulatory activity may consist of at least two components, one of high molecular weight (> 30 kDa) and another of low molecular weight (< 1 kDa), and may be distinct from previously-described TGFs

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INTRODUCTION

1. INTRODUCTION

1.1 Growth Factors

Growth factors, defined as polypeptides that stimulate cell proliferations, are major growth regulatory molecules for cells in culture and probably also for cells *in vivo*. They stimulate cell proliferation by binding to specific high-affinity cell membrane receptors (Rosengurt, 1986). There is some recent evidence for specific nuclear binding sites, but this is controversial (Burwen and Jones, 1987). Growth factors have been purified from a variety of sources including platelets and tissues of adult and embryonic origin. Some growth factors are thought to be released by most cells in culture (Shields, R., 1978). Non-transformed cells show an absolute requirement for growth factors for proliferation in culture and generally more than one growth factor is required (Pledger and Stiles, 1979). Under usual culture conditions, growth factors are more readily depleted than other media components and this becomes rate limiting for proliferation (Barnes and Sato, 1980); (Tsao *et al.*, 1982). A loss of, or a decreased requirement for, specific growth factors is a common feature of neoplastically transformed cells and may lead to a selective growth advantage which might be expected to be a cardinal feature of cancer cells (Temin, 1966; Paul *et al.*, 1971). Membrane receptors for growth factors are ubiquitous with most cells having receptors for more than one growth factor (Wrann *et al.*, 1980; Bowen-Pope *et al.*, 1983).

Recent work with transforming growth factors, platelet derived growth factor and oncogenes, has produced some insight into the mechanisms through which alterations in growth factor-receptor-response pathways could lead to a growth advantage, as found in neoplastically transformed cells. The use of defined growth factors and serum-free methods have been of great use here (Barnes and Sato, 1980). Many oncogene products are similar to growth factor receptors in that they have transmembrane and tyrosine kinase domains (Hunter, 1984). See Section 1.11. Other aspects of the post receptor-growth factor stimulated pathway are also mimiced and lead to growth stimulation (Kelly *et al.*, 1983).

Study of the molecular mechanism(s) of growth factor action has just begun The excitement and attention focused on cellular oncogenes in recent years is now turning toward growth factors, not only as they concern the control of normal cell growth but also the involvement of growth factor-initiated pathways in the etiology of cancer

1 2 1 Epidermal growth factor

Epidermal growth factor was first described by Cohen, 1962, as a peptide which would stimulate precocious eyelid opening and tooth eruption in newborn mice and it was purified on this basis, its ability to stimulate the growth of cultured cells was recognised later (Carpenter and Cohen, 1975, Hollenberg and Cautrecasas, 1973) The active molecule was termed epidermal growth factor due to its capacity to induce cell proliferation in the basal cells of the skin (Cohen, 1962) First purified from male mouse submaxillary glands (Cohen, 1962) and later from human urine as urogastrone (Gregory, 1975, Cohen and Carpenter, 1975), mature EGF is a 6 kDa single polypeptide chain of 53 amino acids displaying 3 internal disulfide bonds (Taylor et al , 1972) (Fig 1 shows the amino acid sequences of growth factors related to EGF)

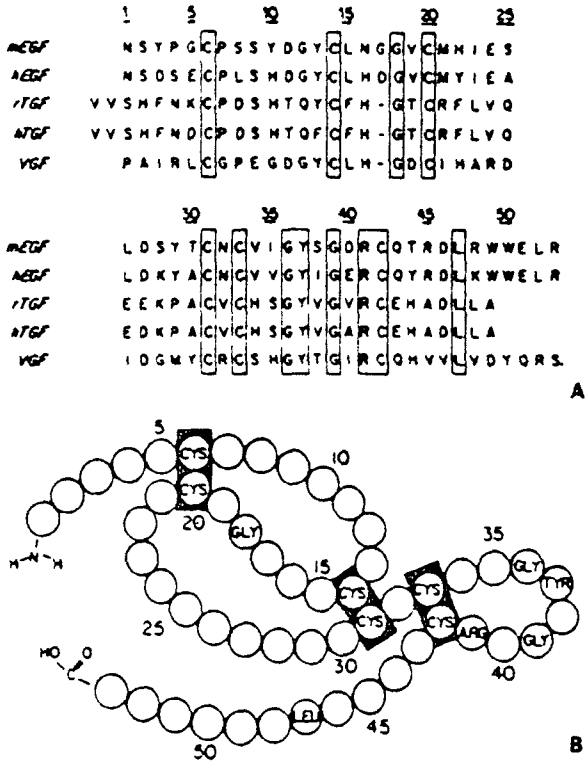


Fig 1 (*A*) Amino acid sequences of growth factors related to EGF The sequences of the individual polypeptides are taken from references cited in the text and are shown in comparison with the sequence of mouse EGF (*B*) Representation of strictly conserved amino acid residues in the EGF family of growth factors and the positioning of disulfide bonds as derived from data on the mouse-derived EGF [6]

EGF and TGF α, a related polypeptide (Derynck et al , 1984), are produced as large precursors In the case of EGF, the protein precursor is approximately 130 kDa in size and contains seven EGF-like sequences in addition to that for authentic EGF (Gray et al , 1983) The available data suggests that EGF (Zabel et al , 1985) and TGF α (Brissenden et al , 1985) are products of unlinked genes that are independently regulated EGF is found in almost all body fluids under normal physiological conditions, but its site of synthesis is uncertain in most species No organ in any species examined contains levels of EGF approaching that found in the mouse submaxillary gland

Examination of a group of EGF-like growth factors like plasminogen activator and fibronectin (Banayi et al , 1983, Doolittle et al , 1984) show that strict sequence conservation in this group is not evident, with the marked exception of the positioning of the cystine residues The precursors of each of these small EGF-like growth factors have a common but unexpected terminus, a hydrophobic sequence suggestive of the 'stop transfer' or membrane anchoring sequence of membrane proteins Based on the occurence of this sequence in the precursor molecules, it has been proposed that the growth factor precursors may exist as integral membrane proteins on the surface of cells It is thought that proteolytic activity at the cell surface may give rise to free growth factor, or that growth factors may interact with the membrane receptors while in the precursor form Rall et al , 1985, has further proposed that in the case of the large EGF precursor, which is produced but not degraded to lower molecular weight products in the proximal tubule cells of the kidney, might also function as a receptor for an unidentified ligand

Radioimmune (Dailey et al , 1978) and radioreceptor (Carpenter et al , 1975) assays have been developed for measuring EGF concentration in extracts the latter assay detects TGF α as equivalent to native EGF Not only do EGF and TGF α recognise the same cellular receptor, they are apparently equally effective on a mole-for-mole basis in most systems TGF α is produced by a variety of transformed cells (Roberts et al , 1983) and is found in acid extracts of tumours (Roberts et al , 1980) and in foetal samples (Proper et al , 1982) It may be the case that EGF is the adult form of the embryonic growth factor TGF α

EGF is mitogenic for a variety of cultured mesenchymal and epithelial cells, its mitogenic activity is strongly potentiated by insulin (Rose et al , 1975, Shipley et al , 1984) EGF also acts in synergism with PDGF on BALB/c - 3T3 cells (Leof et al , 1983) Aspects of differentiation are also induced following EGF treatment in certain cell culture models and in vivo (Oka et al , 1983, Weissman et al , 1983)

Elder et al , 1978, showed, using a fluorescent double-antibody technique, that hEGF was found in the duct cells of the human submandibular salivary glands and in cells of Brunner's glands of the duodenum, but not in many other cell types tested A recent observation by Sato et al , 1985, has found an adenocarcinoma of the human salivary gland which expresses hEGF and TGF β

1 2 2 EGF Receptor

A plasma membrane receptor for EGF was identified in the mid 1970s Early reports showed that cultured cells responded to the mitogenic signal provided by EGF and that radiolabelled EGF bound in a specific, saturable manner and with high affinity for the surface of these responsive cells (Carpenter et al , 1979) The only cells in which EGF receptors have not been demonstrated either in vivo or in vitro, are those of the hemopoietic system

A key element in elucidating the biochemistry of the EGF receptor was the discovery in 1977 that a cell line, designated A-431, over-expresses the EGF receptor (Fabricant et al , 1977) Most cell types express 10^4-10^5 receptor molecules/cell (Todaro et al , 1976), while A-431 cells express 2 5 x 10^6/cell (Haigler et al , 1978) This over-expression of the EGF receptor suggests that it may have a role in the malignant growth of squamous cell carcinomas as suggested by Cowley et al , 1986

EGF was demonstrated to activate a protein kinase in vitro in 1978 (Carpenter et al , 1978) and in 1980 the EGF receptor was found to co-purify with the growth factor-dependent kinase activity (Cohen et al , 1980) The protein kinase activity was subsequently identified as specific for tyrosine residues (Ushiro and Cohen, 1980) Several lines of biochemical and immunologic evidence suggested that the kinase activity was intrinsic to the receptor

molecule and not part of a separate effector system (Cohen et al , 1982, Cohen et al , 1980) The cloning and sequencing of the EGF receptor provided the final evidence that the receptor molecule did indeed possess an intrinsic enzymatic activity, one which might serve as an effector system (Gray et al , 1983)

The EGF receptor is an integral 170 kDa membrane protein exhibiting an extracellular binding domain that serves to bind the ligand, a transmembrane region, and an intracellular domain facing the cytoplasm, exhibiting the tyrosine kinase function and binding sites for ATP and the phosphorylation substrates (Cohen et al , 1982) In response to EGF, the receptor is capable of autophosphorylation on tyrosine residues (Ushiro and Cohen, 1980)

Receptor protein purified from A-431 cells was used for sequencing by Downward et al , 1984, who found that a high level of homology existed between the EGF receptor (170, kDa) and the v-erb B oncogene product (74 kDa) The external part of v-erb-B protein is too small to accommodate a ligand binding site, but the internal domains of both the EGF receptor and the v-erb-B protein contain highy homologous sequences and resemble the sequences described for other tyrosine kinases (Downward et al , 1984) The function of the EGF -dependent protein kinase and the v-erb-B kinase activities in the control of cell proliferation remain to be resolved

1 3 Platelet-Derived Growth Factor

Platelet derived growth factor (PDGF) is a serum protein that is released mainly from the alpha granules of platelets into serum during the clotting process (Stiles, 1983) PDGF is a potent mitogen for connective tissue (smooth muscle cells, fibroblasts and glial cells) and for those cell types possessing receptors for its binding (Ross and Vogel, 1978) Polypeptide growth factors (Deuel and Huang, 1983), in general, appear to have specific target cells and tissues Many cells in culture are serum dependent and require, among other factors, PDGF (Ross et al , 1974, Kohler and Lipton, 1974).

PDGF from human platelets is a cationic glycoprotein of approximately 30 kDa (Antoniades, 1981, Deuel et al , 1981,

Raines, 1982) Each human platelet contains about 1000 molecules of PDGF (Stiles, 1983) PDGF is released during clot formation and human clotted blood serum contains 15-20 ng ml^{-1} of PDGF Reduction of disulfide bonds destroys its mitogenic activity and generates multiple species of 14 to 17 kDa Sequence analysis shows two distinct but related sequences (Antoniades and Hunkapeller, 1983, Waterfield et al , 1983), suggesting that PDGF from human platelets is a heterodimer of two chains, these are termed A and B (Johnsson et al , 1984) Porcine PDGF appears to consist of B-B homodimers (Stroobant and Waterfield, 1984), while human PDGF produced by osteosarcoma cells consists of A-A homodimers (Heldin et al , 1986)

Receptors for PDGF are found on a variety of mesenchymal cells (Bowen-Pope and Ross, 1982, Heldin, 1981) as well as human placental cytotrophoblasts (Goustin et al , 1975) The cytotrophoblasts are part of the human placenta and are the most invasive and proliferative normal cells known, the expression of PDGF receptors in this tissue may help account for their "pseudomalignant" phenotype (Beaconsfield et al , 1980) Stimulation of cells with PDGF induces an autophosphorylation of a 185 kDa protein (Ek and Heldin, 1982) which turns out to be the PDGF receptor (Williams et al , 1984) An antibody to phosphotyrosine has been used in the purification of the receptor from BALB/c-3T3 cells, purified receptors inserted into liposomes reconstitute the growth factor binding characteristics of native receptor (Daniel et al , 1985)

Table 1 shows the variety of cells capable of secreting PDGF and also its possible biological roles (Ross et al , 1986)

Abnormal cell proliferation and its control are primary problems in a number of diseases, including neoplasia and atherosclerosis In many instances, the basis for this cell proliferation, whether normal or abnormal, most likely lies in the response of the cells to a group of polypeptide hormones or growth factors which may act singly or in concert to stimulate the proliferation of particular populations of cells Each growth factor appears to have specific target cells and tissues

The v-sis gene product of simian sarcoma virus (SSV) is nearly identical to the B-chain of human PDGF and the active v-sis product is probably a B-B dimer (Doolittle et al , 1983, Heldin et al , 1983) SSV was the first acute transforming retrovirus to be isolated from a primate Injection of SSV into laboratory test animals induces tumours, but only in those tissues which express the PDGF receptor (Leal et al , 1985) SSV mediates cell transformation by the production of a PDGF like molecule (Gazit et al , 1984) Since all known effects of PDGF are mediated via its interaction with its receptor, it is reasonable to propose that transformation of cells by SSV involves the binding of v-sis encoded PDGF-like proteins with the PDGF receptors of the cell, thereby activating those processes responsive to PDGF

Table 1 : Cells Capable of Secreting PDGF-like Molecules

Cell Type	Agent Inducing PDGF Secretion	Other Growth Factors Produced	Anatomical Considerations	Possible Biological Roles
Circulating Cells				
Platelet	Thrombin, collagen, ADP, adherence	+(FGF, EGF, TGF-β, ?)	9-11 days in the circulation	Wound healing; atherosclerosis
Monocyte/macrophage	Endotoxin, concanavalin A, TPA, foreign agents, adherence	+(FGF, IL-1, ?)	Can reside in tissue for months	Wound healing; atherosclerosis; fibrotic disorders of lung, liver, and kidneys; inflammation; arthritis
Resident Cells				
Megakaryocyte	Thrombin, collagen ADP, adherence	+(?)	Bone marrow	Abundant in some forms of myelofibrosis
Endothelium	Culture, thrombin, factor Xa, injury (endotoxin and phorbois)	+(IL-1, ?)	Permeability barrier between circulating blood cells and underlying cells	Wound healing; athersclerosis
Smooth muscle from:				
Rat pup	"Development"	+(IGF-1, ?)	Rat aorta	Development, growth
Adult rat	Balloon catheter injury culture	+(?)	Rat carotid (neointima), rat aortic media	Atherosclerosis; wound healing
First trimester human placental extracts	"Development"	+(?)	Cytotrophoblasts of the placental cytotrophoblastic shell	Development, growth

Table 1 : Cells Capable of Secreting PDGF-like Molecules (cont'd)

Cell Type	Agent Inducing PDGF Secretion	Other Growth Factors Produced	Anatomical Considerations	Possible Biological Roles
Transformed Cells				
Cells transformed by SSV	SSV	+(?)	Multiple subcutaneous fibrosarcomas, astrocytomas (intracranial)	Neoplasia
Activation of c-sis during transformation	Wide variety of transforming agents			
PDGF-responsive cells		+(TGF-α, ?)	Principally blastomas and sarcomas	Autocrine stimulation in atherosclerosis, neoplasia
PDGF-nonresponsive Cells		+(TGF-α, ?)	Erythroleukamic cells, bladder carcinoma, hepatoma	Paracrine stimulation in myelofibrosis, desmoplasia

Abbreviations : PDGF - platelet-derived growth factor; FGF - fibroblast growth factor; EGF - epidermal growth factor; TGF - transforming growth factor; IL-1 - interleukin-1; IGF - insulinlike growth factor; SSV - simian sarcoma virus; TPA - 12-O-tetradecanolyphorbol-13-acetate.

Ref : (Ross et al., 1986)

1 4 1 Transforming growth factors

Transforming growth factors are hormonally active polypeptides that induce phenotypic transformation in certain normal cells in vitro Two types of transforming growth factors, TGF-α and TGF-β , from both humans (Todaro et al , 1980) and rodents (De Larco and Todaro, 1978, Moses et al , 1981; Roberts et al , 1980, Todaro and De Larco, 1978, Twardzik et al , 1982) have recently been purified, characterized, chemically synthesized and cloned With the identification of cellular receptors for TGF-α and TGF-β , we now have more approaches available to address their mode of action and their role in normal and malignant cell proliferation

TGFs cause the reversible appearance of many characteristics of the neoplastically transformed phenotype in cells that normally grow in an anchorage-dependent manner (connective tissue and epithelial cells) (Moses et al , 1981, Todaro et al , 1980) The phenotypic alterations induced by TGFs include morphologic transformation of cells in monolayer and an induction of multiple rounds of cell division in cells suspended in semi-solid medium (Todaro et al , 1980, Todaro et al , 1981)

1 4 2 Transforming Growth Factor-α

TGF-α is related to epidermal growth factor (EGF) and binds to the EGF receptor, (in contrast to TGF-β , which is a structurally unrelated protein with a distinct receptor) The initial observation that led to the identification of TGF-α was that some retrovirally transformed fibroblasts displayed a strongly reduced number of EGF binding sites at their surface (Todaro et al , 1976) Later it was shown that these cells release an EGF-like factor that is able to bind to EGF receptors, which then become unavailable for binding of an externally added ligand This EGF receptor binding factor was first isolated from murine-sarcoma-virus-transformed fibroblast cultures and was first called sarcoma growth factor (SGF) (De Larco and Todaro, 1978) It was then thought that SGFs (or TGFs as they are now known), were involved in neoplastic cell growth Further investigation of a variety of cell sources showed that this factor was made by many more transformed

cells but not by adult normal cells in culture (Todaro et al , 1980, Roberts et al , 1980)

Following extensive biochemical purification and characterization, it was found that sarcoma growth factor was composed of two structurally unrelated peptides, TGF-α and -β Purified TGF-α alone in serum-containing medium only weakly stimulates soft agar colony formation (Anzano et al , 1983) The apparent colony stimulating ability of sarcoma growth factor was presumably due to the interaction of TGF-α and TGF-β on NRK indicator cells

TGFs-α have been detected in culture supernatants and extracts from several transformed rodent and human cells (De Larco and Todaro, 1978, Marquardt et al , 1983) These TGFs-α, which all bind to the EGF receptor, display upon gel filtration a heterogeneity in apparent molecular weights ranging from a 6 kDa species secreted by several tumour cell lines (Marquardt et al , 1983) to the 34 kDa TGF-α species detected in the urine of cancer patients The low molecular weight TGF-α species has been purified to homogeneity from several cell sources (Massague, 1983) Subsequent amino acid sequencing led to the establishment of the complete amino acid sequence of the 50-amino-acid-long rat TGF-α (Marquardt et al , 1984) These data on the structure of TGF-α have now been confirmed and extended by cDNA analysis The sequence of a human TGF-α cDNA derived from a renal cell carcinoma cDNA library indicates that the 50-amino-acid TGF-α is synthesized as a larger precursor (Derynck et al , 1984), as has now been shown by the sequence analysis of rat TGF-α cDNAs (Lee et al , 1985) To generate the 50-amino-acid TGF-α, proteolytic cleavage of the 160-amino-acid precursor must occur at both the amino and carboxy terminus between an alanine residue and valine dipeptide Fig 2 shows the hypothetical TGF-α precursor model

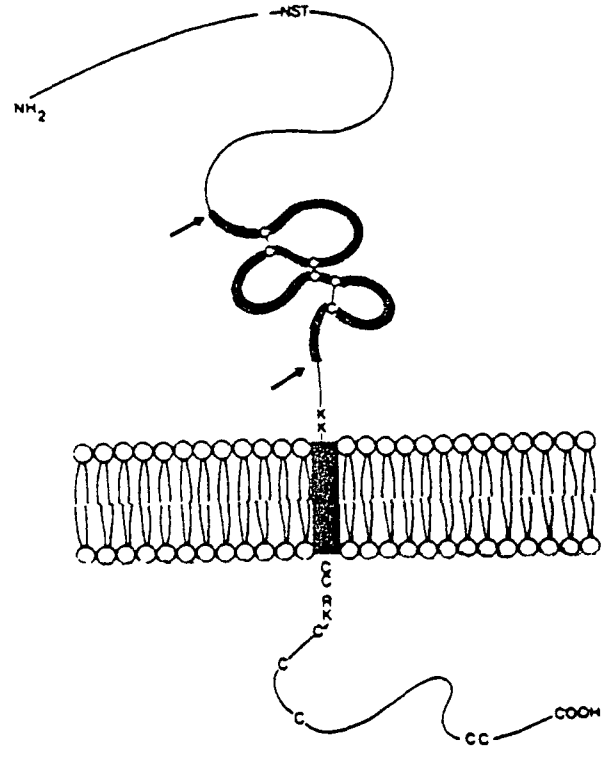


Fig. 2 Depiction of a hypothetical model of the TGF-α precursor as a transmembrane protein. The NH_2-terminal signal sequence is shown as already cleaved from the precursor. The 50-amino-acid TGF-α with its three proposed cystine (C) - disulfide bridges is shown as a heavy fine line, flanked by the proteolytic cleavage sites (arrows). The boxed transmembrane region is flanked at each side by two basic amino acids (KK and RK). The carboxy-terminal cytoplasmic domain shown below the membrane is rich in cystines (C).

The protease cleavage protein required is extremely specific and may not be produced by all cells This could then result in the continuous anchorage of the TGF-α precursor in the membrane and the absence of TGF-α in the medium The rat and human TGF-α precursors show strong homology followed by a long hydrophobic transmembrane region (Derynck et al , 1984) It is in the C-terminal regions that the rat (Lee et al , 1984) and human TGF-α precursors are virtually identical, which reflects an evolutionary conservation of this sequence and suggests an as yet unknown biological function One gene has been shown to code for TGF-α (Derynck et al , 1984) The larger TGF-α species as found by several researchers (Marquardt and Todaro, 1982) are now known to be N-glycosylated and are derived from the same precursor as the smaller 50-amino- acid form (Bringman et al , 1987)

1 4 3 Relationship of TGF-α to other Growth factors

TGF-α and EGF have very similar sequence homology (Derynck et al , 1984) 6 cystines found in human TGF-α and EGF have corresponding sites Rat TGF-α and EGF also have significant homology Since TGF-α and EGF are so similar, it is likely that the three disulfide bonds found in EGF are also found in TGF-α The third cystine loop contains the most significant homology between TGF-α and EGF and it is suggested that this region is important for binding to the EGF receptor (Nestor et al , 1985)

Vaccinia virus encodes a polypeptide precursor of 140-amino-acids with a sequence very similar to TGF-α and EGF This vaccinia virus growth factor competes for EGF receptors as shown by Stroobant et al , (1985)

The initial observation that TGF-α was found in the medium of various virally or chemically transformed cells (Todaro et al , 1985), led to the belief that TGF-α was a key molecule in the malignant transformation of cells TGF-α could not be detected in medium from normal cells in vitro and is not known to be made in normal fully developed tissues However, due to the limited number of obsrvations reported, it cannot be concluded that TGF-α has no role in the physiology of normal adult cells TGF-α is synthesized in early foetal development (Lee et al , 1985), which may indicate that TGF-α may function as an embryonic form of the EGF family

The expression of TGF-α gene at a later stage of growth may occur in the process of malignant transformation and tumour development

In *in vitro* cell growth, it has been shown that anchorage independent growth of NRK cells was due to the combined effect of TGF-α and TGF-β (Anzano *et al* , 1983) It has been postulated that during the transformation process, TGFs exert their action via an autocrine mechanism in which they help sustain the transformed character of the same cells which produced them (Sporn and Todaro, 1980) This hypothesis would also explain the reduced number of EGF binding sites available to externally-added ligand in retrovirally transformed cells (Todaro *et al* , 1976)

It has not yet been reported whether constitutive expression of TGF-α is sufficient to induce malignant transformation via an autocrine mechanism Even though TGF-α expression is quite common in solid tumours and tumour cell lines, it is still debatable if the autocrine mechanisms of transformation by TGF-α or by any growth factors will induce or contribute to the development of human malignancies *in vivo*

The delay in studying the biological activities of TGF-α was due to very low levels produced by cells The determination of the complete sequence of the 50-amino-acid rat TGF-α (Marquardt *et al* , 1984), has enabled the direct synthesis of larger amounts of rat TGF-α by solid phase techniques (Tam *et al* , 1984) The isolation of a human TGF-α cDNA has also led to the synthesis and purification of relatively large amounts of human TGF-α from genetically engineered *Escherichia coli* (Derynck *et al* , 1984, Rosenthal *et al* , 1986)

TGF-α can be quantified by radioreceptor assay in which TGF-α competes with ^{125}I-EGF for binding sites on a mole per mole basis TGF-α also binds to a 60 kDa membrane component but studies have shown that TGF-α binding to this site does not mediate the transformed phenotype (Carpenter *et al* , 1983) EGF and TGF-α exhibit very similar binding to the EGF receptor (Massague, 1983) On binding to the EGF receptor TGF-α activates a receptor associated kinase in the same manner as EGF (Pike *et al*, 1982)

The greater availability of TGF-α in the past few years has allowed comparison of TGF-α to EGF Although they behave almost identically in many systems, but in other cases (as found in bone resorption and angiogenesis), TGF-α is far more potent than EGF See Table 2 for details of TGF-α- (EGF-like) activity

Table 2

Activity	Refs
Soft agar colony formation NRK	(Derynck et al , 1984)
Precocious eyelid opening in new born mice	(Smith et al , 1985)
Accelerate tooth eruption, retard growth rate inhibit hair growth	(Tam, 1985)
Promote calcium release from foetal Rat long bones	(Stern et al , 1985 and (Ibbotson et al , 1986)
Induction of angiogenesis	(Schreibner et al , 1986)

It is unclear how both TGF-α and EGF, which bind to the same receptor, can trigger differential responses It is thought that EGF receptors with high and low affinities respond with different results when binding to TGF-α or EGF Alternatively, there may be differences on internalization of the receptor-ligand complex or different cell types may respond differently to either TGF-α or EGF Further research into the functions of TGF-α is necessary before it can be fully evaluated

1.4.4 Transforming growth factor-β

In spite of its name, transforming growth factor-β is very different to TGF-α in molecular composition, biological response elicited and membrane receptor binding. TGF-α or EGF must be present along with TGF-β to support the formation of soft agar colonies of normal rat kidney fibroblasts (Roberts et al., 1981). TGF-β alone can induce anchorage-independent growth of mouse embryo fibroblasts (AKR-2B) (Moses et al., 1984; Tucker et al., 1983). TGF-β activity is generally assayed by its ability to stimulate soft agar colonies of a clone of NRK-(49F) in the presence of EGF (Roberts et al., 1981). In contrast to these mitogenic effects on some fibroblast systems, TGF-β appears to have a negative growth modulatory role in various other cell systems, particularly normal epithelial cells and several types of malignant cells (Tucker et al., 1984; Roberts et al., 1985; Moses et al., 1985; Heimark et al., 1986).

Initially, TGF-α and TGF-β were co-purified (see section 1.4) until further investigation by HPLC showed that TGF activity on NRK was due to two molecules (Childs et al., 1982; Roberts et al., 1982; Tucker et al., 1983).

TGF-β is a fundamental regulatory molecule widely distributed in different tissues (Goustin et al., 1986; Sporn and Roberts, 1985) and has been purified to homogeneity from four sources; bovine kidney (Roberts et al., 1983); human placenta (Frolik et al., 1983); human platelets (Assoian et al., 1983) and feline sarcoma virus transformed rat cells (Massague, 1984). These sources reveal a 25 kDa disulfide-linked homodimeric molecule. Previous reports have shown that TGF-β from porcine platelets also contain another form of TGF-β with much the same structure and functions as the original TGF-β. Specific receptors have been found for each type which may be a means of modulating the cells response to TGF-β (Cheifetz et al., 1987). TGF-β has numerous regulatory actions on a variety of cell types and virtually all have a specific high affinity receptor for this peptide (Frolik et al., 1984; Tucker et al., 1984; Fanger et al., 1986). The molecular mechanism of action of TGF-β is at present unknown.

TGF-β is derived from a large precursor molecule and on reduction,

the TGF-β dimer yields two identical chains of 112 amino acids (Derynck et al., 1985). TGF-β is highly stable to acid and heat. Derynck et al., 1985 have cloned the gene for TGF-β from a human genomic library and from cDNA libraries derived from human term placenta and from the human fibrosarcoma line HT-1080. Murine TGF-β has also been cloned and the cDNA sequence determined. The high degree of cDNA sequence homology found between human and murine TGF-β coding region suggests that most regions of the TGF-β molecule are necessary for biological activity and that TGF-β probably plays an essential role in normal growth and development (Derynck et al., 1986).

It has recently been appreciated that TGF-β is prototypic of a family of homologous polypeptides that control the development of tissues in organisms ranging from humans to Drosophila. This family includes various inhibins and activins, which regulate the ability of cultured pituitary cells to release follicle stimulating hormone (Mason et al., 1985; Ling et al., 1986); the Mullerian inhibiting substance, which inhibits development of the Mullerian duct in mammalian male embryos (Cate et al., 1986) and the transcript of the decapentaplegic gene complex (DPP-C), which is critical for the development of Drosophila (Padgett et al., 1987). The bioactive domains of these polypeptides share about 25-35% amino acid sequence identity with TGF-β, including conservation of at least seven of the nine cystines. Biological and polypeptide analyses have shown that cartilage inducing factor-A (CIF-A) (Zeydin et al., 1986) is TGF-β.

Three structurally distinct cell surface glycoproteins have been identified that specifically bind TGF-β with affinity constants in the pico molar range. These receptors are found to be ubiquitous like the TGF-β molecule (Tucker et al., 1984) and the receptor number per cell is between 10,000 and 40,000 (Frolick et al., 1984). TGF-β from different species bind to the receptors equally well, suggesting that these growth factor-receptor systems are highly conserved. It was proposed and shown that this family of TGF-β receptors might interact wth a family of TGF-β related polypeptides in a situation similar to that found in the receptors for other families of hormonally active agents (Cheifetz et al., 1987). The TGF-β receptor is unlike those of EGF or PDGF in that, so far, no kinase or other enzymatic activities have been found.

TGF-β enhances the ligand-binding affinity of the EGF receptors of NRK cells (Assoian et al , 1984) and the activation of the EGF-receptor appears to be essential for the action of TGF-β in these cells (Inman and Colowick, 1985) The need for insulin-like growth factors in TGF-β-induced cell transformation has also been proposed (Massague et al , 1985)

It has been shown that various combinations of growth factors can stimulate the formation of soft agar colonies (Van Zolen et al , 1986) and not as was originally thought that transforming growth factors were the sole stimulators However, TGF-β in certain systems can stimulate soft agar colonies of various mouse and rat fibroblasts and also secondary cultures of human foreskin fibroblasts (Moses et al , 1981, Roberts et al , 1981, Moses et al , 1985). The general effects of TGF-β are multifunctional as shown in Table 3 Generally, in the case of cell growth and inhibition, it is thought that TGF-β is primarily an inhibitor (Shipley et al , 1985; Holley et al , 1983), the mechanism(s) of which are largely unknown and that stimulation of fibroblastic cells is mediated by the induction of c-sis followed by the autocrine stimulation of PDGF-like sis-protein and DNA synthesis (Leof et al , 1986) Lawrence et al , 1984 have shown that many cell types release TGF- in an inactive form This inactive form seems to be of a higher molecular weight, which is activated on cleavage from a binding protein e g by acid treatment The mechanism of activation is unknown

Chemically transformed cells gain increased sensitivity to TGF-β with regard to stimulation of growth in soft agar (Moses et al , 1985) It was proposed that on transformation, a newly activated H-ras gene could enhance autocrine stimulation by TGF-β or could be involved in the transduction of the TGF-β signal This would explain how these transformed cells become more responsive to endogenously produced TGF-β without an increase in cellular receptor number (Leof et al , 1986)

Epithelial cells are normally inhibited in the presence of TGF-β It is thought that these cells activate TGF-β found in an inactive state in serum which then inhibits the cells in an autocrine manner The loss of the ability to activate TGF-β in cells that

Table 3

Biological Effects of TGF-β	Refs
1 Inhibits the growth of most normal, especially epithelial cells and several malignant cells	Moses et al , 1985 Roberts et al , 1985 Shipley et al , 1986
2 Mitogenic for mesenchymal cells	Moses et al , 1981 Roberts et al , 1981 Shipley et al , 1985
3 Stimulates anchorage independent growth of non-malignant fibroblasts	Moses et al , 1981 Roberts et al , 1981
4 Inhibits adipogenic differentiation of 3T3 cells	Ignotz et al., 1985
5 Stimulates terminal differentiation of bronchial epithelial cells	Masui et al , 1986
6 Wound Healing	Roberts et al , 1986
7 Regulates the plasminogen activator activity of cultured cells, induces endothelial type plasminogen activator inhibitors	Laiho et al , 1986
8 Enhances the production of connective tissue and components	Roberts et al , 1986
9 Acts as a modifier of different immunological responses	Kehrl, et al , 1986

are normally inhibited could lead to their growth advantage (Moses et al , 1985, Shipley et al , 1986)

TGF-β could play a major role in many disease states involving fibroblastic proliferation and collagen deposition as indicated by its ability to enhance wound healing (Sporn et al., 1983) and its general stimulatory effect on connective tissue (Roberts et al , 1986) in building up the extracellular matrix (Ignotz and Massague, 1986) Atherosclerosis is one of the main diseases in which TGF-β could play an important role (Ross, 1986)

Another effect of TGF-β is in bone resorption although TGF-α is a far more potent factor in the cause of hypercalcemia found in some cancers (Ibbotson et al , 1983, Mundy et al , 1984)

TGF-β is a growth regulatory polypeptide with stimulatory and inhibitory activity on a variety of cell types Further characterization of this molecule will greatly expand our concept of normal and malignant cell control

1 4 5 Other Transforming growth factors

Other transforming growth factors which can stimulate the transformed phenotype are also known TGF-α2 is an acid labile factor that stimulates the growth in soft agar of BALB/c-3T3 cells (Hirai et al , 1983) This growth factor has been purified and an amino acid composition has been determined (Yamnoka et al , 1984) An epithelial tissue derived autocrine growth factor from the carcinoma cell line SW13, has also been described (Halper et al , 1983) Vaccinia Virus-infected cells have been found to release a transforming growth factor functionally related to the known transforming and epidermal growth factors (Twardzik et al , 1985, Stroobant et al , 1985) An acid-sensitive factor from human platelet sonicate has been found to promote soft agar colony formation of human metastatic melanoma cells (Sipes et al , 1985) An autostimulatory melanoma mitogen (melanoma growth-stimulatory activity, MGSA) has also been described by Richmond et al , 1985 The variety of transforming growth factors appears to be greater than at first thought Further characterization will determine the basic mechanism behind transformation and the relationship of all these transforming growth factors

1 5 Insulin-like growth factors (IGF-I and IGF-II)

The insulin-like growth factors (somatomedins) refer to a family of closely related mitogenic polypeptides There are two main groups based on their isolectric points The basic group (IGF-I) with isoelectric point above 7 5 and the acid-neutral group with isoelectric point below 7 5 (IGF-II) IGF-I corresponds to human somatomedin C (Klapper et al , 1983), and IGF-II corresponds to human somatomedin A and rat multiplication stimulating activity (Marquardt & Todaro, 1981) Somatomedin C is the term most often used in the literature Somatomedin C is produced in response to circulating growth hormone, and is one of the important growth factors found in serum and plasma (Svoboda et al , 1980) It is active in stimulating a large number of cultured cells (Van Wyk et al , 1981) Excessive concentrations of insulin (above 100mM) can replace the IGF requirement in defined media through cross-reaction with ubiquitous IGF-I receptors (Van Wyk et al , 1975) Somatomedins apparently circulate in plasma non-covalently bound to a specific carrier protein (Furlanetto, 1980) It is generally thought that somatomedins function in an autocrine fashion (Temin et al , 1982), BRL-3A cells, however, which secrete large amounts of IGF-II into the medium (Dulack & Temin, 1973) do not need IGF-II for stimulation (Nissley et al , 1977) Evidence is accumulating that the stimulation of foetal mouse growth by somatomedin C is in an autocrine or paracrine mode (D'Ercole et al , 1984)

Purification of Somatomedin C (IGF-I) from human serum has shown a single chain of 70 amino acids with 3 internal disulfide bonds This factor has also been sequenced (Rinderknecht & Humbel, 1978) The genes for both human IGF-I and IGF-II have been cloned (Bell et al , 1984) Recombinant somatomedin C, which is almost identical to human somatomedin C except for one amino acid change, is commercially available The human IGF-II gene is found very near the human insulin gene (Bell et al , 1985) Both of the 7 kDa IGFs are processed from larger precursors (Bell et al , 1984), and it is thought that IGF-I may be an adult somatomedin with IGF-II the embryonic counterpart (Adams et al , 1983) Although IGF-I and IGF-II have their own receptors, some cross-reactivity is found at higher growth factor concentrations (Massague & Czech, 1982) Cell receptors for IGF-I (type 1 receptors) show homology to the insulin receptor This is a heterotetrameric 450 kDa

complex consisting of two transmembrane β subunits (98-kDa each), each disulfide bonded to one α-subunit (130 - kDa each) (Pilch et al., 1980). The α subunits provide the insulin (or IGF) binding sites (Pilch et al, 1979) and the β subunit posesses the ATPase and tyrosine kinase activities (Van Oberghen et al., 1983). Much is now known of the insulin receptor. Homology exists between the extracellular domain of the human EGF receptor and the α-region of the insulin receptor. The β domain however, has homology with the oncogenes, in particular the ros oncogene (Ullrich et al., 1985). These homologies strongly suggest that one or more of these oncogenes may encode growth factor receptors. The second type of IGF receptors, Type II, are far more simple. They are a 250 kDa component which may be single chain (Kasuga et al., 1981), and they may not undergo ligand-induced down regulation (Massague, 1985).

1.6.1 Interleukin 2

Treatment of human peripheral blood T-cells with the lectin Con A, causes the release of soluble factors that stimulate the proliferation of activated T-cells (Smith 1984; Morgan et al., 1976). This antigen triggered factor called T cell growth factor or interleukin 2, supported the longterm in vitro culture of clonal populations of normal cytotoxic T-lymphocytes (Morgan et al, 1976). Tanaguchi and co-workers (1983) successfully isolated the first cDNA clone that codes for IL-2. The sequence shows a peptide of 153 amino acids that is cleaved to form the mature 133-residue secreted sialoglycoprotein displaying one internal disulfide bond (Robb et al., 1983).

The human gene for IL-2 spans about 8 kilobases and consists of 4 exons (Holbrook et al., 1984). A cDNA-encoding mouse IL-2 has been cloned which exhibits 76% homology at the amino acid level to human IL-2. The mouse cDNA for IL-2 has a reading frame sufficient to encode a protein of 169 residues (Kashima et al., 1985). The tumour cell line JURKAT has been used in much of the work with IL-2. Treatment of this cell line with Con A induces an IL-2 transcript of 1.5 kilobases (Taniguchi et al., 1983). The lectin phytohemagglutinin stimulates a 30-fold induction of IL-2 transcription in normal human lymphocytes (Efrat and Kaempfer, 1984). However, the immunosuppressive drug cyclosporn A has been

shown to deactivate the IL-2 gene in phytohemagglutinin induced JURKAT cells (Kronke et al , 1984) These observations may suggest a possible role for the activation of the IL-2 gene during T-cell activation

Cell surface receptors for IL-2 have been purified from both normal and transformed lymphocytes (Urdal et al , 1984) The receptors from normal lymphocytes are 55 kDa and from transformed lymphocytes, 60 kDa The reason for the difference in size is not fully known. The receptor is apparently quite different from those of other known growth factors Cloning and sequencing analysis of IL-2 receptor cDNA show only a 33 kDa receptor reading frame with a very short cytoplasmic region. Another functional receptor cDNA sequence may yet be cloned which will contain the full 55 kDa reading frame In the 33 kDa protein, the cytoplasmic domain has one serine and one threonine which can be phosphorylated An alternative receptor species is found in the HuT-102 B2 (human T-cell leukaemia Virus 1 transformed) cells These cells encode an almost normal IL-2 receptor except for a 72 amino acid deletion near the IL-2 binding domain (Leonard et al , 1985) The significance of this truncated receptor species is not clear IL-2 receptor structure and genetic regulation are still not fully understood despite extensive cloning and purification

Functional receptors for IL-2 are not found on resting T-cells (Robb et al , 1981), the action of Con A or antigen in T-cell proliferation thus involves the induction not only of IL-2 production by the T-helper cells, but also of IL-2 receptors on T-killer cells, a two step process (Larsson and Coutinho, 1979) The control of IL-2 receptor presentation in the immune response is, in this way, a key control of normal T-cell proliferation

Future work on the biology of IL-2 will help in the understanding of eukaryotic gene regulation, cell growth and differentiation, the regulation of the immune response, immunodeficiency and leukaemia

1 6 2 Interleukin-1

Interleukin-1 (previously called lymphocyte activating factor or LAF) is released by macrophages and possibly by dendritic cells, and acts on B rather than T lymphocytes IL-1 is membrane

associated but is also secreted from the cells IL-1, however, is as yet poorly characterized and will not be discussed further here

1 7 Fibroblast growth factor (Heparin-binding growth factors)

Bovine neural tissue extracts contain growth factors mitogenic for cultured fibroblasts and vascular endothelial cells (Gospodarowicz et al , 1976) Other sources as recorded by Gospodarowicz include bovine pituitary (Gospodarowicz, 1975), and bovine brain (Gospodarowicz et al , 1978), the molecular characterization of these factors has not been possible until recently It was claimed at one time that FGF was derived from brain myelin protein fragments (Westall et al , 1978), but later this was shown to be incorrect (Thomas et al., 1980) These neural extracts contain several factors which are given the name FGF, they are all apparently single-chain proteins in the 14-18 kDa size Other members of the FGF family include the factors described as endothelial cell growth factor, heparin binding growth factor (Klagsbrun, 1986) and chondrosarcoma factor (Shing et al , 1984) Proteins that are apparently acidic and basic in the neural extracts show similar features (Thomas et al , 1980, Lemmon & Bradshaw, 1983, Lemmon et al , 1982) Several factors have now been purified to apparent homogeneity, an acidic form of FGF from bovine brain (Thomas et al , 1984), and a cationic form from bovine pituitary (Bohlen et al , 1984) have been isolated by multistep procedures and an NH_2- terminal sequence was reported for the cationic form (Gospodarowicz et al., 1984) Both factors have a molecular weight of 16,000

Several factors that have properties similar to those of FGF have been recently purified by heparin affinity chromatography An 18 kDa endothelial growth factor from chondrosarcoma was the first to be purified by this technique (Shing et al , 1984) It was shown that the cationic brain FGF can be purified by this technique and are identical (Gospodarowicz et al , 1985) An 18 kDa pituitary form of the heparin-binding growth factor has also been observed in preparations from bovine pituitary (Shipley et al , 1985) and hypothalamus (Lobb and Fett, 1984). It has been reported that multiple forms of FGF activity can be isolated by heparin binding affinity, including both the cationic and anionic FGFs from brain (Klagsbrun & Shing, 1985) An amino acid composition for both

forms has been reported (Lobb & Fett, 1984) and the acidic form of the molecule isolated by this technique has the same molecular weight and amino acid composition as the molecule isolated by the multistep procedure (Thomas et al., 1984). The complete sequence of bovine pituitary basic FGF is now available (Esch et al., 1985); the sequence describes a molecule of 146 amino acids (16.4 kDa). This sequence agrees with the partial sequence obtained for bovine basic FGF obtained from other tissues, including brain, adrenal gland, retina, corpus luteum, and kidney (Esch et al., 1985). Since this sequence differs substantially with that reported for the bovine brain acidic form (Thomas et al., 1985), there are probably at least two genes encoding FGFs corresponding to the acidic and basic FGFs. There is, however, antigenic and sequence relatedness between these two gene products (Esch et al., 1985). A slight amount of homology exists between acidic FGF and interleukin 1 (Thomas et al., 1985). The factor described as endothelial cell growth factor is related to the FGF family. Purified endothelial cell GF has been radio-iodinated for use in a radioreceptor assay, allowing the estimation of dissociation constant (200-800 pM) and receptor number per cell (20,000-40,000) (Schreiber et al., 1983).

A radioreceptor assay for FGF might allow for a survey of the distribution of FGF content and FGF production by various tissues; no such survey has yet been carried out. The significance of an endothelial cell growth factor concentrated in brain or pituitary is as yet unclear. The possible scenario of FGF as an endocrine growth factor would stand in contrast to patterns of other GFs as locally produced and locally acting paracrine or autocrine growth factors. The production of a FGF by chondrosarcoma is more in keeping with the general scheme, if one imagines a paracrine role for this growth factor in the stimulation of tumour angiogenesis, as has been suggested (Folkman, 1983).

1.8 Nerve growth factor (NGF)

Nerve growth factor has been known for some time; however, its role as a factor for the maintenance and differentiation of sensory and sympathetic neurons argues against its inclusion in a strict list of growth factors in many ways. Indeed, in the most recent research, evidence indicates that NGF may play a role in cultured

rat adrenal chromaffin cells (Lillien & Claud, 1985) NGF was first detected as a factor released by transplanted tumours (Levi-Montalcini & Hamburger, 1951) , it was first purified from snake venom (Cohen, 1959), and then from mouse submaxillary gland (Cohen, 1980) NGF isolated from submaxillary glands is found in a 7S complex, containing three protein subspecies termed α, β and γ (Bradshaw, 1978) NGF activity resides in the β species, a 26 kDa dimer of two identical NGF chains (118 amino acids per chain) which has been sequenced (Angeletti & Bradshaw, 1971) Sequencing of mouse and human cDNA clones suggests that NGF is synthesized as a much larger precursor (Scott et al , 1983), pro NGF is apparently a dimer of 307 residues per chain, with native NGF encoded in residues 188-305 of the precursor

NGF receptors are present on a variety of normal, sympathetic and sensory neurons as well as normal and neoplastic chromaffin cells The rat phaechromocytoma cell line, PC-12 has been used extensively in studies concerning NGF PC-12 cells respond to NGF treatment by an inhibition of proliferation and a stimulation of diffentiation (Green & Tischler, 1976) The mechanisms controlling this response are at present unknown Cross-linking ligand studies have been used to help define the PC-12 receptor It is a single chain protein of 130 kDa, a smaller receptor of 100 kDa, has also been found This is possibly a degradation product (Massague et al , 1981) The receptor in A875 melanoma cells has been partially purified by affinity chromatography, again a 98 kDa species is present, although larger species of 138 and 190 kDa are present (Puma et al , 1983) It is not known how these multiple NGF receptor species correspond to the two receptor species defined by their apparent dissociation constants of 2 pM and 2 nM (Landreth and Shooter, 1980, Costrini and Bradshaw, 1979) Recently, six cDNA clones representing mRNAs induced in PC-12 cells by NGF have been isolated, one of the clones, VGF 8a, encodes a 90 kDa protein, the mRNA of which is induced more that 50 fold by NGF (Levi et al , 1985)

1 9 Colony stimulating factors (CSF-1, CSF-2 and Multi-CSF)

The soft agar colony assay developed by Metcalf & Johnson in 1977 has led to the identification of a number of factors, called CSFs, that regulate the growth and differentiation of hematopoietic

precursor cells. As is the case with other tissue growth factors, CSFs are synthesized at a large number of sites in the body and are active in the low pM level. These factors include CSF-1 [formerly called macrophage CSF (Stanley and Heard, 1977)]; CSF-2 [granulocyte-macrophage CSF (Burgess et al., 1977)]; and granulocyte CSF factor (Nicola et al., 1983) called interleukin 3, (IL-3), is active in stimulating colonies of mixed cell type (Ihle et al., 1983) and has been given the name multi-CSF. This factor is referred to by different names in the literature as a result of its stimulatory effect on various cell types. The activity of IL-3 (multi-CSF) includes the promotion of growth and differentiation of granulocytes, macrophages and multipotential stem cells as well as colony formation from early erythroid, esoinophilic, megakaryocytic and mast cell progenitors (Iscove et al., 1982).

IL-3 was described and purified on the basis of its ability to induce an enzyme (20α-hydroxysteroid dehydrogenase) in mouse spleen T-lymphocytes (Ihle et al., 1981). This method of purification differs greatly to the methods used for other GFs which are usually purified on the basis of a cell growth bio-assay. Multi-CSF has been purified and partially sequenced (Ihle et al., 1983); cDNA clones corresponding to both human and mouse multi-CSF have been isolated (Yokota et al., 1981; Dunn et al., 1985).

CSF-1 has been purified to homogeneity from mouse L-cells (Stanley and Heard, 1977) and human urine (Das and Stanley, 1982) and radio-receptor and radio-immune assays have been developed (Stanley, 1983; Stanley and Gilbert, 1981). Native CSF-1 from mouse L-cells is a 65-80 kDa sialoglycoprotein of two (possibly identical) chains linked by disulfide bonds (Das & Stanley, 1982). The human gene encoding CSF-1 has now been cloned (Kawasaki et al., 1985); sequence analysis of the cDNA clone indicates a pre-pro-CSF-1 of 252 residues with a 32 residue leader peptide. Further proteolytic processing reduces the pro-CSF-1 peptide to a 20 kDa form. Incubation of bovine marrow adherent cells with either CSF-1 or multi-CSF will induce up regulation of the number of CSF-1 receptors (Bartelmez and Stanley, 1985). Recent reports indicate that the c-fms proto-oncogene is closely related to the receptor for CSF-1 (Sherr et al., 1985). This protein is a 170 kDa transmembrane glycoprotein with tyrosine kinase activity (Rettenmier et al., 1985a; Rettenmier et al., 1985b). The c-fms

gene is found on the human chromosome 5 (Heisterkamp et al , 1983), and a deletion in the long arm of this chromosome is associated with a syndrome in which patients are predisposed to certain types of cancer such as myeloid leukaemia (Sokal et al , 1975)

The macrophage-granulocyte factor, (CSF-2), is a glycoprotein that has been purified from endotoxin-treated mouse lung (Metcalf, 1985) The factor has now been cloned from three species, mouse, gibbon ape, and humans A mature protein of 127 residues (14 kDa) is produced by the human and gibbon ape (Wong et al , 1987) and there is 54% amino acid homology between mouse and human CSF-2 (Wong et al , 1985, Cantrell et al , 1985) CSF-2 are widely produced in the body and probably play an important role in resistance to infections

Hematopoiesis, the production of white and red blood cells which are short-lived, is carried out on a complex and enormous scale (There are 100 times more cells in the bone marrow of an adult than there are people in the world) All blood cells originate from a small common population of multipotential stem cells that is formed during one short interval during early embryonic life which maintains hematopoiesis by an extensive capacity for self generation Dearrangement of this complex process of blood cell formation does occur and results in a range of medically important diseases from anaemia to leukaemia However, the hematopoietic system usually functions with remarkable fidelity as a consequence of regulation by an overlapping system of control mechanisms including control by CSFs

1 10 Bombesin

Bombesin is a 14-amino acid peptide, produced and released by most human small cell lung cancers (Moody et al , 1981, Sorenson et al , 1982, Erismann, et al , 1982) Bombesin was initially isolated from the skin of the frog (Anastasi et al , 1971) Bombesin-like immunoreactivity in mammals has been located mainly in the brain, lung and intestine (Walsh et al , 1978, Moody et al, 1978 Wharton et al , 1978) A mammalian analogue of Bombesin has now been isolated from the procine gastrointestinal tract (McDonald et al , 1978), and the cDNA cloned from a human pulmonary

carcinoid tumor (Spindel et al , 1984) Bombesin and its analogue GRP bind to the same cellular receptors (Moody et al, 1983)

Bombesin is a potent mitogen for 3T3 cells (Rosengurt and Sinnett-Smith, 1983) and can stimulate clonal growth of small cell lung cancer cells in serum free medium (Carney et al , 1981)

Monoclonal antibodies specific for the C-terminal region of bombesin not only inhibit the binding of bombesin to its receptor, but also inhibit markedly both the clonal growth of small-cell lung cancer cell lines in vitro and the growth of xenografts of these cells in nude mice (Cuttitta et al , 1985) Both Bombesin and its analogue GRP are both readily found in foetal and newborn lung and significant levels are absent in adult lung This suggests that these growth factors may have an important role in foetal lung function and perhaps development (Cutz et al , 1981) Lung from infants with respiratory distress syndrome show a marked reduction in Bombesin-like immunoreactivity-positive cells (Ghatei et al , 1983) Carney et al , (1987) have shown most recently that several small cell lung cancers lines express high affinity receptors for Bombesin or GRP These cells secrete and respond in a very selective manner to the regulatory peptide, but only in a serum-free defined medium

1 11 Oncogenes

Almost all oncogenes are altered versions of normal cellular genes, and their products are presumed to work at least in part by mimicing the products of the cellular genes from which they arose "Proto-oncogene" is a term used to describe a gene which shares sequences with any of the 20 or so known viral onc genes or genes which have the potential to give rise to cancer cells (Duesberg, 1985) Many, if not all, oncogenes are now perceived as functional components of a mitogenic cascade which is normally controlled by growth factors

Ushiro and Cohen (1980) showed that the receptor for EGF was associated with protein tyrosyl kinase activity It was previously known that the transforming proteins encoded by certain retroviruses are associated with a tyrosine specific protein kinase Gradually, it was discovered that other growth factor

receptors like insulin (Kasuga et al , 1982) somatomedin c (Jacobs et al , 1984) and PDGF (Ek and Heiman, 1982) were also associated with tyrosine-specific protein kinase activity By 1983, the full extent of the overlap between oncogenes and growth factors became apparent (Doolittle et al , 1983, Waterfield et al , 1983)

There are three sites in a growth control pathway at which oncogenic proteins can intervene to deliver a growth stimulus

Firstly, the protein itself might mimic a growth factor The interaction of such a protein with a suitable receptor could stimulate cell growth in an autocrine fashion Secondly, the oncogenic protein might imitate an occupied growth factor receptor and thus provide a mitogenic signal in the absence of exogenous growth factors Thirdly, the oncogenic protein might act on an intracellular growth control pathway, uncoupling it from the need for an exogenous stimulus Oncogenic proteins have been found to interact with all of the above three sites of a growth control pathway

The protein product of the v-sis oncogene of simian sarcoma virus (SSV) and PDGF share strong homology (Waterfield et al, 1983, Doolittle et al , 1983) It is the PDGF B chain which is closely related to the COOH-terminal of the v-sis protein They also share a functional similarity in that the v-sis protein can compete with PDGF for binding to PDGF receptors (Wang and Williams, 1984) All the data available is consistent with the hypothesis that the mature v-sis gene product enters the cellular pathway and interacts with the PDGF receptor to cause the unrestricted growth of SSV-transformed cells in an autocrine fashion To date, no other oncogenic protein is known to be secreted

The EGF receptor purified from the human tumor cell line A-431, shows an almost perfect match with the predicted product of the v-erb-B oncogene of the avian erythroblastosis virus (AEV) (Downward et al , 1984)

Sequence homology has shown that it seems extremely likely that the v-erb-B gene was derived from the chicken EGF receptor gene (Lin et al , 1984) The EGF receptor is the best characterized growth factor receptor (Buhrow et al , 1982) About a 250 amino acid

stretch of the internal domain of the EGF receptor has a striking homology with the catalytic domain of the src gene family of protein tyrosine kinases; this is in keeping with the known enzymic activity of the receptor. The v-erb-B gene product and the EGF receptor show 90% similarity. The v-erb-B protein appears to be a truncated form of the EGF receptor, which lacks most of the external EGF binding domain (Ullrich et al., 1984).

Several types of human tumor show abnormalities in the EGF receptor expression. Many squamous cell carcinomas and gliomas have very high concentrations of EGF receptor protein. In some cases this is apparently a result of amplification of the entire EGF receptor gene. Whether the altered expression of EGF receptor played a part in the tumorigenesis in these cases is unclear. Other growth factor receptors and oncogene products may interact but insufficient work has been done to date to say for certain what these are.

The third type of oncogene product is one that intervenes in a cellular growth control pathway at a post receptor stage. There are several of these oncogenes (CF Table 4.) The family of src oncogene all encode proteins with protein tyrosine kinase activity. These enzymes might phosphorylate proteins which are normally targets for the growth factor-activated receptor protein tyrosine kinase and thus provide a continuous intracellular mitogenic stimulus. The family of ras oncogenes may transduce signals from cell surface receptors by regulating adenylate cyclase and thus cAmp levels. The c-myc (Persson et al., 1984) and c-fos (Curran et al, 1984) genes, which are thought to code for DNA binding proteins, (Abrams et al., 1982) are quickly induced upon PDGF treatment of resting fibroblasts. Since these proteins are located in the nucleus, they may play a role in regulating expression of other genes, which are necessary for the mitogenic response (Muller et al., 1984; Duesbery, 1985). The presence of these various families argues well for the theory of multistage carcinogenesis where activiation of more than one gene is necessary to initiate cancer (Balmain, 1985).

Most of the known growth factors are found to be involved in growth pathways where oncogene transcripts can occur and lead to growth stimulation (Thomas et al., 1982; Kelly et al, 1983; Muller et al., 1984).

Table 4 : Amplification of postreceptor signalling, caused by oncogenes, in pathways mediated by growth factors.

Oncogene	Effect	Reference
myc	Reduced cellular requirement for PDGF	Kelly et al., (1983)
	Enhanced cellular sensitivity to EGF	Armelin et al., (1984)
		Roberts et al., (1985)
ras	Regulation of adenyl cyclase	Hurley et al., (1984)
		McGrath et al., (1984)
	Amplification of EGF signalling	Kamata and Feramisco, (1984)
src	Enhanced cellular sensitivity to EGF and insulin	Balk et al., (1982)

Ref. (Sporn and Roberts, 1985)

1.12 Growth inhibitors

The growth and division of normal cells is usually well regulated by the action of various endogenous stimulators and inhibitors. One of the basic challenges in the study of growth control is to determine the biochemical nature and sites of action of agents that can shift cells from one state to another.

Endogenous inhibitors of cell proliferation have been purified from several sources, including normal tissues (Assoian et al., 1983; McMahon et al., 1982) and conditioned medium from epithelial (Holley et al., 1980) and fibroblast cell lines (Hsu & Wang, 1986). Some growth factors in some instances are also inhibitory; transforming growth factor-β is one such growth factor. It is also closely related to the BSC-1 growth inhibitor purified from the African green monkey kidney epithelial cells (Tucker et al., 1984). The BSC-1 growth inhibitor has a native size of 25 kDa and yields a polypeptide of 12 kDa after reduction, its action is apparently autocrine (Tucker et al., 1984; Holley et al, 1983). TGF-β and BSC-1 growth inhibitor have the same biological activities in that they can stimulate the growth of AKR-2B cells in soft agar, inhibit DNA synthesis in monolayer AKR-2B, BSC-1 and CCL-64 (mink lung) cells, and bind to TGF-β specific receptors on the cell surface.

An inhibitor of hepatocyte proliferation has been purified from rat liver (McMahon et al., 1982). This protein reversibly inhibits the proliferation of non-malignant rat liver cells, but exerts no effect on the proliferation of hepatoma cells.

Different inhibitory factors have been fractionated from 3T3-cell-conditioned medium, in the 13, 11 (Steck et al., 1982) and 45 kDa range (Harel et al., 1983). Secondary cultures of mouse embryo fibroblasts produce growth inhibitors of between 12-15 kDa (Wells and Malluci, 1983). This factor is designated FGR-s (13k), for fibroblast growth regulator secreted or shed in a soluble form. It is not cytotoxic and its effects on target cells are reversible. More recently, Harel et al., 1985, have shown that dense cultures of 3T3 cells secrete both inhibitory and stimulatory autocrine factors of different molecular weights. The inhibitory factor found here is 45 kDa.

A growth inhibitor for Erlich ascites mammary carcinoma cells in vitro has been purified from bovine mammary gland The active preparation consists mainly of a single peptide (13 kDa) The inhibitory protein is found mainly in lactating bovine mammary glands (Bohmer et al , 1985, Bohmer et al , 1984) The growth inhibitory effect on Ehrlich ascites mammary cells could be abolished by EGF and insulin

Other growth inhibitors are also known, like a glycopeptide preparation from bovine cerebral cortex cells that inhibits protein synthesis and cell growth of normal but not transformed cells (Kinders and Johnson, 1982) of 18 kDa, a heparin-like molecule, produced by cultured endothelial cells (Castellot et al , 1981) EGF too, has inhibitory effects on squamous call carcinoma cells in culture, but not on primary cultures of human epidermal keratinocytes and dermal fibroblasts which it stimulated (Kamata et al , 1986)

Most of the work on growth inhibitors has been in tissue culture systems and the activity of these factors needs to be examined in vivo before their physiological importance or practical application can be evaluated

1.13 Autocrine Growth Factors

The relatively autonomous nature of malignant cells has been known for years; that is, they generally require fewer exogenous growth factors for optimal growth and multiplication than do their normal counterparts (Holly, 1975). To explain this phenomenon, it was suggested that cells could become malignant by the endogenous production of polypeptide growth factors acting on their producer cells via functional receptors, allowing phenotypic response to the peptide by the same cell that produces it (Todaro & De Larco, 1978). This process has been termed 'autocrine secretion'.

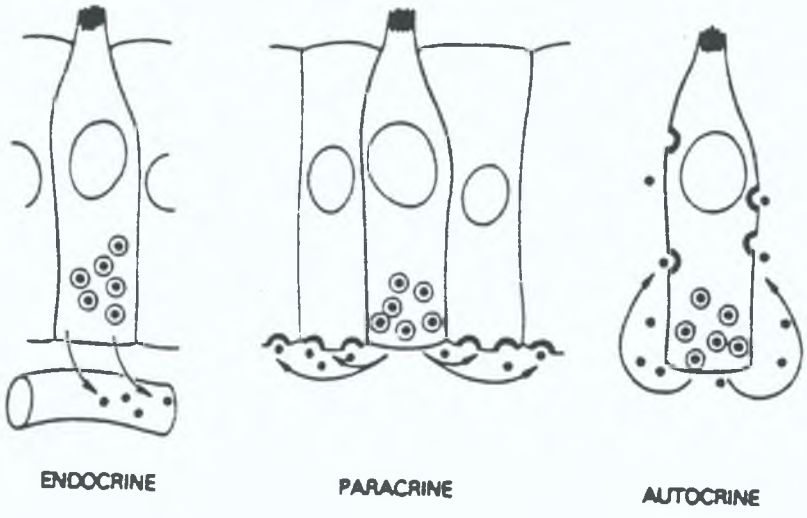


Fig. 3 : Diagrammatic representation of autocrine, paracrine and endocrine secretion. Regulatory chemical messengers such as growth factors are shown in latent form within the cell. The thickened, semicircular regions of the cell membrane represent receptor sites. (Sporn and Todaro, 1980).

Thus the ability of cancer cells to produce and respond to their own growth factors has become a central concept linking oncogene and growth factor research. Oncogenes confer growth factor autonomy on cells not only by coding directly for autocrine peptide growth factors or their receptors, but also by amplifying the mitogenic signals generated by a growth factor at its receptor (Waterfield et al., 1983; Doolittle et al., 1983; Downward et al., 1984).

The first peptides identified as participating in the autocrine pathway are the EGF related TGF-α growth factors There is a lot of data to indicate that human cancer cells produce and release TGF-α and have functional receptors for this peptide (Todaro et al , 1980, Marquardt and Todaro, 1982, Richmond et al , 1983) Other peptide growth factors that function via an autocrine mechanism in cancer cells include peptides related to platelet-derived growth factor (Huang et al , 1984), bombesin (Cuttitta et al , 1985) and TGF-β (Anzano et al , 1985). The autocrine mode of growth action however, is not limited to cancer cells Arterial smooth muscle cells from very young mouse or rat but not from the adult, secrete significant amounts of PDGF-like peptides into their extracellular medium (Seifert et al , 1984)

TGF-β and PDGF are both stored in circulating platelets, and are two of the most active factors regulating both anchorage-independent and anchorage-dependent growth of mesenchymal cells (Assoian et al , 1983) The kinetics of TGF-β monolayer mitogenicity in AKR-2B cells (Mouse embryo derived - normal fibroblasts) are delayed relative to stimulation with serum or other purified growth factors (Shipley et al , 1984) Leof et al , 1986, suggests that the monolayer mitogenic action of TGF-β on AKR-2B cells may be mediated to a large extent through induction of c-sis and PDGF autocrine activity Since most epithelial cells are unresponsive to TGF-β (Moses et al , 1985), and most lack PDGF receptors (Goustin et al , 1985), the lack of stimulation of epithelial cells by TGF-β may thus be explained Autocrine mechanisms for self stimulation may be of critical importance during the repair of tissue injury (Walker et al , 1984), as in wound healing and during the explosive growth of the early embryo (Seifert et al , 1984) only the inappropriate expression of an autocrine peptide may be pathological

The most recent version of the autocrine hypothesis includes the concept that malignant transformation may be the result not only of excessive production, expression and action of positive autocrine growth factors, but also the failure of cells to synthesize, express or respond to specific negative growth factors they normally release to control their own growth TGF-β is a bi-functional factor which can stimulate or inhibit cell growth

(Moses et al , 1985) The set of conditions in a cell determining whether the addition of TGF-β is positive or negative for growth is complex and cannot always be explained by differences in cell type, growth conditions or the concentrations of TGF-β (Roberts et al , 1985) The loss of negative growth control may result from a biochemical lesion in either the growth inhibitor, its receptor or the post receptor signalling pathway (See Section 1 10)

The autocrine hypothesis is therapeutically optimistic, as it allows the possibility of restoring growth control to cancer cells by replacement of a deleted or defective growth inhibitor

1.14 Background and purpose of the work described in this thesis.

The autocrine hypothesis just described has important implications for our understanding of the nature of cancer. It may also have practical implications for the technology of culturing human cells.

Routine methods for the culture of human tumor cells would be very valuable. For example, individual patients' tumor cells could be examined, following surgery, for properties such as response to particular chemotherapeutic agents, growth rate, antigen profile etc. Assessment of such properties for individual patients could help in providing more rational post-operative treatment of metastatic tumour deposits. Routine culture methods could also provide a wider range of established cell lines, which might provide experimental material more relevant to most human cancers than those lines currently available. Primary culture of tumor cells however, currently meets with limited success, (Dobrynin, 1963; Giard *et al.*, 1973), and establishment of permanent cell lines is a rare event; this is in contrast to the sad fact that many of the tumours concerned grow progressively in the patient.

The failure of many human tumors to grow progressively *in vitro* could be due to one or more factors, for example :

(i) Inadequate nutritional/hormonal environment *in vitro*;

(ii) Lack of a perfusion system in culture means build-up of waste, e.g. ammonia, lactate, CO_2, depletion of nutrients and periodic pH/temperature/nutritional shock when the cultures are fed;

(iii) Lack of interaction with other cell types and intercellular matrix *in vitro*.

(iv) Possible selection against tumour cells by aspects of the *in vitro* environment, e.g. subculturing methods.

Another possibility, which forms the basis for the investigations described here, is that progressive tumour growth may in some cases be dependent on substances produced in an autocrine fashion by the tumour cells (Sporn & Roberts, 1985). Such substances might be present at high local concentrations inside the tumor *in vivo*, but

may be diluted below effective levels in cultures of low cell density, as is usually the case in primary cultures

In 1979, Martin Clynes (unpublished observation) demonstrated that the human carcinoma line RPMI 2650 showed a marked density dependence i e , cell growth, as measured by colony forming efficiencies, was virtually eliminated below a certain critical cell density or "cut-off point" This observation suggested that an autocrine factor might be involved, and further support for this idea was provided by the same group in 1982 when McManus and Clynes, (1984) showed that feeder layers of RPMI 2650 growing in monolayer on the bottom of the culture dish markedly lowered the cut-off point of RPMI 2650 cells suspended in agar above the feeders This also showed that direct cell contact was not important in this feeder system

The purpose of the work described in this thesis, was to confirm these preliminary experiments, to develop an assay for detection of the feeder effect in cell-free feeder-conditioned medium, to attempt to purify and characterize the putative autocrine substance(s) and to determine if it/they were identical to any of the known growth factors, in particular the transforming growth factors TGF-α and TGF-β The possibility that the feeder effect was due to an in vitro artifact, such as removal of toxic materials from the medium, had also to be considered

SECTION 2

MATERIALS AND METHODS

2 Materials and Methods

2 1 Ultrapure Water

It is essential to use ultrapure water in cell culture The water used for preparation of all growth media and reagents, was passed through a millipore Milli-Q ultrapure water system This system consisted of a reverse osmosis system, which, with two prefilters removed ionic and non-ionic solutes, and a further purification stage with two-ion exchange filters, a carbon filter and a 0 22μm cellulose acetate filter The resulting water was of reagent grade and was regularly monitored by an on-line conducting meter (10-18 megohms/cm, was the acceptable resistivity)

2 2 Glassware

All items of glassware which came into contact with tissue culture medium and cells were put through a strict washing and rinsing cycle

Glassware containing waste medium from cells was autoclaved and rinsed with tap water Glassware and bottle caps were soaked separately in a non-toxic detergent, RBS-25, at 0 2% (v/v) in warm water 1-2 hours later, bottles were scrubbed out by hand and rinsed in tap water Three rinses in reverse-osmosis purified water were then followed by a final rinse in ultrapure water All glassware was dried and autoclaved ready for use

2 3 Sterilization

Water, glassware and solutions of temperature resistant chemicals (e g PBS A) were sterilized by autoclaving at 120^{o}C for 20 mins

Temperature labile chemicals, (e g enzymes, hormones, growth factors) were filter sterilized through 0 22μm sterile disposable filters (Millipore Millex-GV, for low protein binding) Growth medium and supplements were sterile on receipt

2 4 Growth Medium and Conditioned Medium (CM)

Growth media were made up as shown in Table 2 1 Before use, all media were sterility tested 10-15mls of medium was dispensed into sterile universals and incubated at 37°C for 3 days. This procedure was found adequate to detect for most forms of contamination Antibiotics were not used during routine cell culture 1ml Penicillin/Streptomycin (Gibco 043-05140 H) per 100mls of growth medium was used for all large scale animal cell culture (See Section 2 11)

Conditioned medium (CM) is medium in which cells have been growing for 24-48 hours Initially medium used for conditioning contained 5% foetal calf serum However, for subsequent purification of factors secreted by the conditioning cells, it was necessary to use serum-free medium Cells were generally grown in 25cm^2 flasks for 3-4 days until about 90% confluent at which stage waste medium was removed and cells were extensively rinsed with PBS at 37°C Serum-free medium was then added and conditioned for 24 or 48 hour periods at which time it was collected and centrifuged at 4000 rpm for 15 mins and stored at 4°C Subsequent batches of serum-free conditioned medium were also collected from these cultures for up to 6 days or until the cells began to detach See relevant sections for production of CM by large scale methods

2 5 1 Cell Lines

All cell culture work was carried out in a Class II downflow recirculating laminar flow cabinet (Microflow), with strict aseptic technique See Table 2 2 for details of cells cultivated

Cell lines were grown in 25cm^2 flasks (Cell Cult 32025) or 75cm^2 flasks (Costar 3075) in the recommended medium See Table 2 1 for details of medium and supplements Cells were fed every two to three days, or when a change in pH was observed (colour change of phenol red indicator in the medium) On reaching confluency, cells were subcultured

Table 2 1 . Growth Medium and Supplements

10X Media Stock	Media Supply Source	Sterile Ultrapure Water	1M Hepes** Sigma H-9136	7.5% $NaHCO_3$ BDH 30151	2mM L-glutamine Gibco 043-05030	MEM Vitamins (100X) Gibco 15F-3162	Sodium Pyruvate 100mM Gibco 043-1360	MEM*** NEAA (100X) Gibco 043-01140	Foetal calf serum Gibco 021-6010
MEM - Minimum essential Eagle's medium	Gibco* 042-02501	450 mls	10 mls	11 mls	5 mls	-	-	5 mls	25 mls
DME - Dulbecco's Modified Eagle's Medium	Gibco 042-02501	450 mls	10 mls	11.5 mls	5 mls	-	-	-	25 mls
SLM - Earl's Balanced Salt Solution	Gibco 042-4050	450 mls	-	17.5 mls	10 mls	11 mls	5.5 mls	22 mls	25 mls
RPMI-1640	Gibco 042-2511	450 mls	10 mls	11 mls	5 mls	-	-	5 mls	25 mls

* For details of chemicals and solutions from Gibco, refer to Catalogue or write to Gibco Ltd., Unit 4, Cowley Mill Trading Estate, Londonbridge Way, Uxbridge, UB8 2YG, Middlesex, England. Tel. (0895) 36355.

** Hepes : 4-(2-hydroxyethyl) -1-piperazineethanesulfonic acid

*** NEAA . Non essential amino acids

Note : All media were brought to pH 7.4-7.6 at room temperature with 1.5N NaOH or 1.5N HCl

Penicillin/Streptomycin (Gibco 043-05140H) Penicillin 10,000 IU/ml streptomycin 10,000 UG/ml was not added to growth medium for general cell culturing. P/S was used in Agar assays and large scale cell culture at 1ml per 100 mls medium

2 5 2 Sub-culture of Cell Lines

Animal cells grow attached to the bottom of culture vessels, immersed in growth medium and supplemented with 5-10% foetal calf serum Upon reaching confluency, cells were enzymatically detached using 0 25% Trypsin (Gibco 043-05090) in PBS* (Oxoid BR 14a) All solutions were pre-warmed to 37°C

Method

Waste medium was removed from the cells which were then rinsed with PBS or trypsin (for every 10mls of growth medium, 2mls of PBS or trypsin solution was used) 2mls of 0 25% trypsin was then added and incubated with the cells for 10-15 mins at 37°C Once a single-cell suspension was obtained, growth medium containing foetal calf serum (foetal calf serum contains a trypsin inhibitor) was added to the trypsin in a 1 1 25 ratio of trypsin growth medium

The cell suspension was then transferred to a sterile universal (Sterilin 128A) and centrifuged at 900 rpm for 5 mins The cell pellet was then resuspended in 5mls growth medium and a cell count obtained (See Section below) Cells were then diluted to 10^3 or 10^4 per ml of growth medium 10mls of cell suspension was added to 25cm^2 flasks amd 20mls to 75cm^2 flasks Cells were then incubated at 37°C

* PBS type A, which is calcium and magnesium free

2 5 3 Cell Counting

A sample of single cell suspension was applied with a pasteur pipette to a Waber haemocytometer pipette (improved Neubawer) so that it was held in the counting area between the slide and a cover slip A series of gridded lines were observed at 100 X by microscope (Fig 2 1) All those cells found in the 16 squares of the 4 corner grids were counted Cells found lying on any two of the 4 outer lines were also counted and the total was estimated (X) The average number of cells in the 4 corner grids was then estimated and multiplied by 10^4 ($X10^4$) This gives number of cells per ml of solution

Adjustments for non-viable cells was determined by a dye exclusion test where a 1/5 dilution of trypan blue (Gibco 525) to cell suspension was prepared Those cells which take up the blue dye are non-viable] The area enclosed below the cover slip and the 16 squares is 0 1mm^3

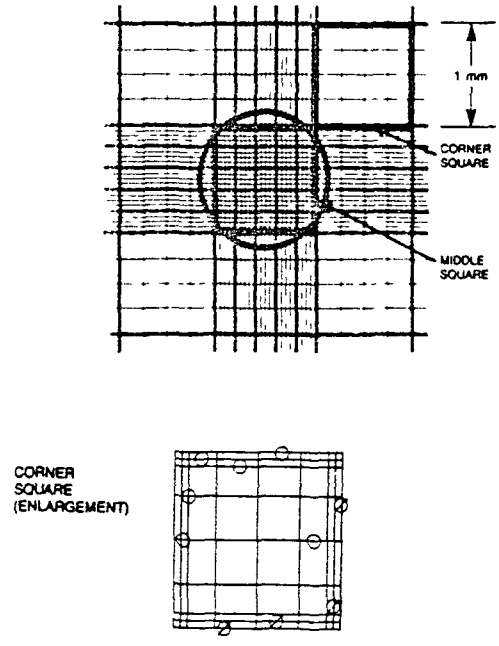


Fig. 2.1 Diagram of standard Haemocytometer gridded area.

Table 2 2 . Cell Lines

Cell Line	Source	Chief Use
RPMI-2650	ATCC (CCL 30)	Production of CM.* Clonogenic/feeder assay Autostimulatory assay Large scale cultivation.
NRK	Ian Pragnall Beatson Institute for Cancer Research Glasgow	TGF assay Mycoplasma test
NRK-49F	Ian Pragnall	TGF-β assay
AKR-2B	Ian Pragnall	TGF-β assay
663 + N	Ian Pragnall	TGF-α production
A431	ATCC CRL-1555	^{125}I-EGF Radioreceptor assay
T47D	Dr. Denis Headon, Dept. of Biochem U.C.G , Galway	^{125}I-EGF Radioreceptor Assay

*CM - Conditioned Medium

Details of Cell Type	Growth Medium (+5% FCS)	Reference
Human squamous epithelial cell carcinoma of the nasal septum. Very small cells which grow in dense clusters	MEM	(Moorhead, 1965) (Moor & Sandberg, 1963)
Normal rat kidney fibroblasts	DME	
Clone of NRK	DME	
Normal embryo fibroblast of the mouse	DME	
Myeloproliferative sarcoma virus transformed NRK cells	DME SLM	(Koury & Pragnall, 1982)
Vulval carcinoma (human)	DME	(Haigler et al., 1978)
Breast carcinoma (human)	RPMI-1640	

2 6 1 Long term storage of Animal Cells

New cell stocks received from the ATCC* (American Type Culture Collection) or other sources, were immediately grown up and then frozen in liquid nitrogen for long term storage Only those cells which were in subconfluent exponential growth phase were suitable for long term storage A cell suspension of at least 2 x 10^6/ml of growth medium was required 1ml of a 10% (v/v) solution of DMSO (BDH 282166) in growth medium was added to 1ml of cell suspension in a slow dropwise manner with continual mixing 1ml of this mixture was transferred by pipette to sterile cyrotubes (Sterilin 35001) The cells were then slowly frozen in the vapour phase of liquid nitrogen for 3 hours, then stored in liquid nitrogen storage container until required

2 6 2 Recovery of Frozen Cell Stocks

The required vials of cell stocks were removed from the liquid nitrogen storage container and quickly thawed at 37°C Once thawed, the cell suspension was transferred to a sterile universal with 5mls of growth medium and centrifuged at 900 rpm for 5 mins The cells were centrifuged to remove the cryo-protective agent DMSO which may be toxic to the cells The cell pellet was resuspended in 5mls of growth medium and transferred to a 25cm^2 flask The cells were incubated at 37°C for 24 hours Waste medium was then replaced with 10mls of fresh growth medium

* American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852

2 7 Monolayer Assays

Monolayer assays were set up to determine the colony forming efficiencies of various cell lines Monolayer assays were also used to test different batches of serum

Serial dilutions of single cell suspensions were prepared in growth medium with 1% or 5% foetal calf serum A range of cell concentrations from 50 to 10^5 cells per plate were usually set up Assays were set up in 30mm plates (Cell Cult 31030) with 3mls of growth medium and incubated at 37°C and 5% CO_2, 95% air for up to 10 days The assays were then stained and colony counts were

estimated manually or by image analysis (CF Section 2.24.1). The colonies were stained using the Leishmanns staining method. See following method.

Note : 35mm plates were also used (Corning 25000)

2.8 Leishmann's Stain (BDH-34042)

Monolayer assays were stained as follows. Waste medium was removed and cells were gently rinsed in PBS. 1ml of Leishmanns stain (0.3% in absolute methanol, filtered through Watmann filter paper No. 1 before use) was added to 30mm plates and left for 5 mins. 1ml of distilled water was added for a further 5 mins. This was then removed from the cells which were rinsed well in distilled water and then dried. Colony counting was carried out manually or by image analysis (CF Section 2.24.1).

2.9 Mycoplasma detection

Unlike contamination by bacteria or fungi, mycoplasma contamination of cells is not obvious. Cells which become infected continue to grow, but their growth rate slows and they appear granular. They can remain like this for some weeks and finally the cells die. Routine mycoplasma examination of cell lines was carried out by a fluorescent staining method, specific for DNA (Chen, 1977). The method used was greatly modified from those in the literature. Initially, each cell line was stained up for mycoplasma, but we found a variation in background between the cell lines. This variation in background may have been due to cell damage caused during fixation. We found that NRK cells were very suitable for the fixation proceedure used.

Method

Mycoplasma-free stocks of NRK cells were grown overnight on sterile cover slips in 1ml of medium at 10^3 cells ml^{-1}, at 37°C and in an atmosphere of 5% CO_2. 2mls of growth medium from cell lines to be tested and 2mls of growth medium from control (mycoplasma-free NRK) cell line were then added to individual cover slips. These cover slips were then incubated for a further 4-5 days at 37°C and 5% CO_2.

Fixing and Staining of Cells

The cells on the cover slips were gently rinsed in PBS A Cells were then fixed to the coverslips by immersion in acetone methanol at a ratio of 1 1, at -20°C, and left for 6 mins The coverslips were removed and thoroughly rinsed in PBS Hoechst 33258, (Sigma) at 0 05 g ml^{-1} in PBS was added to the cells, so that they were completely covered, and left for 10 mins in a light-proof sealed container at room temperature Excess stain was removed by rinsing in water The coverslip was mounted and sealed onto a glass slide (with the cells between the two glass surfaces)

The cells were then observed under oil immersion using 405nm light Definite pin-points of fluorescence in the cell cytoplasm indicated the presence of mycoplasma contamination Contaminated stocks were disposed of and fresh cultures grown up from stock

2 10 Growth Factor Assays

2 10 1 RPMI-2650 Feeder Assay

It was possible to set up two populations of RPMI-2650 cells in a double layer agar assay system One population of cells was grown in monolayer on the base of the plate (feeder layer), while the second population was suspended in the upper layer of the double layer of agar

Method

Feeder layer

Single cell suspensions of RPMI-2650 cells were seeded onto 30mm plates (Cell Cult 31030) in 3mls of growth medium with 5% FCS In a typical experiment, 6 replicates of the following cell concentrations were set up and allowed to attach overnight at 37°C in 5% CO_2

[0, 10^2, 10^3, 5 x 10^3, 10^4, 5 x 10^4, 10^5, 5 x 10^5]

The following day, waste medium was removed from the cells just prior to the addition of a specially prepared agar growth medium mixture

Preparation of agar growth medium and cells for the upper layer

A buffered 2X medium as follows and 100mls of 1 4% agar (Bacto Difco) were prepared in advance

Buffered 2X medium -	MEM [10X]	20 0mls
	Sterile ultrapure H_2O	76 0mls
	1mM HEPES	4 0mls
	7 5% $NaHCO_3$	2 2mls
	1 5N NaOH/1 5N HCl(to ph 7 4)	2 0mls

The agar was melted and transferred to a water bath at 41°C*

The agar growth medium (AGM) was prepared as follows

AGM -	Buffered 2X medium	52 0mls
	FCS	20 0mls
	Penicillin/Streptomycin	1 0mls
	L-Glutamine	1 0mls
	Growth medium	16 0mls

The agar and AGM solutions were allowed to equilibrate to 41°C, then 50mls of 1 4% agar was mixed with 90 mls of AGM. 2mls of this mixture was quickly added onto each plate of RPMI-2650 feeder layers and allowed to set at room temperature (waste medium should be removed) The remaining AGM + Agar was returned to the water bath at 41°C

A single cell suspension of RPMI-2650 cells was then prepared at 9 x 10^4 cells per ml in growth medium Dilution in AGM + Agar in a ratio of 1 25 mls of cells 2 5mls of AGM + agar gave 3 x 10^4 cells per ml 1ml of this mixture was quickly added to the required plate Triplets were set up for each feeder cell concentration with or without cells in the upper layer

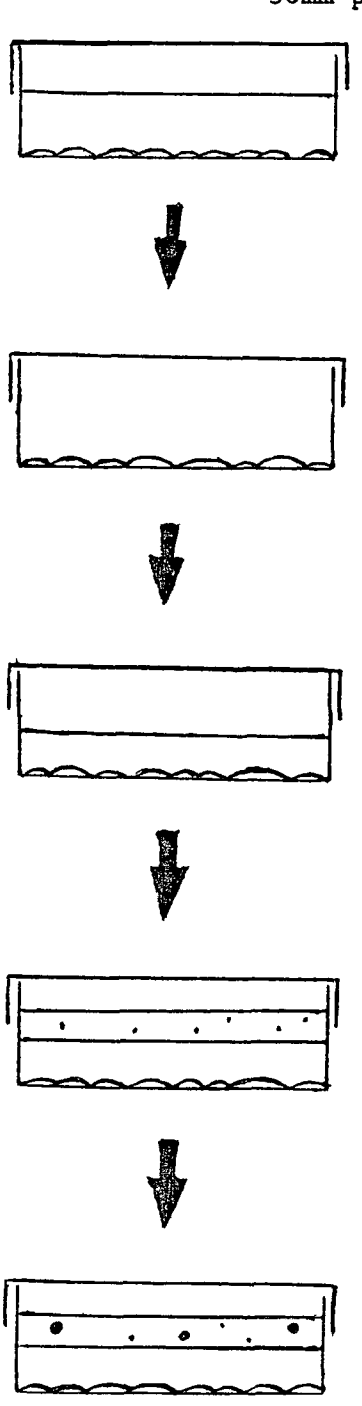
The plates were allowed to set at room temperature for about 10 mins Small dishes of water were included with each assay to prevent drying out of agar, the assay was then carefully covered with tin foil and incubated at 37°C and 5% CO_2 for up to 14 days Colonies greater than 50 m in diameter were counted and the average colony forming efficiencies estimated (CF Section 2 24 2)

* The temperature of AGM is very important At 41°C, the agar remains liquid and will not scald the cells when they come in contact with one another

See Fig 2 2 for diagrammatic representation of Feeder Assay System

See Plate 1 and 2 for photographs of RPMI-2650 cells in monolayer and in agar

Fig. 2.2 : RPMI Feeder Assay



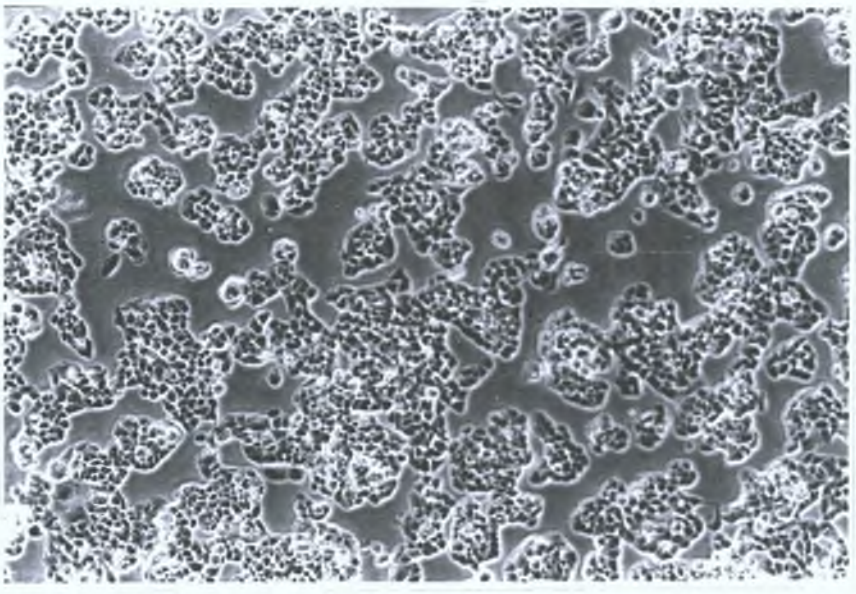


Plate 1 : RPMI-2650 cells subconfluent culture in monolayer Ph 1 (150X) magnification.

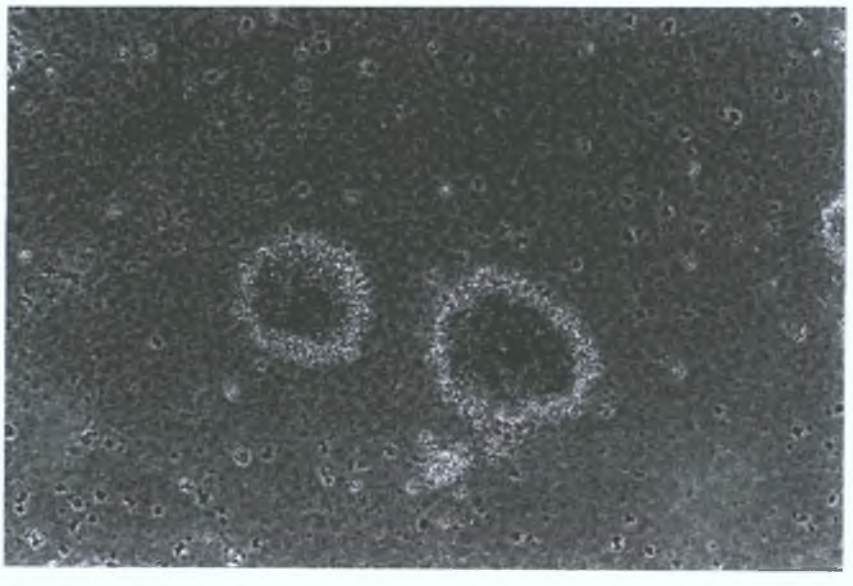


Plate 2 : RPMI-2650 anchorage-independent growth in agar Ph 1 (150X) magnification.

2.10.2 Transforming Growth Factor Assay (TGF)

This assay was set up on the basis that if TGFs are present they will induce non-transformed cells to form colonies while suspended in a semi-solid matrix like agar (De Larco and Todaro, 1978). A series of experiments using various normal fibroblast indicator cell lines and several optimization experiments were set up during the development of the TGF assay. The following protocol is the optimized version of the TGF assay routinely used in this thesis.

Method

50mls of 1.548% Agar (Bacto Difco) was melted and brought to 44°C. It was then mixed with the following agar medium mixture also at 44°C.

AGM -	Buffered 2X medium	50.0 mls
	FCS	10.0 mls
	1mM HEPES	2.0 mls
	Penicillin/Streptomycin	1.0 ml
	L-Glutamine	1.0 ml
	7.5% $NaHCO_3$	1.0 ml
	Growth Medium	14.0 mls

(Buffered 2X medium used here is similar to that used in Section 2.10.1 except that it is made up with DME [10X] instead of MEM [10X]). The agar and AGM was mixed well and 1.5mls was quickly transferred to 30mm plates. The remaining AGM and agar was returned to the water bath at 41°C and the plates were allowed to set at room temperature.

0.6mls of test samples and controls were then added to sterile universals. A single cell suspension of NRK cells at [6.6X] the final concentration per plate was prepared (4×10^4 cells/ml of growth medium). This gave a final concentration of 6×10^3 cells per plate. 0.15ml of 4×10^4 cells per ml was added to 0.6ml of sample in the sterile universal. 0.75 mls of AGM + agar was quickly added to the sample and cells. These were mixed well and the total volume of 1.5mls was transferred to the pre-set base layer. Growth medium was set up as a control. Small trays of water were added to each assay which was then covered with tin foil

and incubated at 37°C and 5% CO_2 for up to 8 days Colonies greater than 50μm were then counted and the colony forming efficiency determined (CF Section 2 24 2)

Note Samples were usually set up in triplicate as follows

2mls of test sample (CM/control)
0 5ml cells
2 5mls AGM + agar

These were mixed well and 1 5mls was quickly added to each of three plates where the base layer was already set It is important to use a suitably tested batch of serum in the TGF assay See Plate 3 and 4 for photographs of NRK in agar and monolayer culture

2 10 3 TGF-β assay

A modification of the TGF assay was developed which was used to determine the presence of TGF-β in samples The indicator cell line used here was NRK-49F It was used at a concentration of 2 x 10^4 cells/plate (i e 1 3 x 10^5/ml stock concentration) The formation of large colonies in the presence of 2ng/ml EGF after 9-10 days incubation at 37°C and 5% CO_2 indicated the presence of TGF-β like growth factor(s) Colonies > 50μm in diameter were counted and the colony forming efficiency determined (CF Section 2 23 2) See Plate 5 for photograph of NRK-49F in monolayer culture

2 10 4 RPMI-2650 Autocrine Assay

RPMI-2650 autocrine assay follows a similar protocol to that for TGF and TGF-β assays However, 3 x 10^4 cells per 30mm plate (2 x 10^5 cells/ml growth medium, stock) were used The assay was incubated at 37°C and 5% CO_2 for up to 10 days at which time colonies > 50μm in diameter were counted as in Section 2 24 2

2 11 Large Scale Cell Culture

Growth factor(s) secreted by RPMI-2650 cells were found at very low levels in CM tested in the autostimulatory and TGF assays For the purpose of purification and characterization of these growth

factor(s), we found it necessary to produce large volumes of CM The following methods were examined

2 11 1 Roller bottle

Bellco roller bottles with an internal surface area of 670cm^2 were washed dried and autoclaved and brought to 37°C before use Roller bottles were first rinsed with about 10mls of growth medium then seeded with 100-120mls of growth medium with 5% foetal calf serum and 1ml of penicillin/streptomycin (P/S) and 5 x 10^5 cells ml^{-1} of the required cell type The roller bottle head space was gassed with a steady flow of 5% CO_2 Air, gas mixture for up to 10 seconds The roller bottle was then transferred to a Bellco roller bottle apparatus at an initial speed of 0 25 rpm for 24 hours and then increased to 0 75 rpm To achieve a satisfactory well-dispersed monolayer, it was essential to bring roller bottle and medium to 37°C before use

These conditions yielded an 85-95% confluent monolayer after 3 days, at which time the culture was set up for collection of CM

Preparation of CM

Waste medium was removed and the cells rinsed twice with 50mls of PBS (at 37°C) 100mls of medium, without foetal calf serum, with 1ml P/S, at 37°C was added and conditioned by the cells for 24 hours The medium was then collected, centrifuged at 4000 rpm for 15 mins and stored at 4°C 5 subsequent 24-hour batches of CM were usually collected, at which time the cells were beginning to detach The remaining cells were trypsinized as described in Section 2 5 2, to remove them before autoclaving and washing of the roller bottle could be carried out prior to subsequent use

2 11 2 Suspension Culture

1 litre and 500ml suspension culture vessels were used (Techne, Cambridge, U K) These employed glass enclosed magnetic bars suspended from the lid of the vessel, this system prevents grinding of the cells on the bottom of the vessel See Fig 2 3 for diagram of suspension vessel

Method

Culture vessel and magnetic stirrer were washed and dried (see Section 2 2) The glass surfaces which would come in contact with the cells were siliconised to prevent cell adherence Sigmacote (Sigma SL-2) was added to the vessel and swirled around, until all internal surfaces and stirrer were fully coated The excess solution was poured off (and stored for subsequent runs) The vessel was dried, rinsed 3 times in ultrapure water, dried and autoclaved Before use, the vessel was rinsed in growth medium and brought to 37°C

100-120mls of MEM with 5% foetal calf serum and 1ml P/S with 5 x 10^5 RPMI-2650 cells was added to the 500ml vessel or 200mls of the above to the 1 l vessel The head space was gassed with a steady flow of 5% CO_2 Air, gas mixture for up to 10 seconds The cells were stirred at 30 rpm at 37°C on a Techne stirrer base (MCS-1044) It was necessary to feed the suspension cultures every 2 days by replacing half the growth medium as follows The vessel was removed from the stirrer base and the cells allowed to settle by gravity It was then possible to remove 50% waste medium from above the cells with a sterile pipette The same volume of growth medium with 5% foetal calf serum and P/S was then added

Cell counting and CM collection

A 2ml homogeneous sample of cells was carefully removed from the suspension culture vessel and centrifuged at 900 rpm for 5 mins Waste medium was removed and 5mls of 0 25% Trypsin was added to the cell pellet This was incubated at 37°C until a single cell suspension was obtained The cells were then counted on a haemocytometer, see Section 2 5 3 This method of counting was found to give consistently accurate results

After 5 or 6 days in culture, the suspension culture yielded > 10^8 cells from a 500ml vessel or twice this concentration from a 1 l vessel At this stage, the culture was ready for the collection of CM The vessel was transferred to the laminar flow cabinet where the cell clumps settled on the bottom of the vessel Waste medium was carefully removed by a pipette until about 20mls remained Care was taken not to disturb the cells The cells were

then transferred to a universal The vessel was rinsed twice with 15mls PBS which was also transferred to a universal The cells were pelleted at 900 rpm for 4 mins The cell pellets were pooled and rinsed twice in 15mls PBS This helped remove most traces of foetal calf serum The cell pellet was finally resuspended in 100mls MEM for a 500ml vessel or 200mls MEM for a 1 litre vessel, without serum, with 1ml P/S per 100mls growth medium at 37°C CM batches were collected at 24 hour or 48 hour intervals for up to 8 days See Plate 6 and 7 for photographs of RPMI-2650 in suspension culture

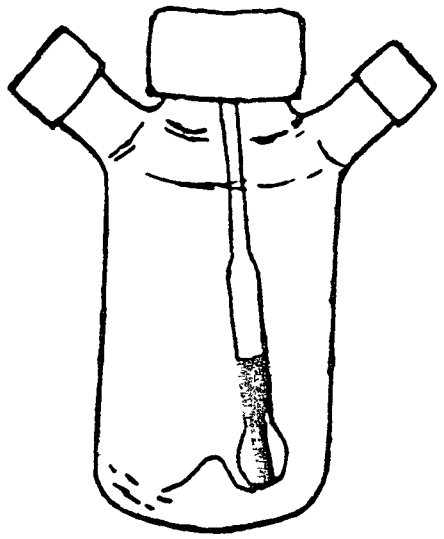


Fig. 2.3 · Suspension Culture Vessel

2 11 3 Microcarrier Culture

The culture of anchorage dependent cells on dextran beads allows a high ratio of cells to medium to be achieved This system consists of a spinner vessel with a stirrer, as in suspension cultures, and small beads kept in suspension by gentle stirring This system was set up on a trial basis, using 500ml spinner vessels CM was collected from RPMI-2650 cells grown on a variety of microcarrier beads available on the market

Cytodex 1	(Pharmacia II 32125)
Cytodex 2	(" KF 35315)
Cytodex 3	(" HL 24905)
Gelı-bead	(KC Biological)

Method

0 3g of each microcarrier was hydrated in 30mls of PBS for 3 hours The beads were washed once with 30mls of PBS and autoclaved The PBS was then replaced with 30mls of growth medium with 5% foetal calf serum at 37°C This was then transferred to the suspension vessel A 10ml single cell suspension of RPMI-2650 cells at 6 x 10^6 per ml in growth medium at 37°C was then added to the beads in the suspension vessel The head space was gassed with a steady flow of 5% CO_2 air for up to 10 seconds The culture vessels was then placed on a stirrer base with intermittent stirring at 37°C (30 seconds at 40 rpm every 4 1/2 mins) This allowed the cells to attach evenly to the beads Intermittent stirring was continued for 3 hours, at which time the volume of medium was brought to 100mls and the culture turned to continuous stirring at 30 rpm (The speed at which the beads were stirred was that speed at which the beads remained in suspension without clumping) A small sample of beads was removed daily to examine the extent of cell growth

Note All glassware surfaces which came in contact with the cells and beads were siliconised (as already described for suspension cultures)

CM Collection

When all the beads were about 90% confluent, they were allowed to settle in the culture vessel About 80mls of waste medium was gently removed with a sterile pipette The beads were then transferred to a universal and the vessel was rinsed twice with 15mls PBS which was also transferred to a universal The beads were gently pelleted at 800 rpm for 4 mins, and all pellets were pooled The beads were rinsed twice with 15mls PBS The pellet was then resuspended in 100mls growth medium without serum with 1ml P/S, at 37°C This was then transferred to the culture vessel and stirred at 30 rpm at 37°C CM was collected at 24 hr and 48 hr intervals for up to 7 days (An accurate method for cell counting was not determined) See Plate 8 and 9 for photographs of RPMI-2650 cells in microcarrier culture

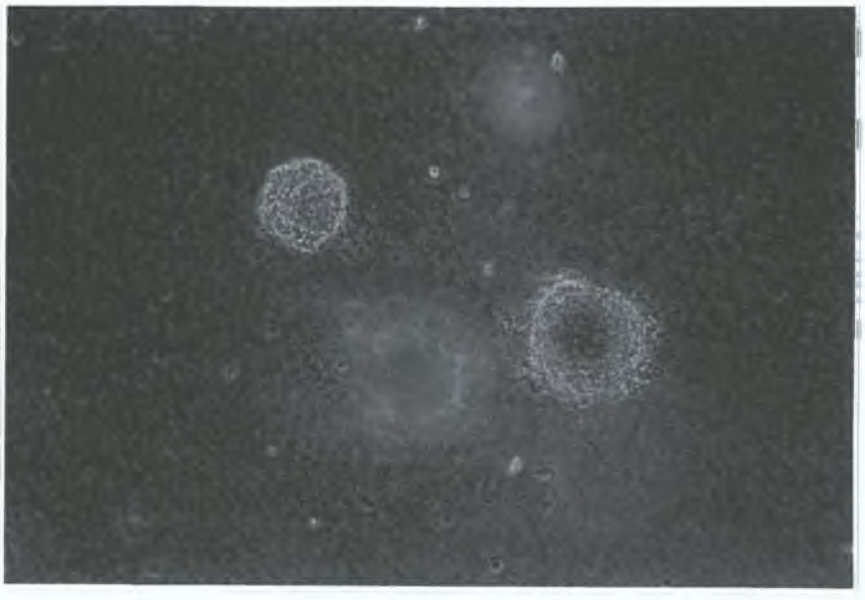


Plate 3 : NRK anchorage-independent-growth in agar (TGF assay) Ph 1 150X magnification.

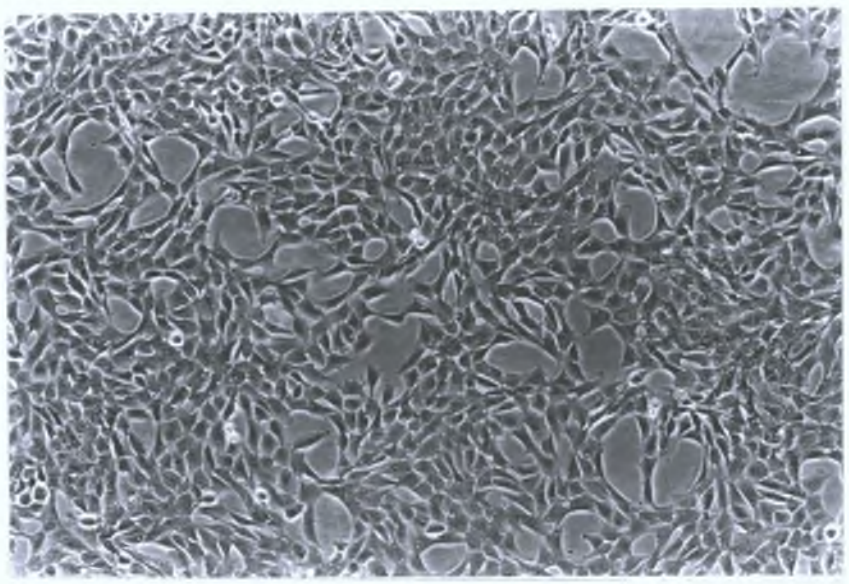


Plate 4 : NRK monolayer culture.
Ph. 1. (150X) magnification.

Plate 5 : NRK-49F monolayer culture.
Ph. 1. (150X) magnification.

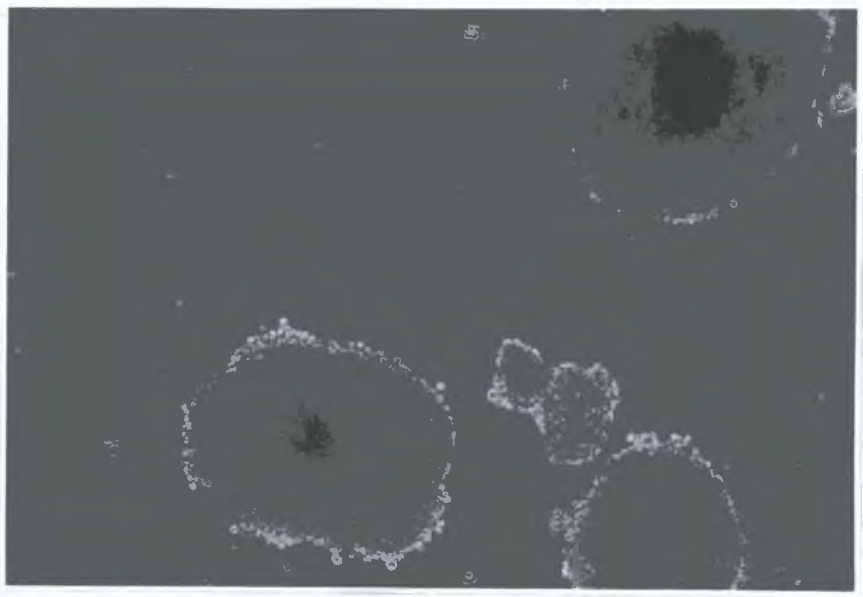


Plate 6 : Growth of RPMI-2650 cells in suspension culture
Ph. 1. (150X) magnification.

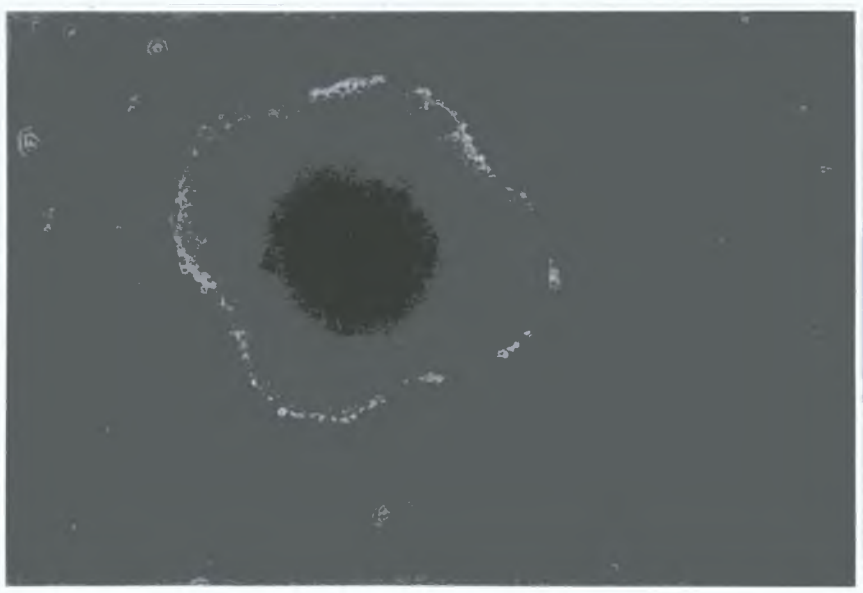


Plate 7 : Large RPMI-2650 colony in suspension culture :-
0.55-0.6mm diameter. Ph. 1. (150X) magnification.

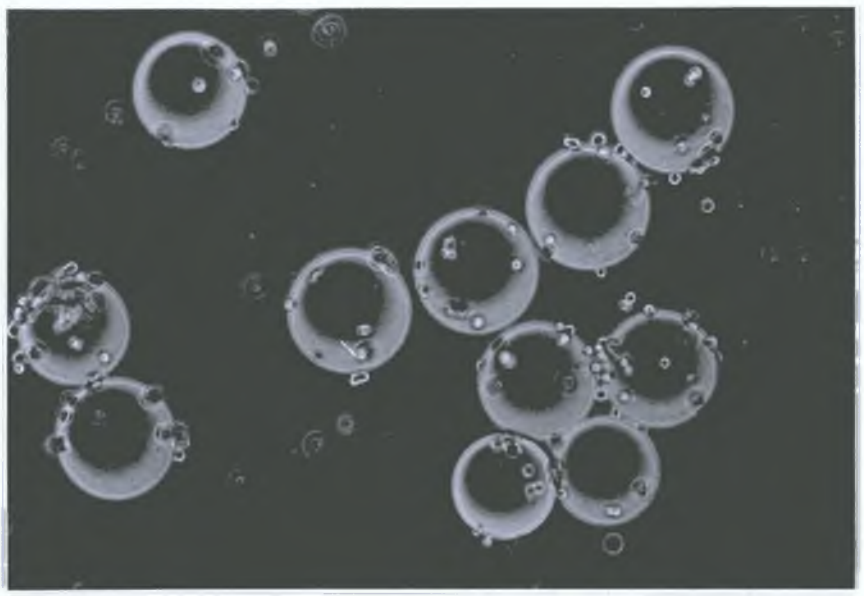


Plate 8 : Growth of RPMI-2650 cells in microcarrier culture (Cytodex 3) - 3 hrs Ph.1. (150X) magnification.

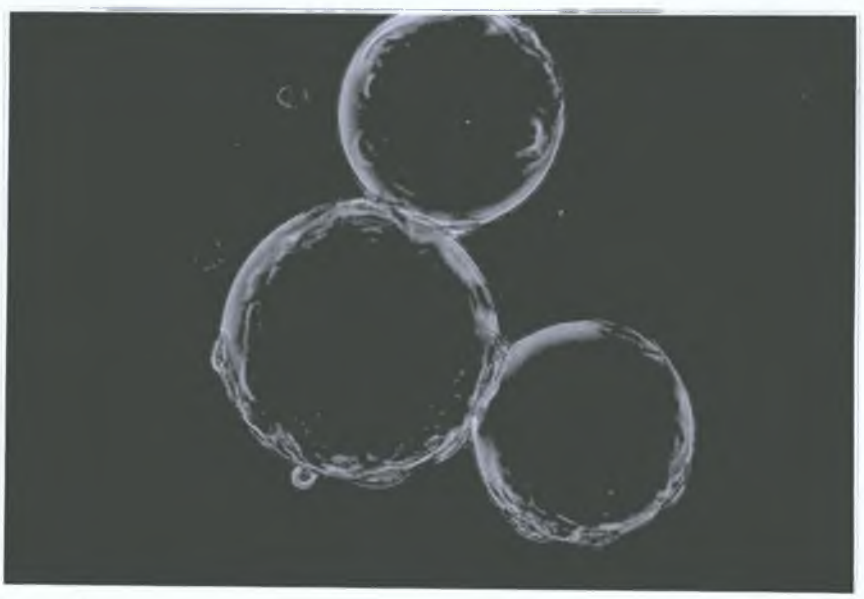


Plate 9 : RPMI-2650 cells in microcarrier culture (Cytodex 3) 24 hrs. Ph. 2. (300X) magnification.

2 12 Ultrafiltration

Concentration of large volumes of CM was carried out by ultrafiltration The stirred cell method was used (Amicon No 8499 - 400ml capacity chamber) Standard procedures were used in setting up the ultrafiltration cell Various molecular-weight cut-off membranes were used

YM 30	(Amicon)	30,000 cut-off
YM 10	(Amicon)	10,000 cut-off
YM 5	(Amicon)	5,000 cut-off
YM 2	(Amicon)	1,000 cut-off

Method

CM batches were ultrafiltered through a YM 2 membrane until a [10X] concentrate was obtained The retentate and samples of filtrate and unconcentrated CM were then filter-sterilized and assayed for autocrine and TGF activity The 1000 retentate, IR, was used for most purification procedures

Ultrafiltration of RPMI-2650 CM through a series of membranes of various molecular-weight cut-offs was used to help determine the relative size of the autocrine and TGF activity molecule(s)

Method

Each of 4 batches of 130mls of CM was ultrafiltered through one of the 4 membranes [10 X] retentates were obtained Samples of retentates and filtrates were collected A fifth batch of CM was processed as follows

The YM 30 filtrate was passed through YM 10 from which a [10 X] retentate was collected The YM 10 filtrate was then passed through YM 5 and a [10 X] retentate was collected The YM 5 filtrate was finally passed through YM 2 until a [10 X] retentate was obtained

All of the above retentates and filtrates were centrifuged at 4000 rpm for 15 mins and filter sterilized Samples were then stored at 4°C and subsequently assayed for TGF, TGF β and autocrine

activity, and for protein content

The following abbreviated versions of ultrafiltered CM fractions have been used in experimental detail

Conditioned Medium	-	CM
30,000 retentate	-	30 R
30,000 filtrate	-	30 F
10,000 - 3000 retentate	-	10 - 30R
10,000 retentate	-	10 R
10,000 filtrate	-	10 F
5,000 - 10,000 retentate*	-	5 - 10 R
5,000 rententate*	-	5 R
5,000 filtrate	-	5 F
1,000 - 5,000 retentate	-	1 - 5 R
1,000 retentate	-	1 R
1,000 filtrate	-	1 F

Note All retentates were ultrafiltered to 10 X, unless otherwise stated

* It was found that the protein content of 5 R retentate was lower than the protein content of 30 R, 5 R and 1 R retentates This indicated that this particular batch of YM 5 membranes may have been leaky to proteins

2 13 Dialtrafiltration

Before fractionation of CM by column chromatography could be carried out, it was necessary to make the CM up in 1M acetic acid, the elution buffer for Bio-Gel P-60 and P-30 This was generally achieved by dialysis against 1% acetic acid to reduce the salt content, followed by lyophilization The sample was then dissolved in 1M acetic acid This was a lengthy process The time involved in dialysis was reduced by dialtrafiltering the CM

Method

A 10ml sample of [>50 X] CM was brought to 100mls with 1% acetic acid The sample was ultrafiltered to 10mls through YM 2 The 10ml retentate was again brought to 100mls with 1% acetic acid and ultrafiltered. This process was repeated up to 4 times The final 10ml retentate was then lyophilized and reconstituted in 1ml 1M acetic acid The sample was centrifuged at 4000 rpm for 15 mins to remove acid insoluble protein and fractionated by column chromatography

2 14 Dialysis

10mls samples of concentrated CM was extensively dialysed against 10 volumes of 1% acetic acid A low molecular weight, benzoylated, 1,200 cut-off tubing was used (Sigma D7884)

Before use, the required length of tubing was boiled for about 4 mins in 10mM EDTA It was then washed in distilled water Dialysis was carried out at 4°C The acetic acid was changed twice daily for 3 days

CM was then removed and centrifuged at 400 rpm for 15 mins to remove insoluble protein and lyophilized The protein was then reconstituted in the required buffer For column chromatography 1M acetic acid was used, for HPLC 0 1% TFA and for all bioassays and ^{125}1-EGF radioreceptor assay, growth medium

2 15 Lyophilization

Lyophilization of CM at various stages of purification was carried out, using a 4 5 L Consol freeze dryer (Virtis) Samples were routinely frozen to -30°C overnight The freeze drying cycle was then commenced and generally lasted for 36 hours

A solvent trap, based on liquid nitrogen, removed harmful solvents from the vacuum before they reached the pump All HPLC fractions contained a mixture of TFA and acetonitrile and were freeze dried using the liquid nitrogen solvent trap

2 16 Bio-Rad Protein Microasay

0 2ml dye reagent concentrate (Bio-Rad 500-0006) was added to 0 8ml of standards and appropriate dilutions of samples with either MEM or 1M acetic acid in clean Eppendorfs Reagent and samples were then mixed thoroughly

A standard curve using BSA (Sigma A7031) 1- 25 g ml^{-1} of working buffer was prepared for each assay Absorbance at OD_{595} was read for all standards and samples. Readings were taken after 5 mins and before 60 mins Protein concentration of samples were read from the standard curve

2 17 1 Acid Stability

Samples of CM were brought to pH 3 6 with 1 5N HCl and left for 1 hour at 37°C They were then brought to pH 7 5 with 1 5N NaOH Acid unstable protein was removed by centrifugation at 4000 rpm for 15 mins

2 17 2 Dilutions

Samples of CM were diluted as required with growth medium without foetal calf serum Samples of CM concentrated to [10X] by ultra-filtration were filter sterilized and diluted to [X] before assaying as required

2.18 Column Chromatography

Fractionation of RPMI-2650 and 663+N CM was carried out by column chromatography using Bio-Gel P-60, 100-200 mesh (Bio-Rad 150 1640) and Bio-Gel P-30, 100-200 mesh (Bio-Rad 150 1340) gel bed. This procedure gave an approximate molecular weight of the growth factor(s) present in CM. 1.5 x 90cm columns were set up according to standard requirements using a downward flow system, equilibrated with 1M acetic acid at room temperature. Bio-Gel P-60 was run at 5mls per hour and Bio-Gel P-30 was run at approximately 10mls per hour, fractions of 8mls were collected on a fraction collector (LKB 2070 Ultrorack II). A steady flow of buffer was maintained by use of a peristaltic pump (Pharmacia P-1). The void volumes were determined using Blue Dextran (Sigma 0-5751) and the column calibrated with the following molecular weight markers : BSA (Sigma A-8531) 66kDa; Carbonic anhydrase (Sigma C-5024) 29kDa; Cytochrome C (Sigma C-7150) 12.4kDa; and Bovine aprotinin (Sigma A-3886) 6.5kDa. (See Fig. 2.4 for standard curve).

Sample preparation

CM from RPMI-2650 or 663+N cells was concentrated by ultrafiltering through a YM 2 (1000 cut-off) membrane. [500 X] samples were prepared from RPMI-2650 CM and [200 X] samples were prepared from 663+N CM. Samples were then extensively dialysed against 1% acetic acid, lyophilized and reconstituted in 1M acetic acid. The sample was then centrifuged at high speed to remove particulate and insoluble protein before being applied to the column.

Note : The concentration factor of CM samples differed for most column runs.

Fraction analysis

Up to 30 fractions (8 mls) were collected. 0.8ml samples were removed for protein analysis using the Bio-Rad Microassay (Section 2.16). All remaining fractions were then lyophilized and reconstituted in 3mls growth medium - FCS. The fractions were serially filter-sterilized through a Millex-GV filter and assayed for TGF, TGF-β and autocrine activity.

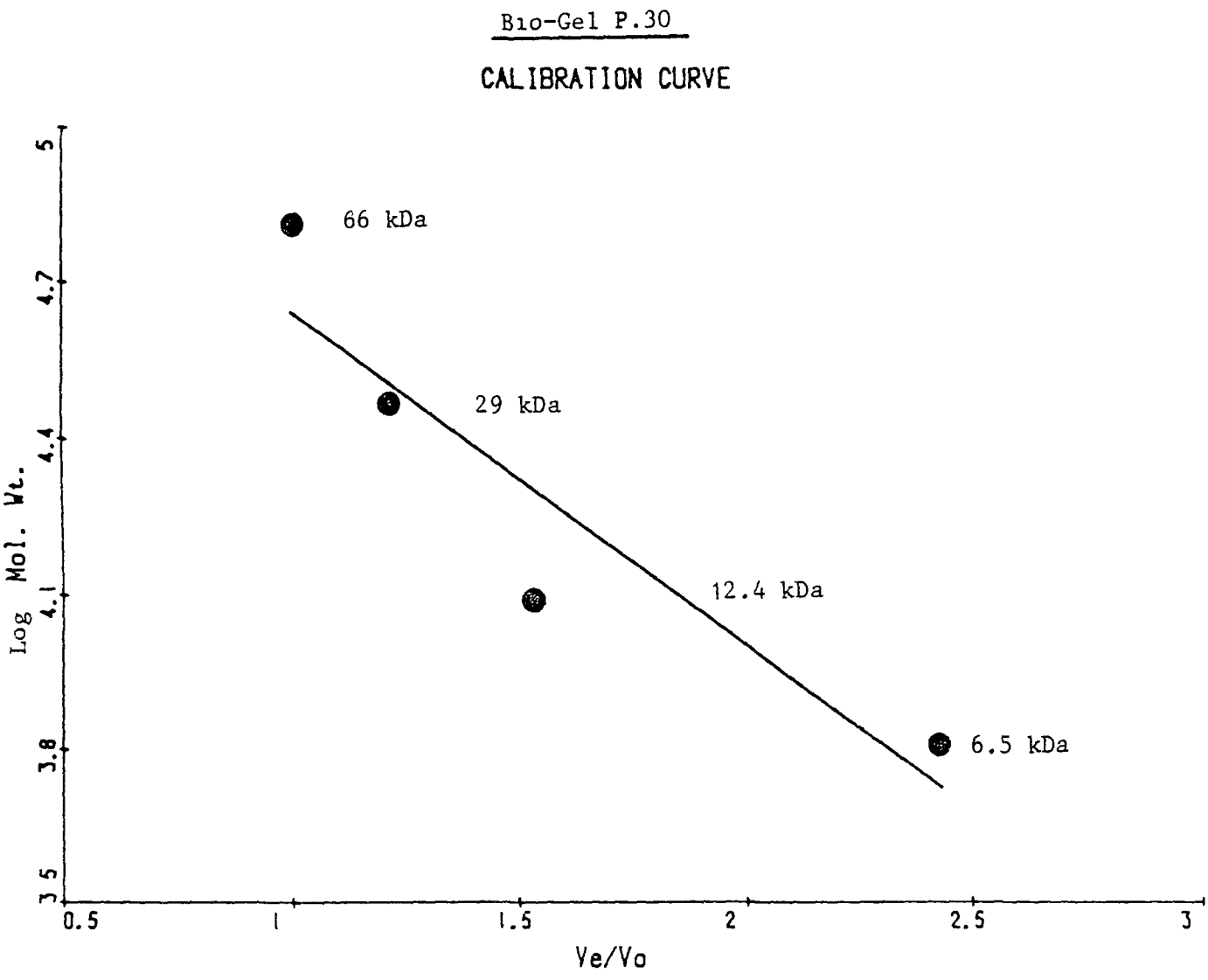


Fig. 2.4 Bio-Gel P 30 calibration curve

Average fraction vol = 7.83 mls

Void volume = 52 mls

Analysis of the fractions in the ^{125}I-EGF radioreceptor assay was carried out on lyophilized fractions which were reconstituted in about 200µl of growth medium without FCS (Section 2 20)

2 19 ^{3}H-Thymidine incorporation Assay

Cell growth was measured by the extent of ^{3}H-thymidine (Amersham AD438D) incorporation into DNA by various treatments NRK cells were used in a standard assay to determine the elution profile of RPMI-2650 CM mitogenic activity from Bio-Gel P-30

Method

Cells were set up at 10^{4} cells per 16mm well in 24 well plates (Costar 3524) in 1ml growth medium with 5% foetal calf serum Plates were incubated at 37°C and 5% CO_2 for 3 days Waste medium was then removed and the cells rinsed with 1ml growth medium without foetal calf serum Cells were then refed with 1ml growth medium without foetal calf serum and incubated for 2 days at 37°C and 5% CO_2 100µl of test samples or controls and 20µl of ^{3}H-thymidine (6 6 Ci ml^{-1}) was added to each well and incubated for a further 24 hours at 37°C and 5% CO_2

Method of Counting .

Waste medium was removed and stored for radioactive waste disposal Wells were rinsed with 1ml cold PBS and 4 rinses with 1ml cold 5% trichloroacetic acid, followed by two rinses with 1ml EtOH (absolute alcohol) Plates were then dried at 37°C 1ml of 0 3N NaOH was added to each well and the plates were incubated at 37°C for 30 mins 0 5ml of this solution was removed from each well and transferred to a scintillation vial (Beckmann 158715) containing 100 1 of 1 5N HCl 6mls of scintillation cocktail [Toluene Triton X-100, 2 1 v/v, containing 0 4% 2,5-diphenyloxazole (PPO) (BDH 14615)] was then added to the vials which were shaken well before counting on a Beckmann Scintillation Counter (69 3409Y)

2 20 ^{125}I-EGF Radioreceptor Assay

EGF and TGF-α bind to the EGF receptor on a mole per mole basis (Todaro et al , 1980) Concentrated samples of CM from RPMI-2650 and 663+N (a known TGF-α producer) were compared in this assay

Method

10^5 A431 cells were inoculated in 16mm wells in 24 well plates (Costar 3524) in 1ml DME + 10% FCS and incubated overnight at 37°C in an atmosphere of 5% CO_2

Waste medium was removed and the cells rinsed twice with cold binding buffer (DME) + 1% BSA (Sigma A7030)

Standards and test samples were prepared in cold binding buffer and kept on ice until required ^{125}I-EGF (Amersham 1M 124) was prepared to give a final activity of 0 01μCi per 100μl of binding buffer The concentrations of EGF (Sigma E1257) for the standards were as follows 10μg ml^{-1}, 500 ng ml^{-1}, 100 ng ml^{-1}, 50ng ml^{-1}, 10ng ml^{-1}, 5ng ml^{-1}, 1ng ml^{-1}

100 1 of each standard was added to triplicate wells with 100 1 of ^{125}1-EGF Total volume of medium per well was 200μl All samples were mixed well by swirling and incubated at 20°C for 1 hour 100μl of test samples were also set up as for standards with 100μl ^{125}I-EGF and incubated at 20°C for 1 hour

The supernatant was removed and the cells rinsed three times with 1ml cold binding buffer The cells were then dissolved with 1ml 0 5N NaOH and transferred to counting vials (Rohren tubes 55 44 Starsted) The wells were rinsed out twice with 0 5ml of 0 5N NaOH which were also transferred to counting vials

The samples were then counted for 1 min on a mini gamma counter (LKB 1275)

A standard curve was drawn up of counts versus concentration of EGF Unknowns were then read from this curve

Note

(1) Stocks of EGF were prepared in 450μl quantities in PBS at 10 g ml^{-1} and stored at -20°C A freshly thawed stock was used in each experiment.

(2) Results were estimated as percentage inhibition of each sample over total ^{125}I-EGF bound in the absence of a competitor

$$\% \text{ inhibition} = 1 - \left[\frac{\text{CPM bound per sample - non-specific CPM}}{\text{Total CPM - non-specific CPM}} \times 100\right]$$

2 21 Hormones and growth factors used in ^{125}I-EGF radio-receptor assay

Three hormones, Progesterone, Dexamethasone and β-estradiol were obtained from Dr Denis Headon from University College Galway*, were incorporated in the ^{125}I-EGF radioreceptor assay They were examined for their effect on EGF receptors on A431 and T47D cells

Method

A431 cells and T47D cells were set up at 10^5 cells per 16mm well (Costar 3524) in 1ml DME with 10% foetal calf serum The following concentrations of hormones were set up for each cell type

Progesterone**	(stock 20µg ml^{-1} EtOH)	3 1ng ml^{-1}
Dexamethasone	(stock 100µg ml^{-1} EtOH)	100ng ml^{-1}
β-Estradiol	(stock 20µg ml^{-1} EtOH)	2 7ng ml^{-1}

The plates were incubated for 24 hours at 37°C and 5% CO_2

Waste medium was removed and all cells were rinsed twice with 1ml cold binding buffer (DME) with 1% BSA 100µl 1µCi ml^{-1} ^{125}I-EGF (370 kilo BQ) was added to each well 100µl of DME was added to each well Non-specific binding of ^{125}I-EGF was determined in the presence of excess unlabelled EGF where 100µl of 10µg ml^{-1} EGF was added to the well The assay was completed as described in Section 2 20

* Dept of Biochemistry, University College, Galway

** Stocks were reconstituted in a small amount of EtOH and growth medium to 20µg ml^{-1} Progesterone, 100µg ml^{-1} Dexamethasone, 20µgml^{-1} β-Estradiol

See Plate 10 and 11 for photographs of A431 and T47D in monolayer culture

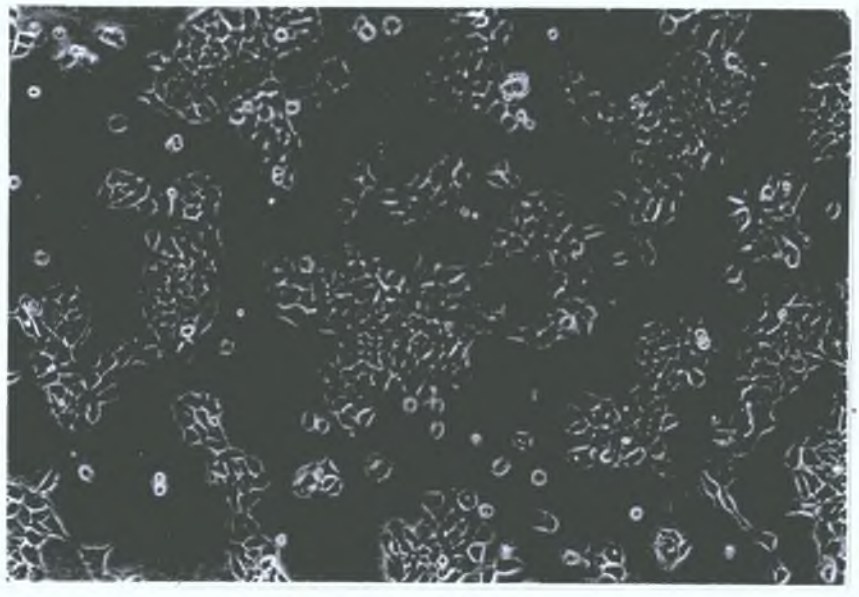


Plate 10 : A431 monolayer culture
Ph. 1 (150X) magnification.

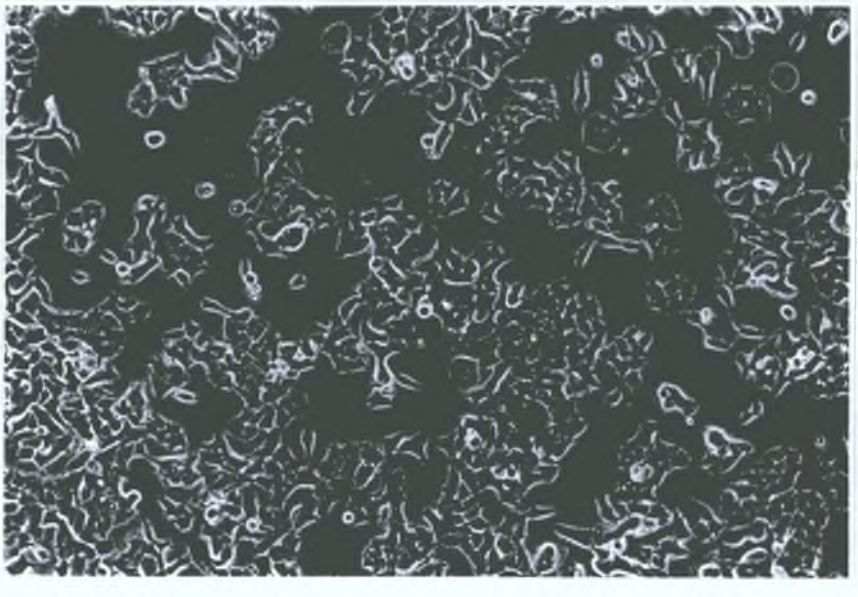


Plate 11 : T47D monolayer culture
Ph. 1 (150X) magnification.

2 22 Reverse Phase HPLC

Concentrated CM from RPMI-2650 and 663+N cells were dialysed, lyophilized and reconstituted in 0 1% trifluoro acetic acid (TFA). Insoluble particulate matter was removed by centrifugation at 4000 rpm for 15 mins

Method

Samples filtered through°0 45 m filters (Millipore SJ HV LOLNS) were injected onto a $C_{18}\mu$-Bondapak column (30cm x 3 mm, Waters Associates) The column was equilibrated with 20% acetonitrile/ 0 1% TFA and run at a flow rate of 0 8ml/min A gradient of CH_3 CN from 20-40% for 100 mins, was followed by a 15 min linear gradient to 60%, then to 80% for over 5 mins Fractions of 1 6ml were collected, lyophilized and reconstituted in growth medium All fractions were assayed for ^{125}I-EGF activity (use of solvent trap during lyophilization) Fig 2 5 shows gradient profile of acetonitrile

Bio-Gel P-30 fractionation of RPMI-2650 CM showed two peaks of activity in the TGF-β assay These two pools of activity were lyophilized and independently run on HPLC Every third fraction from each run was then reconstituted in growth medium and assayed for autocrine, TGF-β and ^{125}I-EGF competition activity

Note All solvents were analar HPLC grade and were filtered and degassed before use (Organic solvents through sartorious SM 1107 and aqueous through Sartorious SM 11106 filters)

2 23 Known growth factors in TGF, TGF-β and autocrine assays

A number of known growth factors were incorporated into the TGF, TGF-β and autocrine assays, at the concentrations shown in Table 2 3 800mls of concentrated RPMI-2650 CM was fractionated on Bio-Gel P-30

Fractions 10-18 were pooled, lyophilized and reconstituted in 5mls DME + 1mg % BSA (100%) [X], [1/5X] and [1/10X] dilutions were also prepared and tested in the assays Combinations of growth factors and RPMI-2650 CM were assayed, using the following concentrations

TGF-α	5ng ml^{-1}
TGF-β	0 2ng ml^{-1}
EGF	2 0ng ml^{-1}
PDGF	1 0ng ml^{-1}
RPMI-2650	1/5

Table 2 3 Known Growth Factors in TGF, TGF-β and autocrine assays

Growth Factor	Reconstitution Buffer*	Working Concentration in Assays
TGF-αGenentech 55 1 g	50μg ml^{-1} DME	50, 5 and 0 5ng ml^{-1}
TGF-β R & D Research (PD-1)	10μg ml^{-1} 4mM HCl	2, 0 2, 0 02ng ml^{-1}
EGF Sigma E4127)	10μg ml^{-1} PBS	2ng ml^{-1}
PDGF (Sigma P8147)	200ng ml^{-1} DME	1ng ml^{-1}
Bombesin (Sigma B5508)	308ng ml^{-1} DME	10nM
FGF (Sigma F1881)	100μg ml^{-1} DME	100, 10ng, ml^{-1}

* Reconstitution buffer contained 1mg % BSA Controls contained 1mg % BSA,

2 24 Colony Counting

2 24 1 Monolayer

Monolayer assays stained with Leishmann's stain as in Section 2 8 were counted by hand or by image analysis

The image analysis system consisted of placing the stained plate on a light source The image of the plate was then relayed to a television screen by camera The image analyser (AMS 40-10) was calibrated to pick up those colonies with more than 20 cells The conditions used for counting were stored in a memory recall bank for subsequent assays All counts were collected on a print-out

2 24 3 Agar Colonies

Colonies were counted on an inverted microscope (CK Olympus Tokyo) at 100X or 40X All colonies greater than 50µm in diameter after at least 8 days in culture were counted An eye-piece graticule was graduated using a stage micrometer and was then used to size colonies

All agar plates were viewed, superimposed upon a transparent gridded disc with $4mm^2$ grids An average of 30 grids were viewed for each 30mm plate The total number of colonies per plate was then estimated and percentage of the colony forming efficiency determined

$$\text{CFE } (\%) = \frac{\text{No colonies per plate}}{\text{No. cells plated}} \text{ X } 100$$

The mean CFE (%) for each sample, which was usually set up in triplicate, was determined and the standard error calculated

$$\text{SEM} = \pm \frac{\sqrt{\frac{\text{E } x^2 - \frac{(\text{E}x)^2}{n}}{n-1}}}{\sqrt{n}}$$

Where n = number of replicates per sample

x = CFE of each replicate per sample

E = sum of the samples from 0 to n

SEM = standard error of the mean

Because of the long assay period involved and the many parameters which had to be examined, most experiments were performed only in triplicate. Standard error of the mean is recorded to give an idea of variability; because only triplicate cultures were examined, some of the results would not be significant in students t test, but they provided valuable information on trends, which allowed further progress.

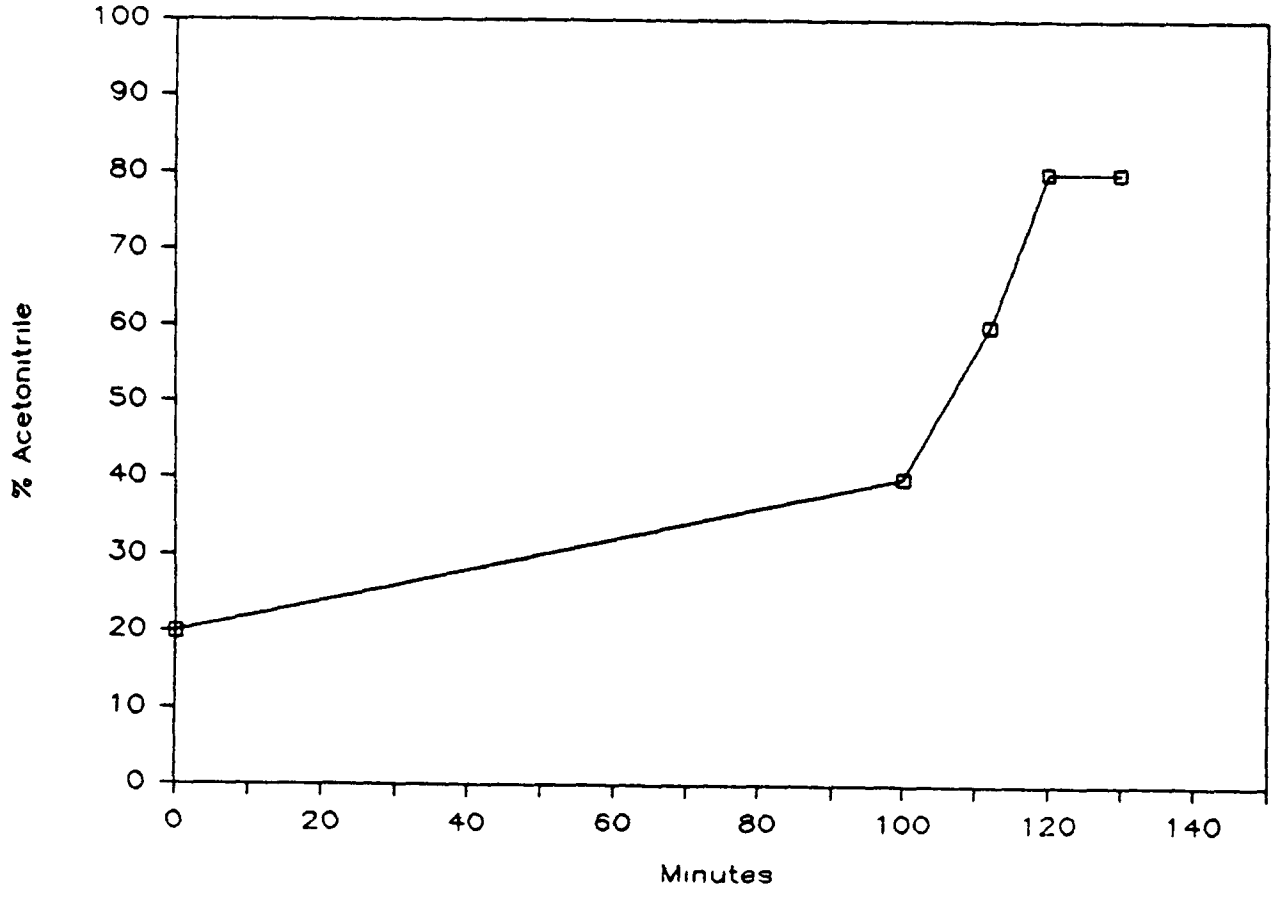


Fig. 2. Graph of Acetonitrile gradient used in HPLC Analysis

Flow rate 0.8ml/min.
Collected 1.6ml fractions

SECTION 3

RESULTS

3. Results

3 1 Effect of RMPI-2650 feeder layers on growth of RPMI-2650 cells

A double layer agar assay system as described in Section 2 10 1 was set up in which one population of RPMI-2650 cells - feeder cells, formed the feeder layer and a second population of RPMI-2650 cells was suspended in the upper layer

The data in Fig 3 1 and Table 3.1 show that the presence of a feeder layer of RPMI-2650 cells influences the CFE of similar cells in the double layer assay system At low feeder cell concentrations, no enhancement is found although a slight inhibition over the control showed up a number of times At 5 x 10^3 to 5 x 10^4 cells a stimulation of cell growth is observed in the upper layer At higher feeder concentration, the level of stimulation decreases Since the upper cell layer is physically separated from the feeder layer, these results imply that a diffusible autostimulatory substance was produced in proportion to the number of cells present in the feeder layer Removal of an inhibitory substance by the feeder cells could also be an explanation for the stimulation of RPMI-2650 colony formation

Table 3 1 RPMI-2650, double layer agar feeder assay

RPMI-2650 feeder layer concentration	CFE (%) RPMI-2650*	
	3 x 10^{4}**	2 x 10^{4}**
0	1 02 ± 0 18	1 36 ± 0 39
10^2	0 42 ± 0 12	0 56 ± 0 22
10^3	0 76 ± 0 08	1 0 ± 0 48
5 x 10^3	2 19 ± 0 12	2 46 ± 0 42
10^4	2 71 ± 0 12	3 6 ± 0 26
5 x 10^4	2 5 ± 0 60	3 7 ± 0 89
10^5	1 1 ± 0 32	4 17 ± 0 72
5 x 10^5	0 96 ± 0 73	3 27 ± 0 27

* ± S E M , (n = 3)

** Conc cells plated in the upper layer per 35mm plate

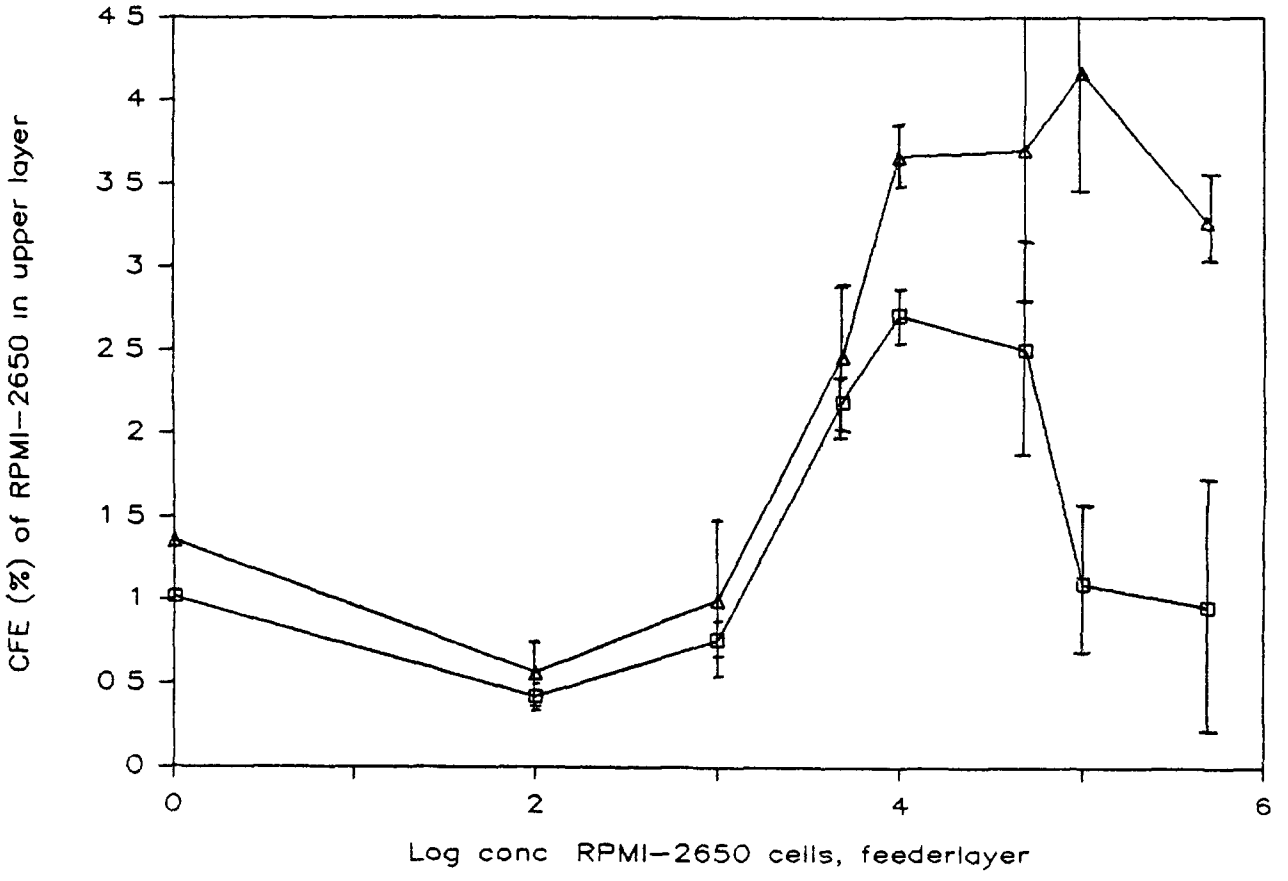


Fig. 3.1 RPMI-2650, double layer agar feeder assay

△ 2 x 10^4, □ 3 x 10^4 cells plated in the upper layer.

3 2 Effect of RPMI-2650 conditioned medium on RPMI-2650 cells in a double layer agar assay system

Before we could eliminate the possibility that RPMI-2650 cells were removing an inhibitory substance from the assay system described in Section 3 1, it was necessary to show that the feeder effect could be transmitted via a cell-free conditioned medium (CM) collected from RPMI-2650 cells RPMI-2650 cells were grown in monolayer for up to 4 days in 25cm^2 flasks Waste medium was removed when the flasks were 90% confluent and replaced with growth medium This conditioned medium was collected from these cultures at 18, 22 and 26 hours Unconditioned medium was used as a control at time 0 These samples were tested in the double layer assay system as in Section 2 10 4 and the results of this experiment are shown in Table 3 2

Table 3 2 Effect of RPMI-2650 conditioned medium on RPMI-2650 cells in a double layer agar assay system

RPMI-2650 CM Conditioning time	CFE (%) RPMI-2650*	
	4 x 10^{3}**	1 5 x 10^{3}**
0	0	0
18 hrs	9 35 ± 1 87	6 94 ± 2 32
22 hrs	13 71 ± 3 31	9 61 ± 3 67
26 hrs	10 15 ± 0 96	11 48 ± 1 22

* ± S E M (n = 3)

** Cell concentration (per 35mm plate) in upper layer

These results confirmed that RPMI-2650 cells were secreting a stimulatory factor into their extracellular environment, and that the physical presence of feeder cells was not necessary for the feeder effect The slight reduction in CFE found in 26 hr CM compared to 22 hr CM have been due to nutrient depletion found at higher cell concentrations or to production of inhibitors This assay was termed the RPMI-2650 "autocrine" assay

3 3 The effect of RPMI-2650 indicator cell concentration in the double layer agar assay

A serial dilution of RPMI-2650 cells was set up in the double layer agar assay system to determine a suitable indicator cell concentration as described in Section 2 10 4 The results of this experiment are shown in Table 3 3

Table 3 3 The effect of RPMI-2650 indicator cell concentration in the double layer agar assay

Conc RPMI-2650 cells per plate	CFE (%) RPMI-2650*
10^5	TNTC**
3×10^4	2 38 ± 0 40
10^4	1 80 ± 0 40
3×10^3	0 90 ± 1 20
10^3	0
0	0

* ±S E M (n=3)

** Too numerous to count

The above results show that at concentrations above 10^5 cells per plate, it becomes difficult to make accurate colony counts The colonies are too numerous with many merging to form clumps of 3 and 4 colonies At 10^3 cells per plate no colonies grew For subsequent autostimulatory assays, we decided that the most suitable indicator concentration for RPMI-2650 cells was 10^4 - 3 x 10^4 cells per plate This gave a low background which should not mask the autocrine activity of RPMI-2650 CM

3 4 The effect of Agar and Agarose on the CFE of RPMI-2650 cells in the double layer assay system

A series of concentrations of RPMI-2650 cells were set up in the double layer assay using two anchorage independent support matrices. The assays were set up using agar (Bacto Difco) and Agarose Type II (Sigma A677) using the procedure described in Section 2 10 4 The results from this experiment are shown in Table 3 4 1

Table 3 4 1 Clonal growth of RPMI-2650 cells in Agar and Agarose

Conc RPMI-2650 cells per plate	CFE (%) RPMI-2650*	
	Agar	Agarose
3×10^4	1 93 ± 0 5	3 7 ± 0 3
10^4	2 20 ± 0 3	3 3 ± 1 1
3×10^3	2 50 ± 0 8	3 2 ± 0 8
0	0	0

* ± S E M (n = 3)

These results showed that RPMI-2650 cells grew more efficiently in agarose than in agar This was an important finding which showed that the use of agar in the autostimulatory assay would provide a reduced background CFE of RPMI-2650 cells in comparison with agarose We also found that high concentrations of colonies in agarose tended (in a random manner, in some assays) to disintegrate, thus making accurate counting difficult We therefore concluded that agar was a more suitable matrix than agarose for the autostimulatory assay

3 5 Collection and ultrafiltration of RPMI-2650 CM in serum-free media

RPMI-2650 CM was initially collected in MEM with 5% foetal calf serum However, for the purpose of purification of the autocrine factor, it was necessary to collect serum-free CM from RPMI-2650 cells In this experiment, RPMI-2650 CM was concentrated to [10X] by ultrafiltration in Section 2 12 The retentate and filtrate were assayed in the autocrine assay and the results are as shown in Table 3 5 1

Table 3 5 1 RPMI-2650 autocrine assay

Test Sample	CFE % RPMI-2650*
RPMI-2650 CM control	0 68 ± 0 44
RPMI-2650 30,000 retentate [10X]	2 80 ± 0 09
RPMI-2650 30,000 filtrate	0 64 ± 0 20
Unconditioned MEM-FCS	0 42 ± 0 20

* ± S E M (n = 3) of 10^4 cells per 35mm plate

This experiment was important because it demonstrated that RPMI-2650 CM could be collected in serum-free media thus facilitating further purification. It also illustrated that the autostimulatory substance(s) had a molecular weight in excess of 30 kDa and excluded trivial explanations of the CM effect, such as removal of the conditioning cells of toxic substances

3 6 Effect of growth conditions and passage level of indicator cells on RPMI-2650 autocrine assay

RPMI-2650 CM collected from roller bottle cultures as described in Section 2 11 1 was ultrafiltered to [10X] through YM 30 and YM 2 Amicon membranes as described in Section 2 12 Samples of the retentates and filtrates were collected The remaining YM 30 filtrate was passed through the YM 2 membrane to give a [10X] retentate with all those molecules ranging in sizes between 1 and 30 kDa The samples were filter sterilized and the retentates 1R, 30R and 1-30R were then diltued in growth medium All the samples were subsequently assayed in the autocrine assay using two passage levels of RPMI-2650 cells In Table 3 6 1 the RPMI-2650 indicator cells were taken from 3-day log-phase cultures and in Table 3 6 2 the RPMI-2650 indicator cells were taken from confluent cultures

The results as shown in these experiments indicate that the growth phase at which the indicator cells are used is very important The cells taken from the log phase of growth as shown in Table 3 6 1 showed the dilution effect of RPMI-2650 CM, while the cells taken from confluent cultures in the stationary phase gave mixed stimulation results with the dilution curves of RPMI-2650 cells It seems therefore, that the growth phase from which the indicator cells are taken is more important than the passage level of the cells The careful pretreatment of RPMI-2650 autocrine indicator stocks is very important in this assay

Table 3 6 1 The effect of RPMI-2650 CM on different passage levels of log phase RPMI-2650 cells

RPMI-2650 CM Test sample	CFE (%) RPMI-2650 P 40**	P 58**
30,000 retentate [10X]	1 41 ± 0 09	1 85 ± 0 24
" " [5X]	1 49 ± 0 37	1 51 ± 0 08
" " [X]	0 56 ± 0 05	0 94 ± 0 08
30,000 filtrate	0 18 ± 0 01	0 31 ± 0 12
1,000 retentate [10X]	2 14 ± 0 13	1 64 ± 0 24
" " [5X]	1 74 ± 0 07	1 42 ± 0 25
" " [X]	0 31 ± 0 08	0 77 ± 0 17
1,000 filtrate	0 10 ± 0 04	0 21 ± 0 03
1,000-30,000 retentate [10X]	0 16 ± 0 06	0 19 ± 0 01
" " " [5X]	0 21 ± 0 03	0 37 ± 0 06
" " " [X]	0 25 ± 0 09	0 39 ± 0 04
Control CM untreated	0 53 ± 0	0 65 ± 0 05
Control medium	0 15 ± 0 02	0 37 ± 0 01

* ± S E M (n = 3) of 3 x 10^4 cells per 30mm plate

** Passage level of indicator cells

Table 3 6 2 The effect of RPMI-2650 CM on different passage levels of confluent indicator RPMI-2650 cells

RPMI-2650 CM Test sample	CFE (%) RPMI-2650*		
	10^4	2 x 10^4	4 x 10^4
	Passage No - 36		
1,000 retentate [8X]	0 06 ± 0 02	0 10 ± 0 04	0 05 ± 0 02
" " [4X]	0 20 ± 0 00	0 28 ± 0 10	0 02 ± 0 10
" " [X]	0 16 ± 0 02	0 20 ± 0 05	0 56 ± 0 05
Control CM untreated	0 01 ± 0 02	0 02 ± 0 00	0
Control medium	ND**	ND	0
	Passage No - 56		
1,000 retentate [8X]	0 15 ± 0 05	0 38 ± 0 10	0 38 ± 0 09
" " [4X]	0 43 ± 0 05	0 33 ± 0 11	0 34 ± 0 09
" " [X]	0 58 ± 0 02	0 04 ± 0 02	0 30 ± 0 05
Control CM untreated	0 11 ± 0 02	0 01 ± 0 02	0 17 ± 0 02
Control medium	ND	ND	0

* ± S E M (n = 3) of the concentration of indicator cells shown

** Not determined

3 7 1 Effect of serum on RPMI-2650 cell growth in monolayer

The ability of RPMI-2650 cells to detect differences in foetal calf serum concentration was determined with monolayer cultures of RPMI-2650 cells The cells were grown in 35mm plates in growth medium with 5% and 10% foetal calf serum Various cell concentrations were set up The results for 3 x 10^3 and 10^3 cells per plate are shown in Table 3 7 1

Table 3 7 1 Effect of serum concentration on the growth of RPMI-2650 cells in monolayer

Conc RPMI-2650 cells per plate	Number of colonies per plate (X10^2)	
	5% FCS	10% FCS
3 x 10^3	15 72 ± 1 4	18 4 ± 1 8
10^3	9 60 ± 1 9	10 9 ± 1 5
0	0	0

These results show that RPMI-2650 cells grow well in medium supplemented with 10% foetal calf serum Cell growth is in 5% foetal calf serum is almost as good as growth in 10% serum. This indicated that for routine cell maintenance it was more economical to use 5% foetal calf serum, thus halving the cost of serum while only reducing cell growth by about 10%

3 7 2 Serum batch testing with RPMI-2650 cells in monolayer

Foetal calf serum is an important component of growth medium in cell culture Batches of serum differ greatly in their basic components so it is important to work with a batch which has been proven to work well for the particular cell line and assay system involved

We received several batches of foetal calf serum One of these batches was tested in the TGF-assay (See 3 8 4) and found to be suitable for work with RPMI-2650 cells and the TGF assay Table 2 7 2 shows the difference in growth of RPMI-2650 cells, between a good working batch - 30Q, and a new batch B6 7, at 5% serum concentration

Table 3 7 2 Serum batch testing with RPMI-2650 cells in monolayer

Conc RPMI-2650 cells per 16mm well	RPMI-2650 cell growth*	
	B7 6	30Q
10^4	94 00 ± 15 7	161 20 ± 64 67
10^3	1 50 ± 0 9	10 37 ± 0 98
10^2	1 02 ± 1 4	2 26 ± 1 53

* Cells were stained with Leishmann's stain 2, and counted by image analysis 2 Counts are expressed as area in "picture cells'

These results show that the new batch B6 7 is not as effective as the working batch 30Q in stimulating cell growth

3 8 1 Development of the TGF assay

Since there has been much recent interest in the so-called "transforming growth factors" as autocrine regulators, it was of interest to determine if RPMI-2650 CM with autocrine activity contained such factors Three normal fibroblast cell lines as described in Table 2 2 were examined for their suitability in the TGF assay which was set up as described in Section 2 10 2 Positive TGF samples were prepared in CM collected from a known TGF producer cell line, 663+N. This CM was collected in two media types DME and SLM (see Table 2 1) The results of this experiment are shown in Table 3 8 1.

Table 3 8 1 Different CM collection media and different cell lines in the TGF assay

Test sample	CFE (%) of indicator cell line*		
	NRK	NRK-49F	AKR-2B
663+N CM (SLM + 5% FCS)	47 0 ± 8 3	1 20 ± 0 05	3 16 ± 0 29
" " (DME + 5% FCS)	6 3 ± 2 2	0 83 ± 0 04	1 26 ± 0 36
Control (SLM + 5% FCS)	21 3 ± 0 7	0 28 ± 0 09	0 98 ± 0 10
" (DME + 5% FCS)	0	0 36 ± 0 10	1 83 ± 0 28

* S E M (n = 3) of 1 2 x 10^3 NRK, 5 x 10^4 NRK-49F and 10^5 AKR-2B indicator cell concentrations

These results indicated that NRK cells responded better to the presence of TGF activity than NRK-49F and AKR-2B indicator cells These results also showed that SLM gave an undesirably high background in comparison to DME when examined in the NRK assay

NRK was chosen as the indicator for routine TGF assays since it was felt that the low background (in the case of DME) would provide a more reliable assay

3 8 2 Agar versus Agarose as a semi-solid matrix in the TGF-Assay

The TGF assay was prepared as described in 2 10 2 using two semi-solid matrix types agar (Bacto Difco) and agarose type II (Sigma-A6877). The effect of CM prepared from 663+N cells in DME was determined for TGF activity as shown in Table 3 8 2

Table 3 8 2 Agar versus agarose in the TGF assay

Test Sample	CFE (%) NRK*	
	Agar	Agarose
663 + N CM (DME)	13 66 ± 8 5	11 01 ± 7 06
Control medium (DME)	0	4 83 ± 0 58

* ± S E M (n = 3) of 1 2 x 10^3 cells per plate

In this experiment, the most significant finding was that the background in agar is non-existent, while in agarose the background is quite high Here again as in 3 4 1, we found that colonies in agarose tended to disintegrate and become very diffuse The reason for this is not understood

3 8 3 The effect of passage level of NRK indicator cells in the TGF assay

Various passage levels of NRK cells were examined in the TGF assay 663+N CM was used as a positive indicator in the assay as shown in Table 3 8.3

Table 3 8 3 Effect of NRK passage level in the TGF assay

Test sample	CFE (%) NRK*			
	Passage 21	24	26	32
663+N CM	20 62 ± 0 60	9 43 ± 0 29	9 32 ± 1 00	4 11 ± 0 88
Control medium	0 02 ± 0 04	0	0 02 ± 0 04	0

* ± S E M (n = 3) of 6 x 10^3 cells per 30mm plate

This experiment showed that the passage level of NRK used in the TGF assay can be very important We found in general that NRK between passage 17 and 23 was most suitable for the TGF assay Also, use of stocks, freshly thawed from liquid nitrogen ensured relative freedom from contaminants such as mycoplasma

3 8 4 Serum batch testing in the TGF assay

Two batches of foetal calf serum were examined in the TGF assay A sample of 663+N CM and RPMI-2650 CM were compared for TGF activity as described in 2 10 1 The results are shown in Table 3 8 4

Table 3 8 4 Serum batch testing in the TGF assay

Test Sample	CFE (%) NRK*	
	20F**	30Q**
663+N CM	1 00 ± 0 7	5 2 ± 0.0
RPMI-2650 CM	0 53 ± 0 3	2 0 ± 0 7
Control medium	0	0

* ± S E M (n = 3)

** Batch number

These results showed that the batch of serum used in the TGF assay was extremely important All batches of serum differ in their basic components It is, therefore, important to test a number of batches of serum and to use the most suitable batch for all subsequent work In this experiment, it was noted at the time of counting that the colony size formed in the presence of serum batch 30Q was far greater than those formed in 20F serum All subsequent TGF work and autocrine work was carried out using 30Q serum Other batches of serum were used for general cell maintenance

3 8.5 Estimation of a suitable NRK indicator cell concentration in the TGF assay

Many of the TGF assays using NRK as the indicator were set up using 1 2 x 10^3 NRK cells per plate However, it became obvious later that CM from RPMI-2650 cells was far less effective than 663+N CM in the TGF assay At 1 2 x 10^3 cells per plate, the background CFE (%) was 0 This meant that the presence of very low levels of TGF activity would not be seen Some batches of RPMI-2650 CM were not active in the TGF assay and this may have been the reason It was, therefore, necessary to use concentrated samples of CM. This was not practical when testing many batches of RPMI-2650 CM, so we decided to try to increase the background CFE (%) of NRK in the assay and in this way to attempt to detect low TGF activity in RPMI-2650 CM. Table 3 8 5 shows the general background CFE of various concentrations of NRK cells in the TGF assay

Table 3 8 5 Effect of plating density on NRK colony formation in the TGF assay

Number of cells plated	CFE (%) NRK*
6 x 10^3	0 36 ± 0 10
5 x 10^3	0 21 ± 0 06
4 x 10^3	0 38 ± 0 23
3 x 10^3	0
2 x 10^3	0 11 ± 0 15

* ± S E M (n = 3)

These results show that below 4 x 10^3 cells per plate the concentration of NRK cells is at a critical level (low number of colonies or no colonies form) Unless the activity of the CM used is more than borderline, it may not always be detected 6 x 10^3 cells per plate was subsequently chosen as the cell concentration in the TGF assay.

3 8 6 Effect of duration of conditioning on TGF activity of 663+N CM

CM was collected from two passage levels of 663+N cells grown in 25cm^2 flasks at 48, 72, 96 and 120 hours These batches of CM were then assayed for TGF activity as described in Section 2 10.2 The results of this experiment are shown in Fig 3 8 6 and Table 3 8.6

Table 3 8 6 Effect of duration of conditioning on TGF activity of 663 + N CM

Test Sample		CFE (%) NRK*	
		Passage 31	Passage 60
663+N	0 hrs	0	0
"	48 hrs	0 80 ± 0 30	1 0 ± 0 4
"	72 hrs	2 20 ± 1 10	3 5 ± 0 6
"	96 hrs	0 60 ± 0 34	2 7 ± 1 0
"	120 hrs	0 06 ± 0 10	1 9 ± 1 0

* ± S E.M (n = 3) of 3 x 10^3 NRK cells per 35mm plate

The results described in Fig 3 8 6 and Table 3 8 6, shows that 663+N CM secretes optimum TGF activity up to 72 hours after setting up From then on, the production of TGF decreases rapidly in P 31 and 60

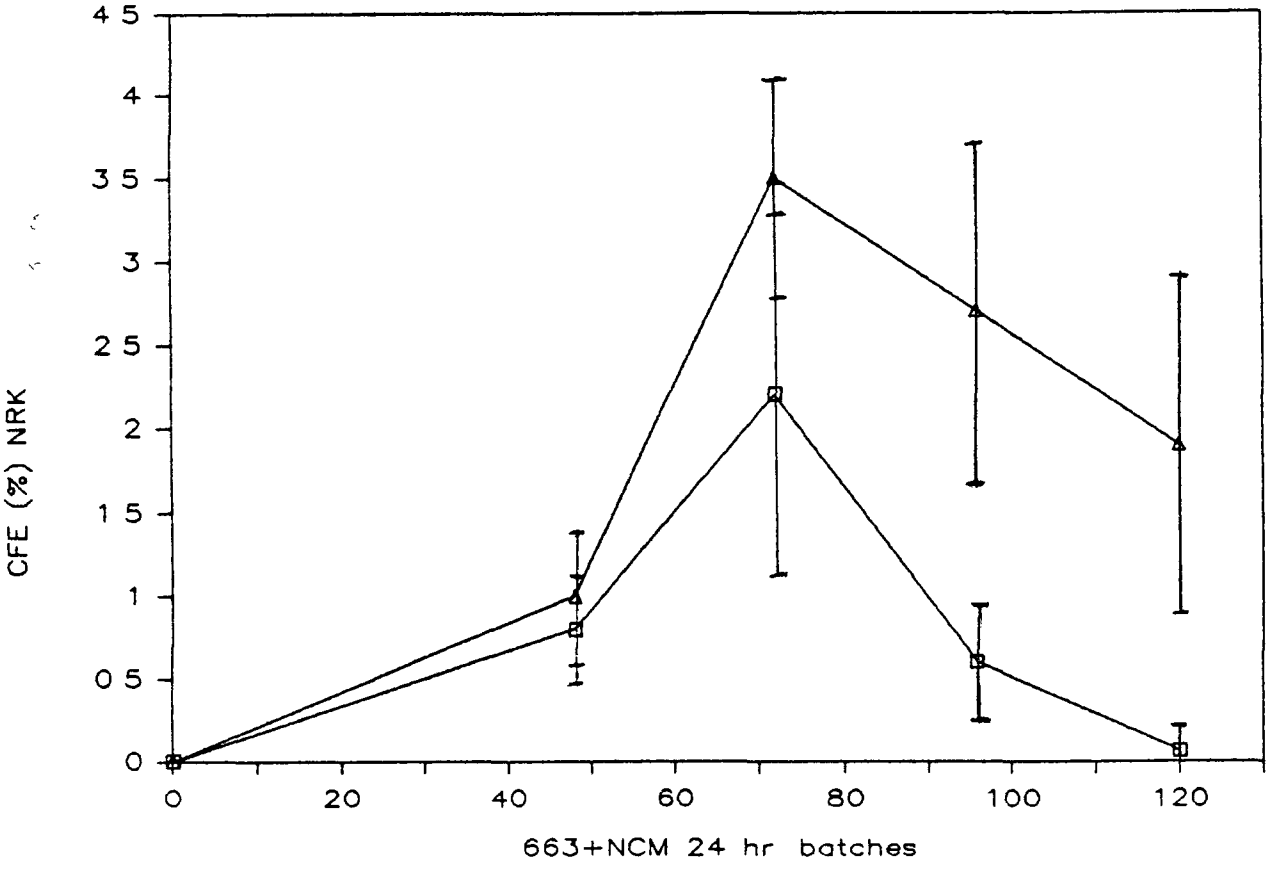


Fig. 3.8.6. TCF activity of CM collected from 663+N cells at the indicated times.

CM collection from 663+N cells grown to passage 31-□, and passage 60-△

3 9 1 Effect of serum in RPMI-2650 CM in the TGF assay

Stocks of RPMI-2650 cells were grown to 90% confluency for 48 hours in 25cm^2 flasks in MEM with 10% foetal calf serum Waste medium was removed and the cells were set up with MEM with 5% foetal calf serum This medium was conditioned for 48 hours Control MEM with 5% foetal calf serum was incubated at 37°C for 48 hours These two batches of medium were ultrafiltered through YM2 membranes to give the following concentrates CM - [5 16X] and unconditioned MEM to [6 6X] A control of sample of MEM with 10% foetal calf serum was also ultrafiltered to [4 14X] Samples of retentates, filtrates and non-ultrafiltered CM and medium were assayed for TGF activity as shown in Fig 3 9

Table 3 9 1 Effect of serum in RPMI-2650 CM in the TGF assay

Test Sample	CFE (%) NRK*		
	Control MEM + 5% FCS	Control MEM + 10% FCS	CM + 5% FCS
Control	0	0 83 ± 1 04	0 33 ± 0 28
5,000 retentate	0 5 ± 0 5	2 50 ± 0 00	18 03 ± 0 50
5,000 filtrate	0	0	0

* ± S E.M (n = 3) of 1 2 x 10^3 cells per plate

These results showed that serum influenced activity in the TGF assay The stimulatory effect caused by foetal calf serum in the 5,000 retentate was small when compared to the effect caused by CM from RPMI-2650 cells The presence of even a small amount of concentrated serum factors in CM during purification could cause misinterpretation of some of the results

3 9 2 Absorption to membrane filters of RPMI-2650 CM TGF activity during filter-sterilization

Samples of RPMI-2650 CM concentrated by ultrafiltration were examined in one TGF assay after sterilization Two sterile disposable filters were examined It was found that Millex-GS filters (Millipore-SLGS 0250S) absorbed up to 80% of the TGF activity from the sample while Millex-GV filters (Millipore-SLGV 025 BS) absorbed 28% of the TGF activity Results are shown in Table 3 9 2 The GS filters are composed of cellulose acetate while the GV filters are composed of Polyvinylidene difluoride Because of the low levels of growth factor secreted into CM, it is important to avoid undue loss of activity caused by binding to filter membranes as shown here

Table 3 9 2 Absorption of RPMI-2650 CM TGF activity to membrane filters during filter sterilization

Test Sample	CFE % NRK*
Millex-GV	5 50
Millex-GS	1 56
Control CM	7 70
Control Medium	0 20

* 3 x 10^3 NRK cells per 35mm plate

3 10 Comparison of 663+N and RPMI-2650 CM in the TGF assay

Samples of CM was prepared from 663+N cells in SLM and from RPMI-2650 cells in MEM The CM was ultrafiltered through either the YM5 or YM30 membranes as described in Section 2 12 and assayed in the TGF assay, as shown in Table 3 10 1 Samples of the 663+N YM5 filtrate were taken at three stages during ultrafiltrations, after 70mls (1), 170mls (2), and 195mls (3) of filtrate had passed through the membrane The 5,000 retentates were diluted to half their concentrated form with 5,000 filtrate The 30,000 retentate as shown in Table 3 9 2 was ultrafiltered through the YM5 membrane(5-30R) The retentates in this experiment were concentrated approximately [10X]

The results in Table 3 10 1 show that 663+N cells secrete more TGF activity than RPMI-2650 cells The dilution effect of 663+N CM does not appear to be linear This may mean that the upper sensitivity limits of the assay have been reached The most concentrated sample of RPMI-2650 CM in this case is well within the limits of the assay Table 3 10 2 indicates that 663+N CM and RPMI-2650 CM contain different proportions of transforming growth factors of different molecular weights The 663+N TGF activity is primarily in the 5-30 retentate, while the RPMI-2650 TGF activity is in the 30 retentate The results in Table 3 9 1 also show that the YM5 membrane is not absolute The efficiency of this membrane to retain molecules greater than 5 kDa decreases as the sample is concentrated

Experiment 1 as shown in Table 3 10 1 was repeated one week later (Expt 2) and showed that the activities obtained in this assay can vary considerably from assay to assay, but that the profile of results are generally similar This underlines the importance of including control unconditioned and conditioned media in each experiment, and the desirability in future work, when stable purified forms of the growth factors have been obtained of including a standard of known activity in each experiment

Table 3 10 1 Comparison of 663 + N CM and RPMI-2650 CM in the the TGF assay

Test Sample	CFE (%) NRK*	
	Expt 1	Expt 2
663+N CM Control	11 68 ± 1 25	15 3 ± 1 92
" 5F (1)	0	0
" 5F (2)	0 33 ± 0 28	0 16 ± 0 28
" 5F (3)	0 66 ± 0 76	1 16 ± 0 28
" 5R [5 6X]	30 22 ± 2 36	48 43 ± 5 80
" 5R [2 8X]	24 04 ± 3 78	40 91 ± 4 94
Control medium (SLM)	0	0
RPMI-2650 CM Control	0 66 ± 0 28	0
" 5F	0	0
" 5R [5 3X]	2 16 ± 0 57	3 66 ± 0 76
" 5R [2 6X]	1 00 ± 0 50	1 33 ± 0 76
Control Medium (MEM)	0	0

* ± S E M (n = 3) of 1 2 x 10^3 cells per 35mm plate

Table 3 10 2 Comparison of 663+N CM and RPMI-2650 CM in the TGF assay

Test Sample	CFE (%) NRK*	
	663+N CM	RPMI-2650 CM
5R	0 75 ± 0 36	0 44 ± 0 21
5F	0 08 ± 0	0
30R	0 27 ± 0 04	0 69 ± 0 04
30F	0 64 ± 0 19	0 10 ± 0 04
5-30R	0 47 ± 0 12	0 11 ± 0 12
CM Control	0 33 ± 0	0 38 ± 0 19
Medium Control	0	0

* ± S E M. (n = 3) of 6 x 10^3 NRK cells per plate

3 11 Development of the TGF-β assay

3 11 1 Examination of NRK-49F as an indicator for the TGF-β assay

In order to specifically detect the presence of TGF-β in conditioned media, we used NRK-49F cells in a TGF assay with 2ng ml^{-1} EGF as described in Section 2 10 3 (NRK cells are sensitive to TGF-α and to TGF-α and TGF-β together, but not to TGF-β NRK-49F cells are sensitive to TGF-β in the presence of EGF) Table 3 11 1 shows the effect of various batches of CM on NRK-49F cells with and without EGF

Table 3 11 1 Examination of NRK-49F as an indicator for the TGF-β assay

Test Sample	CFE (%) NRK-49F*	
	+ EGF**	-EGF
RPMI-2650 CM 5R [6X]	9 40 ± 0 70	0 66 ± 0 15
" 5R [10X]	20 50 ± 0 70	3 40 ± 0 26
663+N CM	0 96 ± 0 10	1 66 ± 0 05
NRK-49F CM 5R [10X]	8 10 ± 0 60	0 63 ± 0 15
" Control	0 50 ± 0.00	0
Control Medium	0 43 ± 0 05	0

* ± S E M (n = 3) of 2 x 10^4 cells per 30mm plate

** 2ng ml^{-1}

These results show that RPMI-2650 CM contains a TGF-β like growth factor which is strongly potentiated by EGF 663+N CM activity is inhibited by the addition of EGF A sample of NRK-49F CM ultrafiltered through YM5 to [10X] was also tested in this assay and shown to be potentiated by the presence by EGF

NRK cells (± EGF) may also be used to detect TGF-β, but the CFE in the presence of CM without added EGF is often so high that it becomes difficult to reliably detect EGF induced stimulation NRK-49F (± EGF) is therefore the indicator system of choice for detection of TGF-β, while NRK is more sensitive in the detection of TGF (α + β)

3 11 2 Effect of EGF on the RPMI-2650 autostimulatory assay

RPMI-2650 cells were set up for an autocrine assay as described in Section 2 10 4 2ng ml^{-1} EGF was added and samples of CM were assayed. A control experiment without EGF was also set up The results from this experiment are shown in Table 3 11 2

Table 3 11 2 Effect of EGF on the RPMI-2650 autostimulatory assay

Test Sample	CFE (%) RPMI-2650*	
	+ EGF**	-EGF
RPMI-2650 CM 30R (MEM)	1 74 ± 0 12	2 80 ± 0 09
" " 30F (MEM)	1 18 ± 0 63	0.64 ± 0 20
" CM (MEM)	0.33 ± 0 57	0 68 ± 0 44
Control MEM	0 28 ± 0 09	0 42 ± 0 25
663+N CM (SLM)	1 83 ± 0 04	3.00 ± 0 06
Control SLM	1 92 ± 0 42	2 44 ± 0 96

* ± S E M (n = 3)

** 2ng ml^{-1} ,

This experiment clearly shows that EGF does not stimulate the growth of RPMI-2650 cells in agar, in fact, there is an indication of a possible inhibitory effect, although more data would be needed to establish statistical significance

This experiment also shows that SLM medium gives a very high background in the presence of RPMI-2650 cells. This masks the stimulatory activity of CM prepared in it; MEM was therefore used routinely for RPMI-2650 CM preparation in subsequent experiments.

3 12 Mitogenic activity of RPMI-2650 CM prepared from suspension culture

Samples of RPMI-2650 CM collected from a 100ml suspension culture system at 24 hour intervals over a 7 day period (see Section 2 11 2) were assayed for their ability to influence incorporation of ^{3}H-Thymidine into NRK cells as described in Section 2 19 The remaining CM was pooled and ultrafiltered to [28X] through a YM5 amicon membrane and was also tested in this assay Control medium (MEM) and control medium with 10% foetal calf serum were included in the assay The results of this experiment are shown in Table 3 12

Table 3 12 NRK Mitogenic activity of RPMI-2650 CM collected from suspension culture

Test Sample	^{3}H-Thymidine incorporation (CPM)*	**
RPMI-2650 CM 24 hr	4,520 ± 522	88
" 48	4,937 ± 149	101
" 72	4,403 ± 620	83
" 96	4,514 ± 670	88
" 120	6,065 ± 990	153
" 144	7,045 ± 1,800	194
" 168	5,085 ± 692	112
" [28X]	12,154 ± 1,853	407
Control MEM	2,396 ± 361	0
Control MEM + 10% FCS	8,576 ± 135	257

* ± S E M (n = 3)

** % increase of CPM over control MEM

These results indicated that RPMI-2650 cells contained a mitogen active on NRK cells in monolayer and was concentrated by ultrafiltration through a YM5 amicon membrane It is not clear if the factor(s) involved are identical to, or different from, the factor(s) which stimulate NRK growth in agar

3 13 1 ^{125}I-EGF competition activity in RPMI-2650 and 663+N CM

The ^{125}I-EGF radioreceptor assay was set up to detect for EGF like molecules in CM collected from RPMI-2650 cells A lengthy method was used in the initial experiments This method was later shortened to the method described in Section 2 20 663+N CM had been reported to contain TGF-α (Dr Ian Pragnall, personal communication) A concentrated sample of 663+N CM was incorporated into the ^{125}I-EGF radioreceptor assay and was found to compete with ^{125}I-EGF for EGF receptor sites Batches of CM from RPMI-2650 cells were ultrafiltered through different cut-off membranes to [10X] concentrates, as described in 2 12 These were shown to have no effect in the ^{125}I-EGF radioreceptor assay Subsequent experiments were set up with 663+N CM at a top concentration of [15X] with 50%, 25% and 10% dilutions, and RPMI-2650 CM with a top concentration of [300X] with similar dilutions, and were assayed in the ^{125}I-EGF radioreceptor assay Fig 3 13 1 and Table 3 13 1 shows the results of this experiment

These results show that RPMI-2650 CM and 663+N CM contain ^{125}I-EGF competing activity However, it would appear from these results that 663+N CM contains up to 20 times more ^{125}I-EGF competing activity than an equivalent sample of RPMI-2650 CM For example, the results in Table 3 13 1 show that for a [300X] concentrate of 663+N CM, a similar dilution curve is given At 25% dilution of both 663+N CM and RPMI-2650, they both inhibit about 62% ^{125}I-EGF binding. This is converted to pg from the standard curve i e 3162 pg approx When the concentration factors are taken into consideration, it would appear that in this particular case 663+N cells secreted approximately 8 4ng equivalent EGF binding factor per ml of growth medium and RPMI-2650 cells secreted 0 42ng equivalent EGF binding factor per ml of growth medium Figure 3 13 2 shows the standard curve of inhibition of binding of ^{125}I-EGF by EGF

Table 3.13 1 Effect of RPMI-2650 and 663+N CM in the ^{125}I-EGF radioreceptor assay

Conc CM (%)	% inhibition of ^{125}I-EGF binding*	
	RPMI-2650 CM **	663+N CM
100	86 27	84 10
50	78 27	74 27
25	62 29	63 99
10	45 08	57 94
0	00 00	00 00

* All S E M were less than 10%

** Conc RPMI-2650 CM [300x] and 663+N CM [15X]

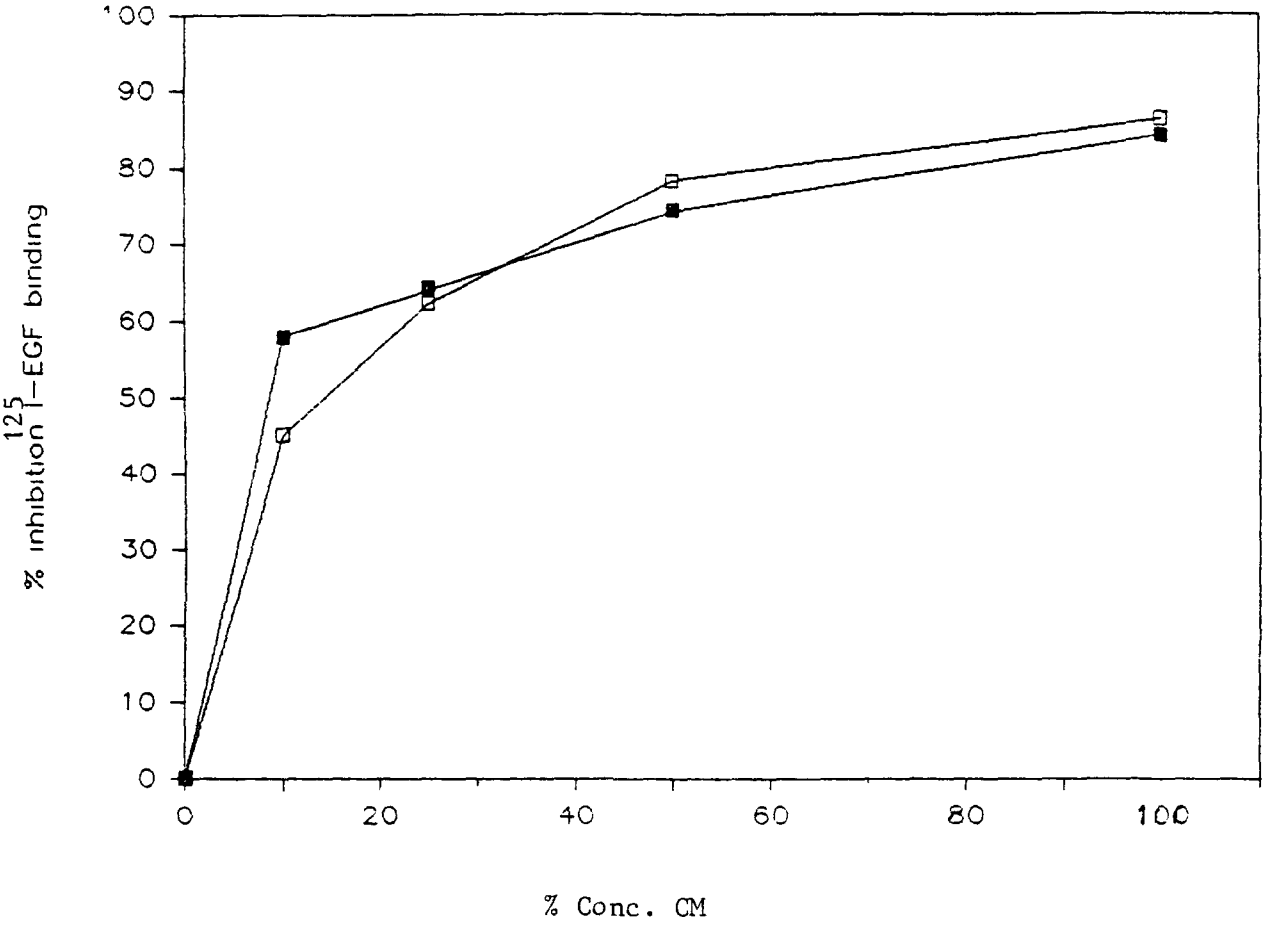


Fig. 3.13.1 ^{125}I-EGF binding competition by CM from RPMI-2650 □, and 663+N cells ■.

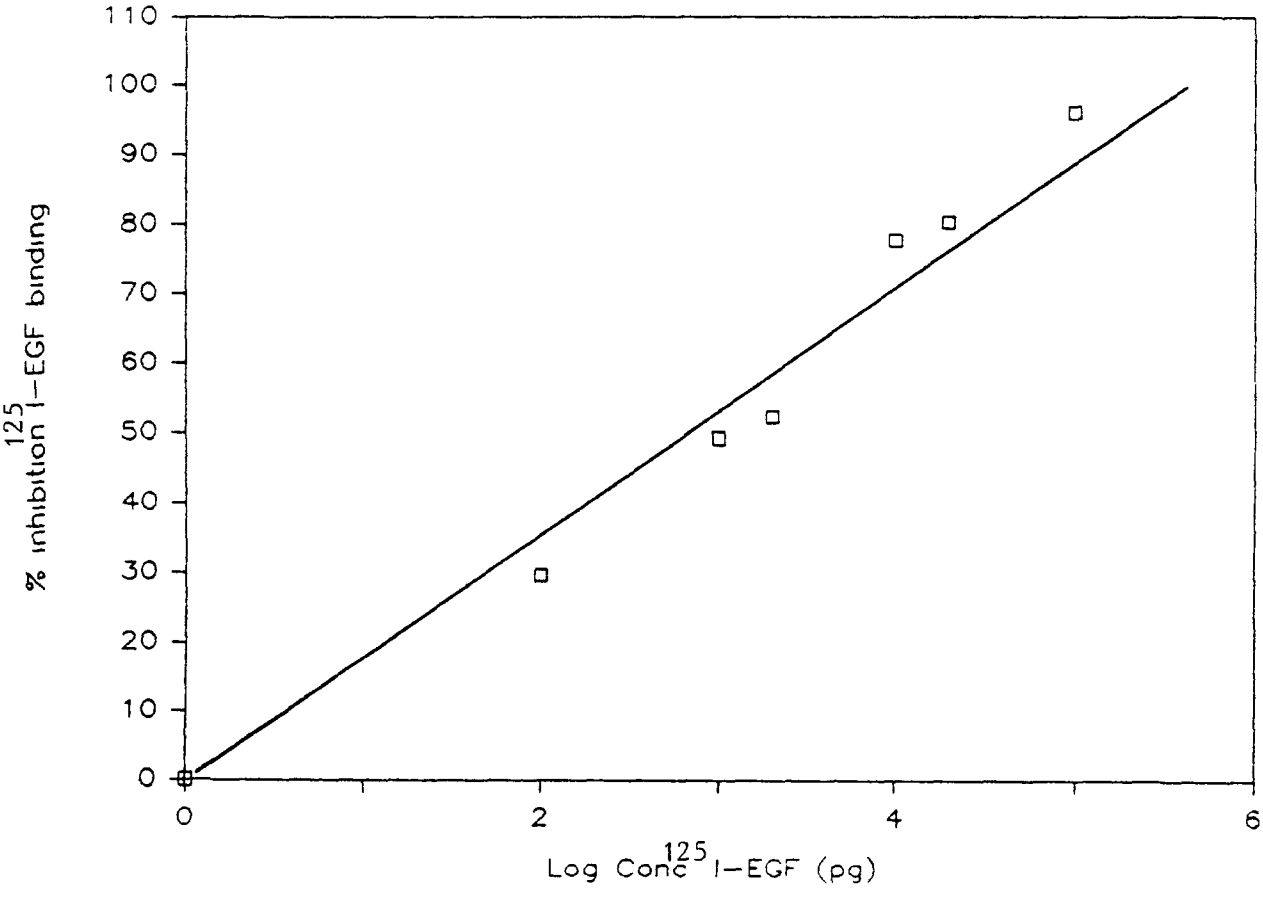


Fig. 3.13.2 Standard curve of inhibition of binding of ^{125}I-EGF by EGF.

3 13 2 Presence of EGF receptors on some cell lines

An ^{125}I-EGF radioreceptor assay was set up as described in Section 2 20 A431 cells and 3 other cell lines (NRK, RPMI-2650 and SCC9) were compared for their ability to bind ^{125}I-EGF Non-specific binding was determined in the presence of excess unlabelled EGF (10μg ml^{-1}) The results from this experiment are shown in Table 3 13 2 These results show that A431 cells have the most EGF receptor sites NRK and SCC9 cells have less and RPMI-2650 cells have a relatively small number of EGF receptor sites

Table 3 13 2 ^{125}I-EGF binding to various cell lines

Cell line*	Relative ^{125}I-EGF counts per min Adjusted to allow for non-specific binding
A431	2759 37
RPMI-2650	45 33
NRK	984 66
SCC9**	642 97

* 10^5 cells per 16mm well were plated for each cell line At this concentration virtually all the cells attached

** Squamous carcinoma cell line (Tongue)

3 14 1 Dialysis of 663+N and RPMI-2650 conditioned media

It was necessary to dialyse CM prior to fractionation by Bio-Gel P 60 in order to change the medium to acetic acid for column chromatography 663+N CM was dialysed against acetic acid and PBS as described in Section 2.14 Table 3 14 1 shows the results from this experiment

Table 3 14 1 Effect of dialysis on TGF activity of 663+N CM

Test Sample	CFE (%) NRK*
Dialysis 663+N CM 1% Acetic Acid 4°C	10 5***
" " " " " R T **	4 2
" " " PBS 4°C	2 9
Control CM untreated	6 5
Control medium	0 0

* 3 x 10^3 cells per 35mm plate

** Room temperature

*** Raw data mislaid, S E M s not available

Some TGF activity is lost by dialysis against PBS at 4°C and by dialysis against acetic acid at room temperature A slight activation of TGF activity occured during dialysis of 663+N CM at 4°C It would appear therefore, that dialysis of 663+N CM against acetic acid at 4°C is the method of choice

The autocrine TGF and TGF-β activities of RPMI-2650 CM were shown to be stable to dialysis and this procedure was widely used in the preparation of RPMI-2650 CM before fractionation by column chromatography

3 14 2 Dilution curve of 663+N conditioned medium

Since column chromatography involves dilution of applied samples, it was necessary to establish that the activity detected in the TGF assay was not lost through excessive dilution A sample of 663+N CM was diluted in growth medium and assayed for TGF activity as shown in Fig 3 14 1

These results show that 663+N CM TGF activity dilutes in a linear manner This assay shows however, that at high concentrations of 663+N CM, a saturation level is reached and above this concentration, a mildly inhibitory effect is seen

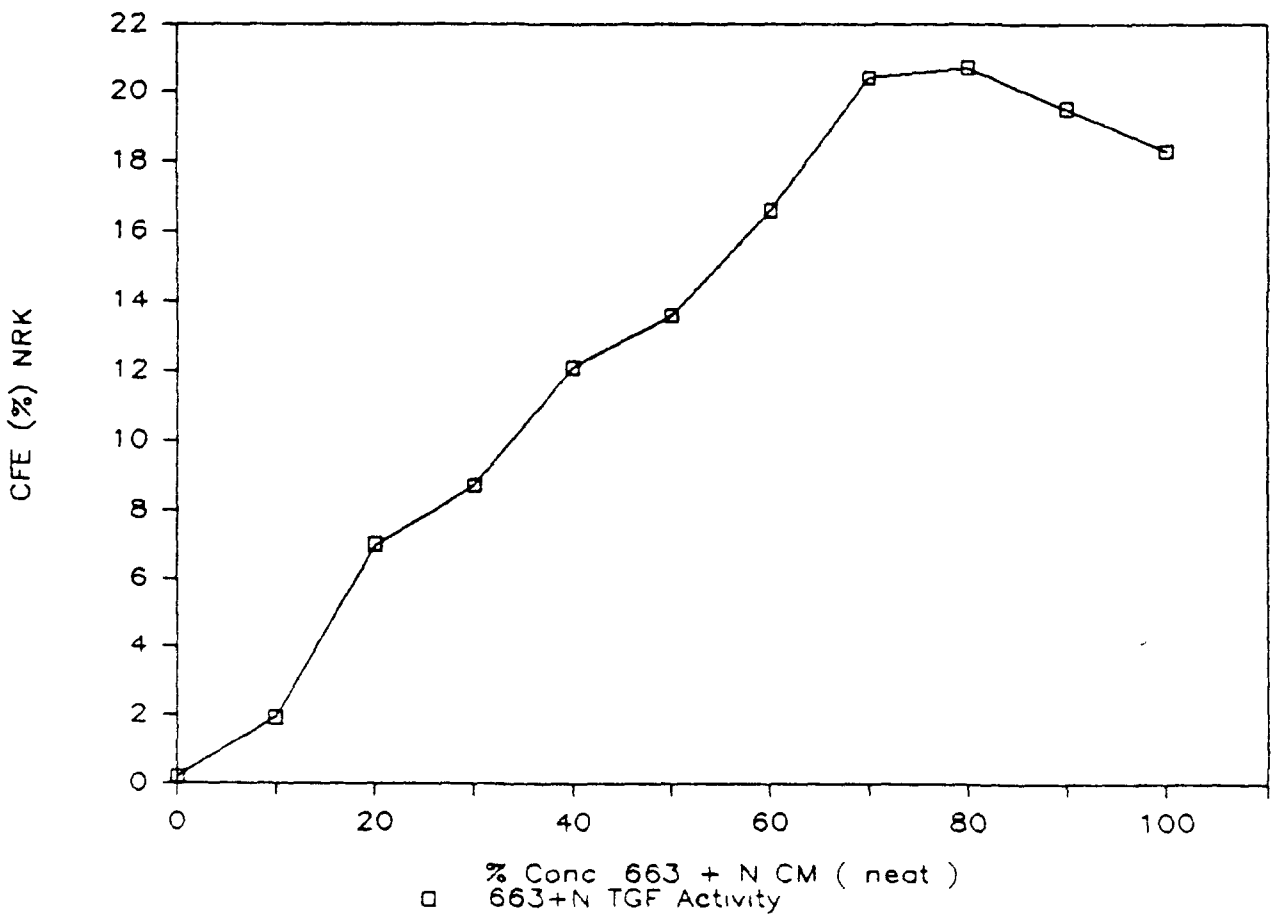


Fig 3 14.1 : Dilution curve of 663+N CM TGF activity (1.2×10^3 NRK cells)

3 14 3 Dilution effect of RPMI-2650 conditioned medium in the TGF and TGF-β assays

A sample of RPMI-2650 was concentrated to [10X] by ultrafiltration through a YM2 cut-off membrane as described in Section 2 12 This sample of CM was then assayed for TGF and TGF-β activity as shown in Fig 3 14 2 and Table 3 14 2

Table 3 14 2 Dilution effect of RPMI-2650 CM in the TGF and TGF-β assays

Conc RPMI-2650 CM	TGF CFE (%) NRK*	**	TGF-β CFE(%) NRK-49F*	**
[10X]	7 45 ± 0 99	100	1 92 ± 0 3	100%
[4X]	7 09 ± 1 33	95 1	1 2 ± 0 2	62 5
[2X]	4 41 ± 1 98	59 1	0 51 ± 0 06	26 5
[1 33X]	0 58 ± 0 55	7 7	0 23 ± 0 06	11 9
Control medium	0	0	0 02 ± 0 04	1 04

* ± S E M (n = 3) of 6 x 10^3 NRK cells per 30mm plate and 2 x 10^4 NRK-49F cells per plate

** Results shown as % change over 100% control

These results show that RPMI-2650 CM is sensitive to dilution in growth medium when assayed for TGF and TGF-β activity The TGF assay shows that RPMI-2650 CM has reached saturation levels at its highest concentrations in the TGF assay The TGF-β activity is represented by a linear dilution curve

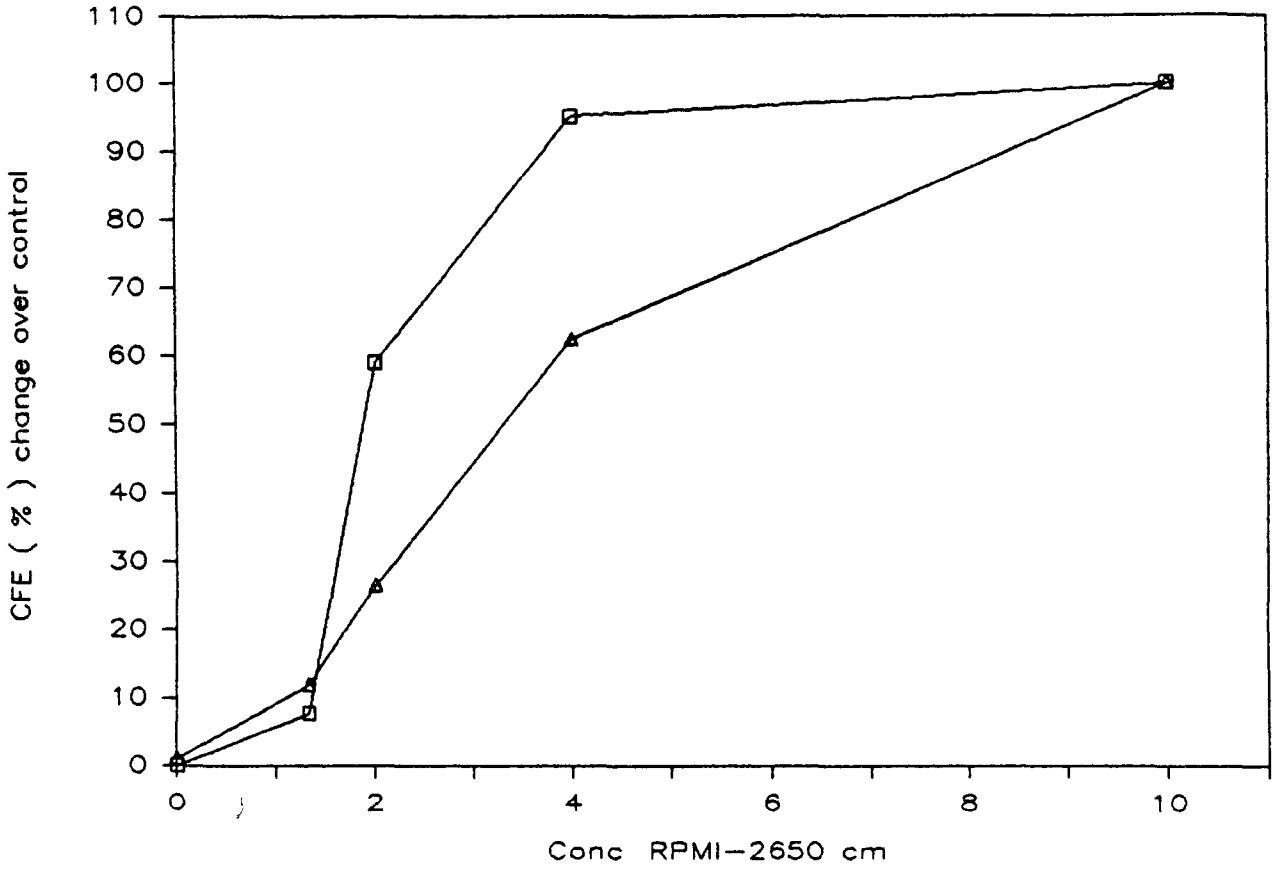


Fig. 3.14.2 : Dilution effect of RPMI-2650 CM on NRK □ and NRK49F △ cells.

3 14 4 Dilution curve of RPMI-2650 CM in the autocrine assay

A [10X] concentrated sample of RPMI-2650 CM by ultrafiltration through a YM2 amicon membrane was diluted in growth medium and assayed for autocrine activity The dilution curve results are shown in Fig 3 14 3 and Table 3 14 3 These results show that the autocrine activity of RPMI-2650 CM is sensitive to dilution in growth medium

Table 3 14 3 Dilution effect of RPMI-2650 CM on RPMI-2650 cells

Test Sample	CFE (%) RPMI-2650*	
		**
RPMI-2650 CM [10X]	2 14 ± 0 13	100.-
" [5X]	1 74 ± 0 07	81 3
" [X]	0 31 ± 0 08	14 48
Control medium 0	0 15 ± 0 02	0 07

* ± S E M (n = 3) of 3 x 10^3 cells per 30mm plate

** Growth expressed as percentage of [10X] control

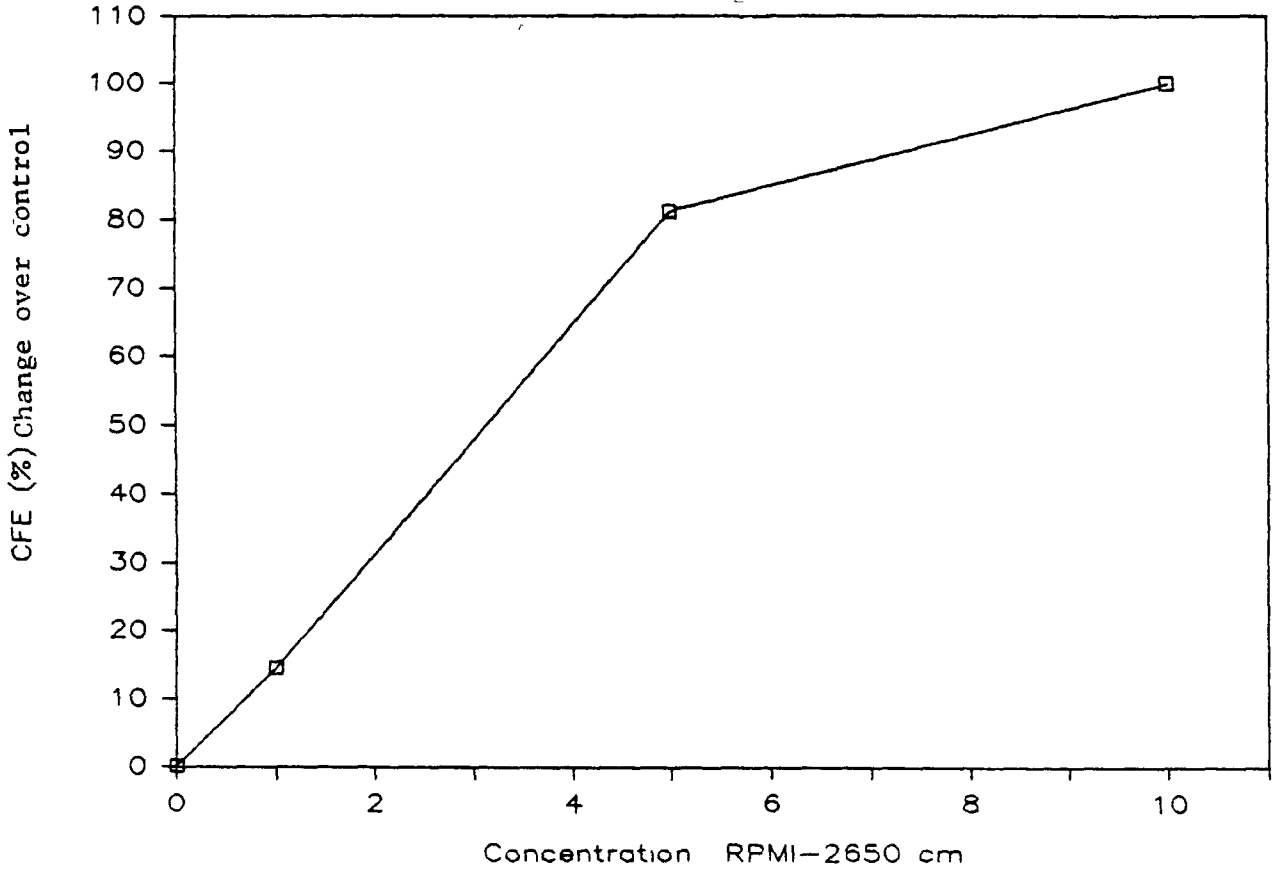


Fig. 3.14.3. Dilution curve of RPMI-2650 CM on RPMI-2650 cells

3 14 5 Dilution effect of RPMI-2650 CM collected from suspension culture in the TGF and autocrine assays

A sample of RPMI-2650 CM collected from suspension culture as described in Section 2 11 2 was ultrafiltered through a YM2 amicon membrane to [21X] This sample of retentate was diluted in growth medium and assayed for TGF and autocrine activity The dilution curve of TGF activity is shown in Fig 3 14 4 and Table 3 14 4 The autocrine activity is shown in Table 3 14 5

Table 3 14 4 Dilution curve of TGF activity of RPMI-2650 CM [10X] from suspension culture

Test Sample	CFE (%) NRK*
RPMI-2650 CM [21X] 100	19 5 ± 4.0
90	18 5 ± 1 7
80	14 2 ± 5 6
70	18 8 ± 3 7
60	10 8 ± 5 9
50	9 0 ± 5 5
40	4 9 ± 2 5
30	4 8 ± 1 0
20	2 18 ± 0 08
10	0 95 ± 0 3
Control Medium MEM	0

* ± S E M (n = 3) of 6 x 10^3 NRK cells per 30mm plate

Table 3 14 5 Dilution curve of autocrine activity of RPMI-2650 CM [21X] from suspension culture

Test Sample	CFE (%) RPMI-2650*
RPMI-2650 CM [21X] 100	0 24 ± 0 20
90	0 21 ± 0 09
80	0 15 ± 0 04
70	0 14 ± 0 02
60	0 02 ± 0 03
50	0 04 ± 0 01
40	0 09 ± 0 07
30	0 05 ± 0 02
20	0 10 ± 0 04
10	0 05 ± 0.04
Control Medium MEM	0

* ± S E M (n = 3) of 5 x 10^4 cells per 30mm plate

The dilution curve results of RPMI-2650 CM in the TGF assay show poor stimulation at low CM concentrations The stimulatory effect of RPMI-2650 CM is very obvious up to 70% where it peaks Above this concentration, the optimum sensitivity of the TGF assay has been reached The autocrine dilution curve shows that RPMI-2650 cells are less sensitive to stimulation by RPMI-2650 CM than NRK cells RPMI-2650 cells are insensitive to the presence of low concentrations of RPMI-2650 CM Colonies form in some plates and very few colonies in other plates A definite stimulatory effect is seen only above 70% of a 21X concentrate

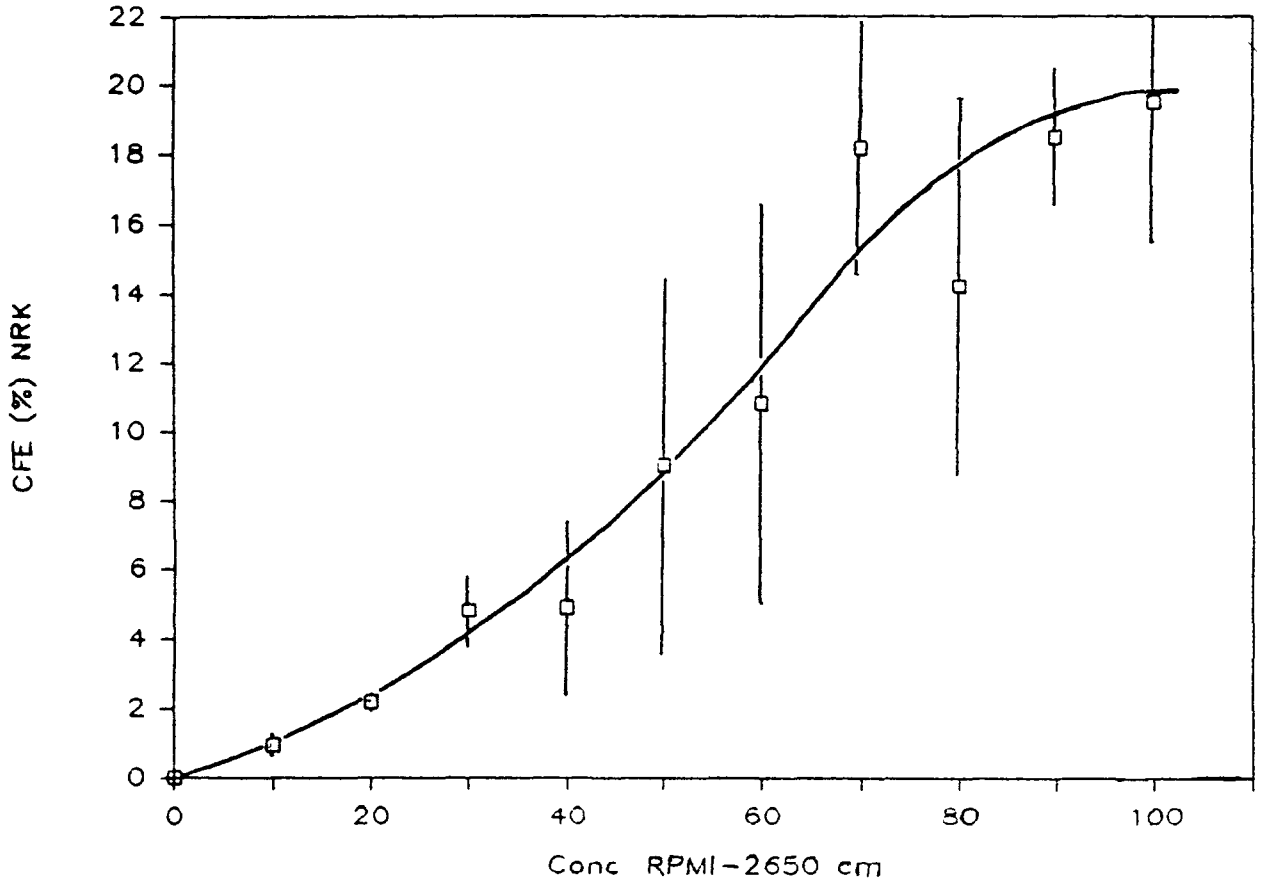


Fig. 3.14.4 Dilution curve of RPMI-2650 CM from suspension cultures in the TGF assay.

3 15 1 Large scale production of RPMI-2650 CM

It was necessary to culture RPMI-2650 cells on a larger scale than with 25cm^2 flasks (10mls per flask) if the large volumes of CM (>500mls) needed for purification/fractionation were to be obtained routinely in a reasonable length of time

The trend of TGF activity of conditioned media was shown to be dependent on cell concentration (see Table 3 15 1, experiment using 25cm^2 flasks for collection of RPMI-2650 CM) and time of conditioning (see Table 3 15 3, CM from roller bottles)

Roller bottles, microcarrier cultures and suspension cultures were set up as described in Sections 2 11 1, 2 11 2, and 2.11 3 Tables 3.15 2 and 3 15 3 compare activities of CM produced by RPMI-2650 cells grown in 25cm^2 flasks, roller bottles, suspension and microcarrier cultures Suspension cultures seem to provide the best results, roller bottles are also a very simple and convenient method for scale-up The microcarrier method is long and tedious to carry out, and the data presented indicate that it has no special advantage for this particular application Subsequently, large scale production of CM was performed exclusively with roller and suspension cultures

Table 3 15 1 TGF activity of RPMI-2650 produced from 25cm^2 flasks

Concentration of RPMI-2650 cells per 25cm^2 flask	CFE (%) NRK * CM collection after 48 hours
3 x 10^5	0 53 ± 0 50
1 x 10^6	0 66 ± 0 75
1 5 x 10^6	1 0 ± 0 50
2 x 10^6	1 66 ± 0 28
6 x 10^6	1 16 ± 1 60
9 x 10^6	1 83 ± 0 57

* ± S E M (n = 3) of 1.2 x 10^3 cells per 35mm plate

Table 3 15 2 TGF activity of RPMI-2650 CM from large scale cell culture methods

Test Sample	CFE (%) NRK*
RPMI-2650 CM Suspension culture	2 48 ± 0 12
" Microcarrier Cytodex 1	1 86 ± 0 12
" Roller bottle	1 62 ± 0 56
Control Medium	0

* ± S E M (n = 3) of x 10^3 cells per 35mm plate

Table 3 15 3 TGF activity of RPMI-2650 CM produced by various culture methods

Test Sample	CFE (%) NRK*
RPMI-2650 CM roller bottle 24hr	0 26
" " " 48hr	1 40
" " " 72hr	1 60
Microcarrier Cytodex 1 12hr	0 26
" " 24hr	0 13
25cm^2 flask 12hr	0 06
" " 24hr	0 06
" " 48hr	0 26
Control medium (SLM)	0

* 3 3 x 10^3 cells per 35mm plate

3 15 2 RPMI-2650 CM production by suspension culture

RPMI-2650 cells were set up in suspension culture as described in Section 2 11 2 Initial experiments were set up to determine the parameters of RPMI-2650 cells in suspension culture Inoculation density, spinner speed, feeding and counting methods were examined It was found that RPMI-2650 cells grew well in suspension culture The growth curve showed an initial lag period of 24-48 hrs which was followed by a period of rapid growth Feeding at 4hr intervals was necessary to maintain steady growth, which was greatly reduced by nutrient depletion Fig 3 15 1 shows a flow chart of RPMI-2650 suspension culture systems. A 100ml suspension culture was used as a starting culture (S_1) , which was subcultured after 8 days into two more 100ml suspension cultures (S_2 and S_3), these were used to inoculate a 200ml suspension culture L_1) using half the total cell number from S_1 and S_2 S_2 was subsequently used for CM preparation which was collected for 7 days at 24 hour intervals S_3 was used to set up 200ml suspension cultures L_2 and L_3 L_1, L_2 and L_3 were used for CM preparation collected for 8 days at 4 hour intervals

In 100ml suspension culture system 4 x 10^6 cells/ml were inoculated and grew to about 1 4 x 10^7 cells/ml by day 10 Thereafter, some decrease in cell numbers occurred In a 200ml suspension culture system, 3 0 x 10^6 cells/ml were inoculated and grew to about 2 x 10^7 cells per ml after 10 days in culture and thereafter some decrease in cell numbers occurred

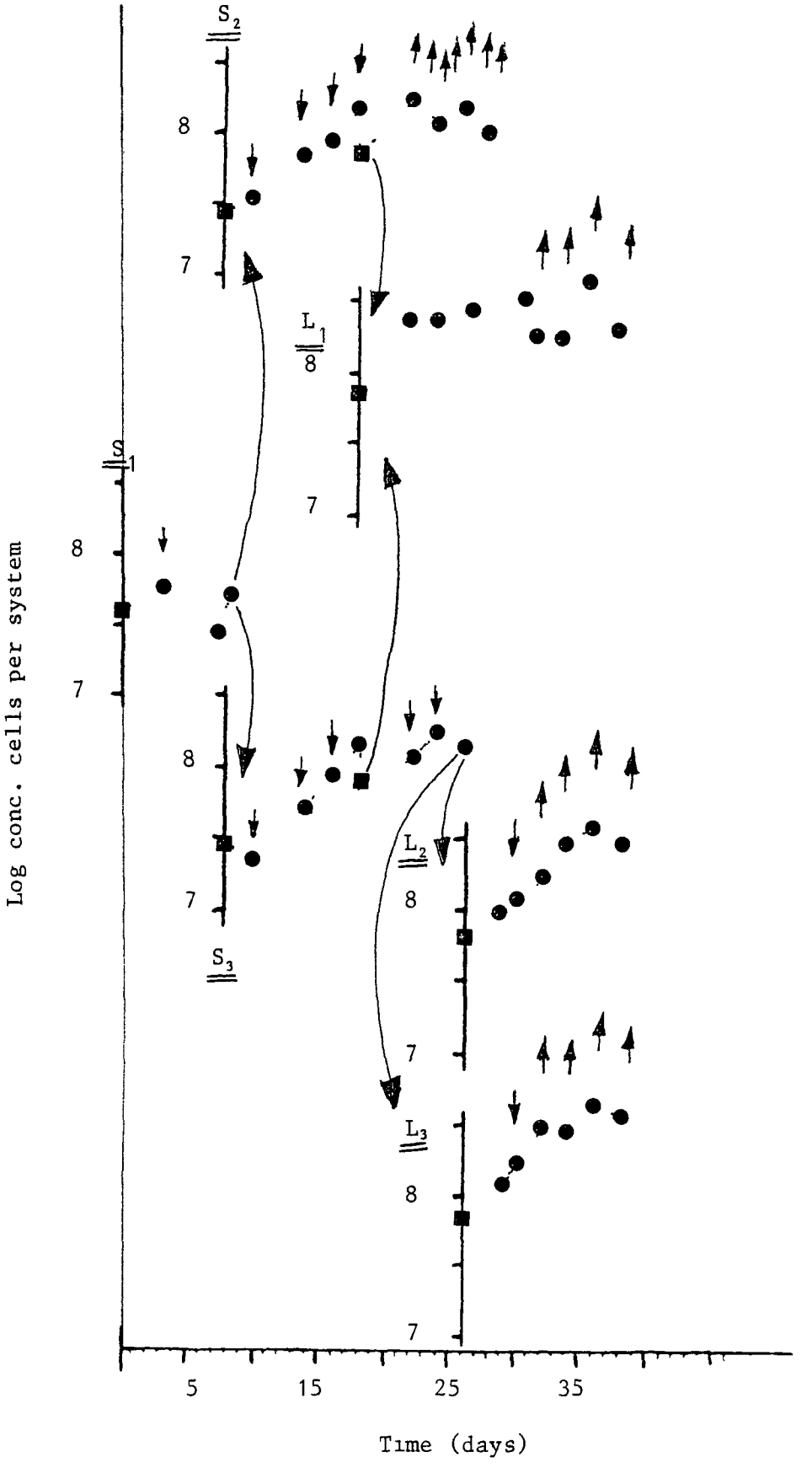


Fig. 3.15.1. Growth of RPMI-2650 cells in suspension culture

Log conc. cells per culture system ●

S - 100ml culture, L - 200ml culture

↓ - feed, ↑ - collection of CM

■ Inoculation concentration

3 15 3 TGF activity of RPMI-2650 CM prepared from suspension culture

Table 3 15.4 shows the TGF activity of RPMI-2650 CM collected from a 100ml suspension culture S_3, as shown in Fig 3 15 1 100ml batches of CM were collected at 24hr intervals for 7 days Table 3 15 5 shows the TGF activity of RPMI-2650 CM collected from 3 200ml suspension cultures L_1, L_2 and L_3 200ml batches of CM were collected at 48hr intervals for 4 days Samples of each of these batches of CM were assayed for TGF activity and the remaining CM pooled and ultrafiltered through YM5 to [28X] for the 24 hour batches and [21X] for the 48 hour batches

These results show that RPMI-2650 cells produce a steady flow of TGF activity The [21X] and [28X] concentrates gave very high activity as expected These concentrates were used for dilution curves (Table 3 14 4 and 3 14 5) and for ^{3}H-Thymidine assay (3 12)

Table 3 15 4 TGF Activity of RPMI-2650 CM batches from a 100ml suspension culture system at 24hr intervals

Test Sample	CFE (%) NRK*
RMPI-2650 CM(S_2)** 24hrs	0 78 ± 0 09
" 48hrs	0 55 ± 0 17
" 72hrs	0 47 ± 0 09
" 96hrs	0 55 ± 0 04
" 120hrs	0 69 ± 0 17
" 144hrs	0 75 ± 0 14
" 168hrs	0 69 ± 0 21
[28X] CM concentrate (5,000 retentate)	27 48 ± 3 12
Control medium (MEM)	0 24 ± 0 15

* ± S E M (n = 3) of 6 x 10^3 cells per 30mm plate

** S_3 as in Fig 3 15 1

Table 3 15 5 TGF activity of RPMI-2650 CM prepared from 200ml suspension cultures

Test Sample			CFE (%) NRK*
RPMI-2650 CM	L_1**	48 hrs	0 92 ± 0 34
"		96 hrs	0 61 ± 0 25
"		144 hrs	0 36 ± 0 05
"	L_2	48 hrs	0 38 ± 0 17
"		96 hrs	1 25 ± 0 33
"		144 hrs	0 92 ± 0 08
"	L_3	48 hrs	0 78 ± 0 09
"		96 hrs	0 83 ± 0 23
"		144 hrs	1 08 ± 0 43
[21X] CM concentrate (5,000 retentate)			25 99 ± 2 29
Control Medium (MEM)			0 24 ± 0 15

* ± S E M (n = 3)

** L_1, L_2 and L_3 200mls cultures as in Fig 3 15 1

3 16 1 Bio-Gel P-60 Chromatography of 663+N CM

A sample of 663+N CM was ultrafiltered to [50X] through a YM5 Amicon membrane as described in 2 12 The [50X] sample was dialysed against 1% acetic acid and lyophilized as described in 2 15 The sample was reconstituted in 1M acetic acid and clarified by centrifugation at 5,000 rpm for 10 mins and manually applied to a Bio-Gel P-60 column 28 x 6ml fractions were collected 1 5ml lots of each fraction were lyophilized and reconstituted in 1ml growth medium, filter sterilized and assayed for TGF activity 0 8 ml samples of each fraction were assayed for protein content by the Bio-Rad Microassay method as described in 2 16 The protein profile of 663+N CM eluted from Bio-Gel P-30 is shown in Fig 3 16 1 and the TGF profile is shown in Fig 3 16 2

These results show that 663+N CM TGF activity elutes into two peaks of activity Pool II is completely separated from bulk protein while Pool I is not fully separated from bulk protein Pool I is found mainly in fractions [10-12] and Pool II mainly in fractions 15-18

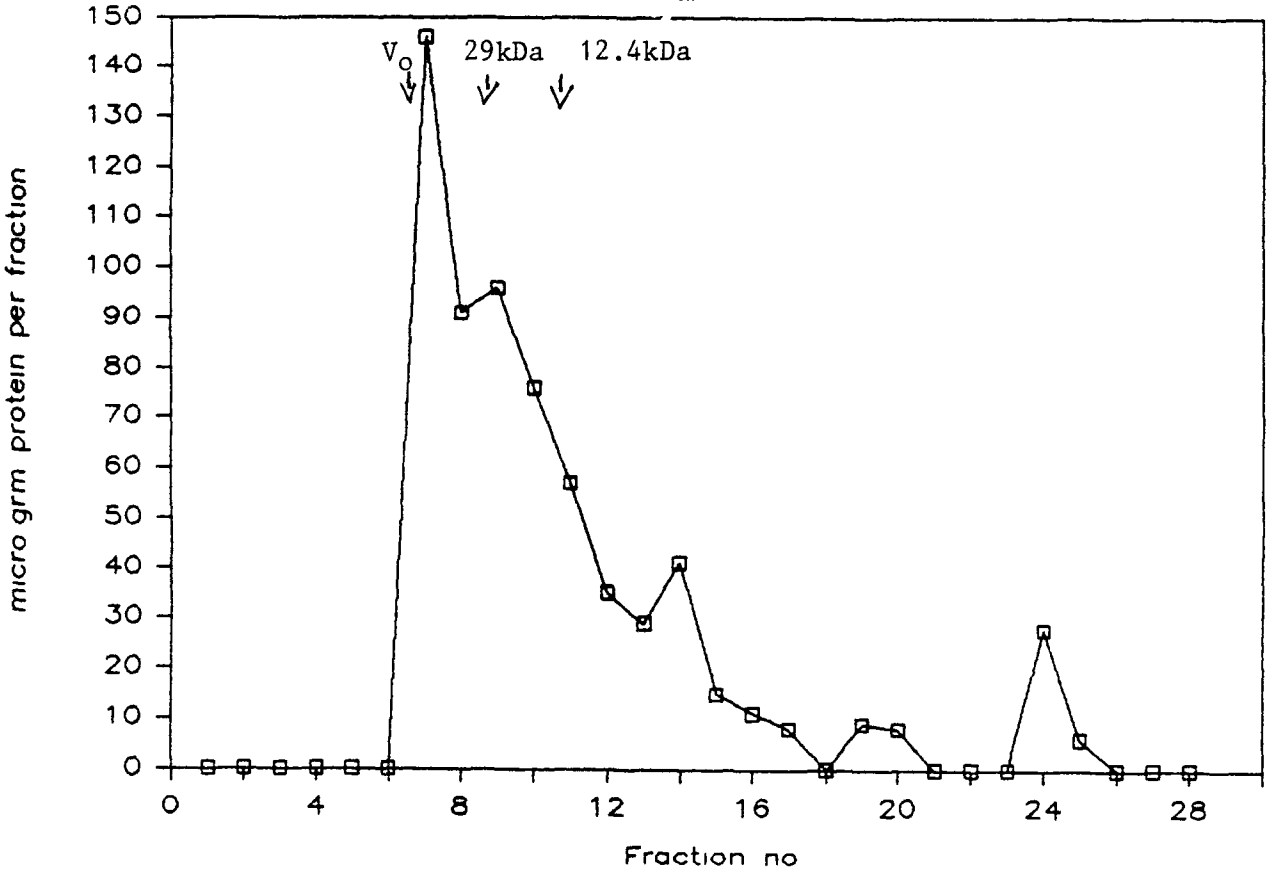


Fig. 3.16.1 Protein analysis of Bio-Gel P-60 Chromatography of 663+N CM

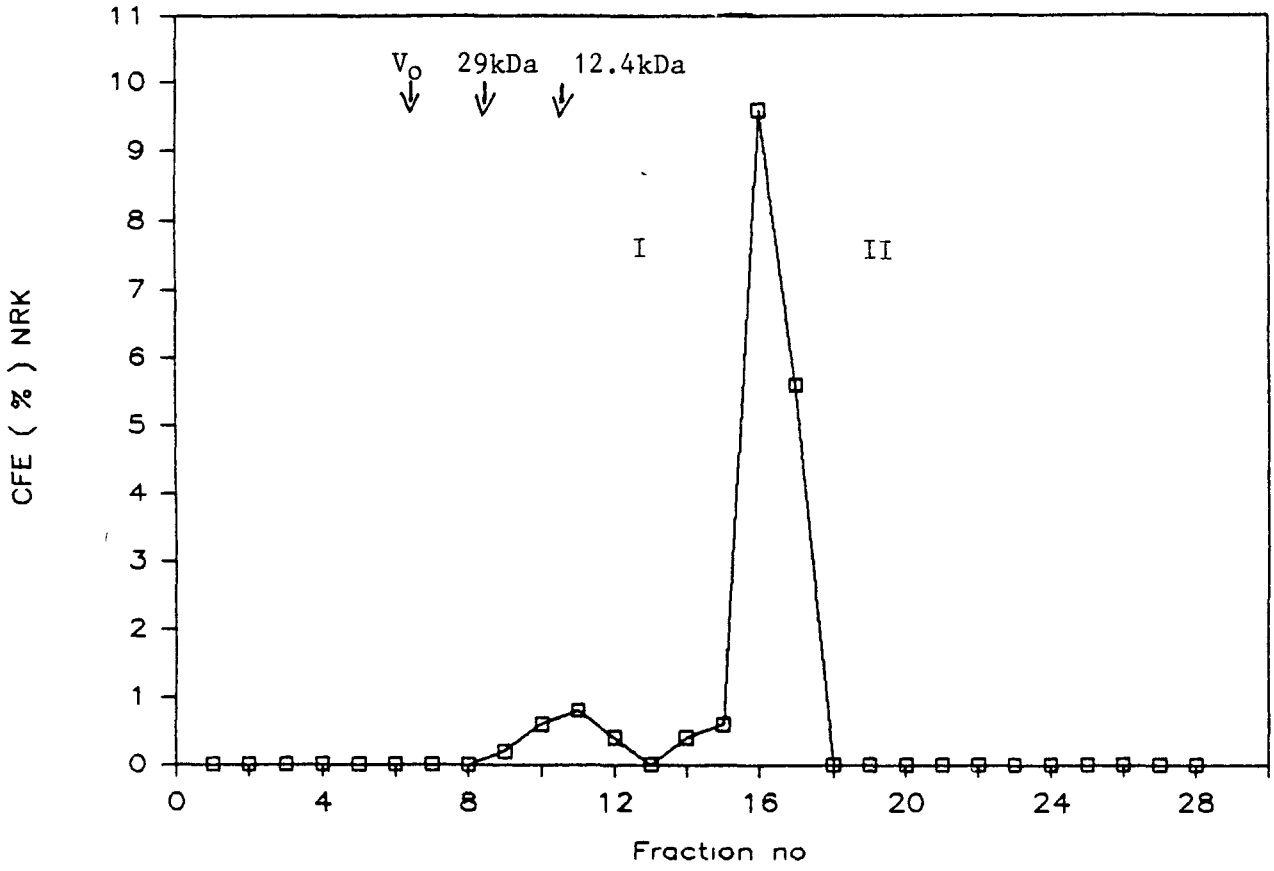


Fig. 3.16.2 Bio-Gel P-60 Chromatography of 663+N CM TGF Activity

3 16 2 Bio-Gel P-60 Chromatography of RPMI-2650 CM

A sample of RPMI-2650 CM was ultrafiltered to [300X] through a YM5 Amicon membrane This [300X] 5,000 retentate was dialysed against 1% acetic acid, lyophilized, reconstituted in 1ml 1M acetic acid and clarifed by centrifugation at 5,000 rpm for 10 mins as described in Sections 2 12 and 2 15

This sample was then applied to a Bio-Gel P-60 column 30 x 8ml fractions were collected 1 5ml samples were removed from each fraction, lyophilized and reconstituted in growth medium These samples were filter sterilized and assayed for TGF activity Samples were also assayed for TGF activity in the presence of 2ng ml^{-1} EGF Protein determination was by the Bio-Rad Micro-assay (2 16) The protein profile is shown in Fig. 3 16 3 and the TGF and TGF + EGF assays are shown in Fig 3 16 4 and Fig 3.16 5

These results show that RPMI-2650 CM contains TGF activity which is eluted by Bio-Gel P-60 chromatography A distinct peak of activity was located around fractions 9 and 10 A calibration curve for approximate molecular weight determination showed that this activity was approximately 7 8 kDa and on analysis of the 663+N TGF activity profile, this peak appeared to elute in a similar molecular weight range (differences in fraction volume were taken into consideration) The broad band of TGF activity for RPMI-2650 CM in the presence of EGF may imply that RPMI-2650 CM contains a strongly potentiated EGF factor(s) which is present in high amounts in RPMI-2650 CM The TGF and TGF + EGF activity may be different An alternative chromatography gel with a narrower fractionation range than Bio-Gel P-60 separated the RMPI-2650 TGF factor(s) more clearly Bio-Gel P-30 was subsequently set up This gel has a fractionation range from 40 kDa to 2 5 kDa Bio-Gel P-60 has a fractionation range of 60 kDa to 3 kDa

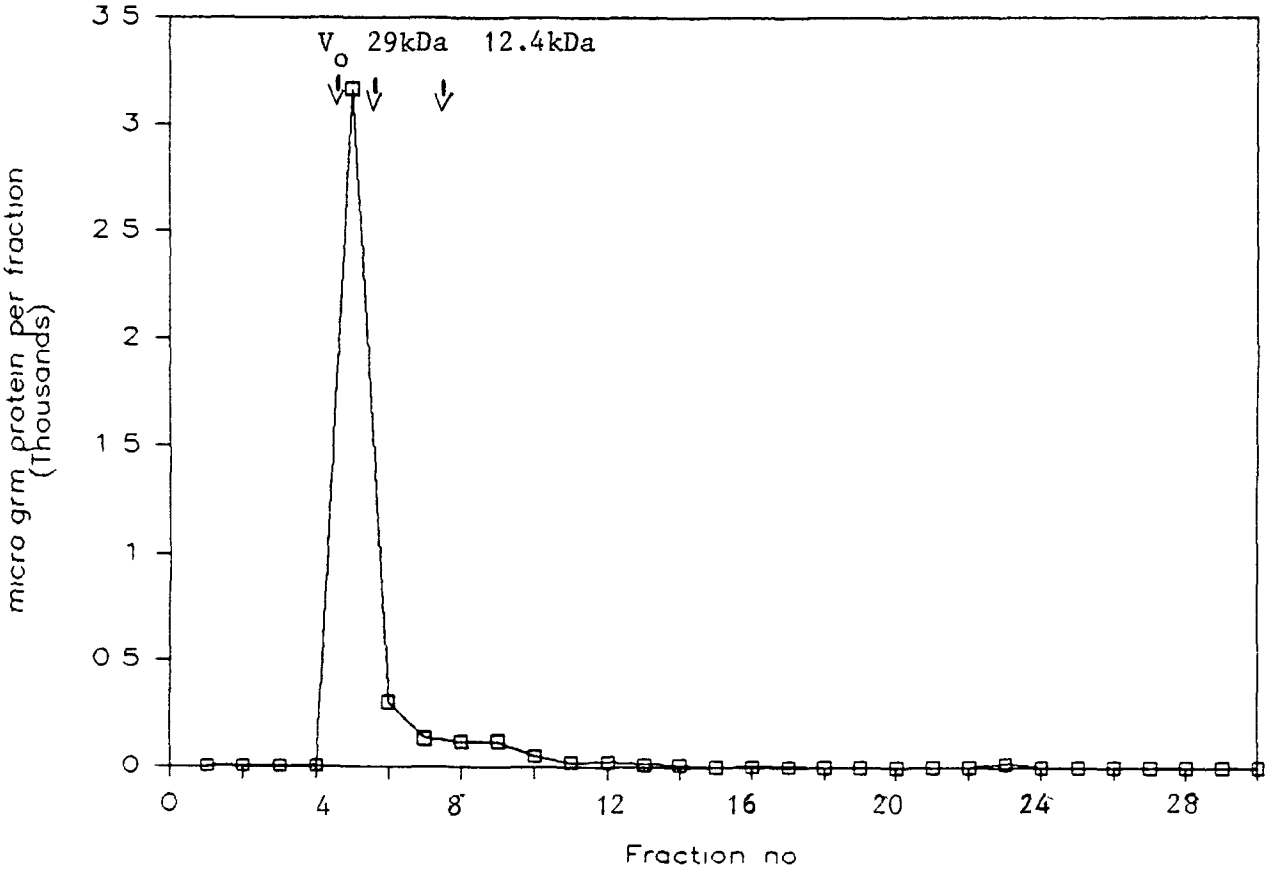


Fig. 3.16.3 Protein analysis of Bio-Gel P-60 Chromatography of RPMI-2650 CM

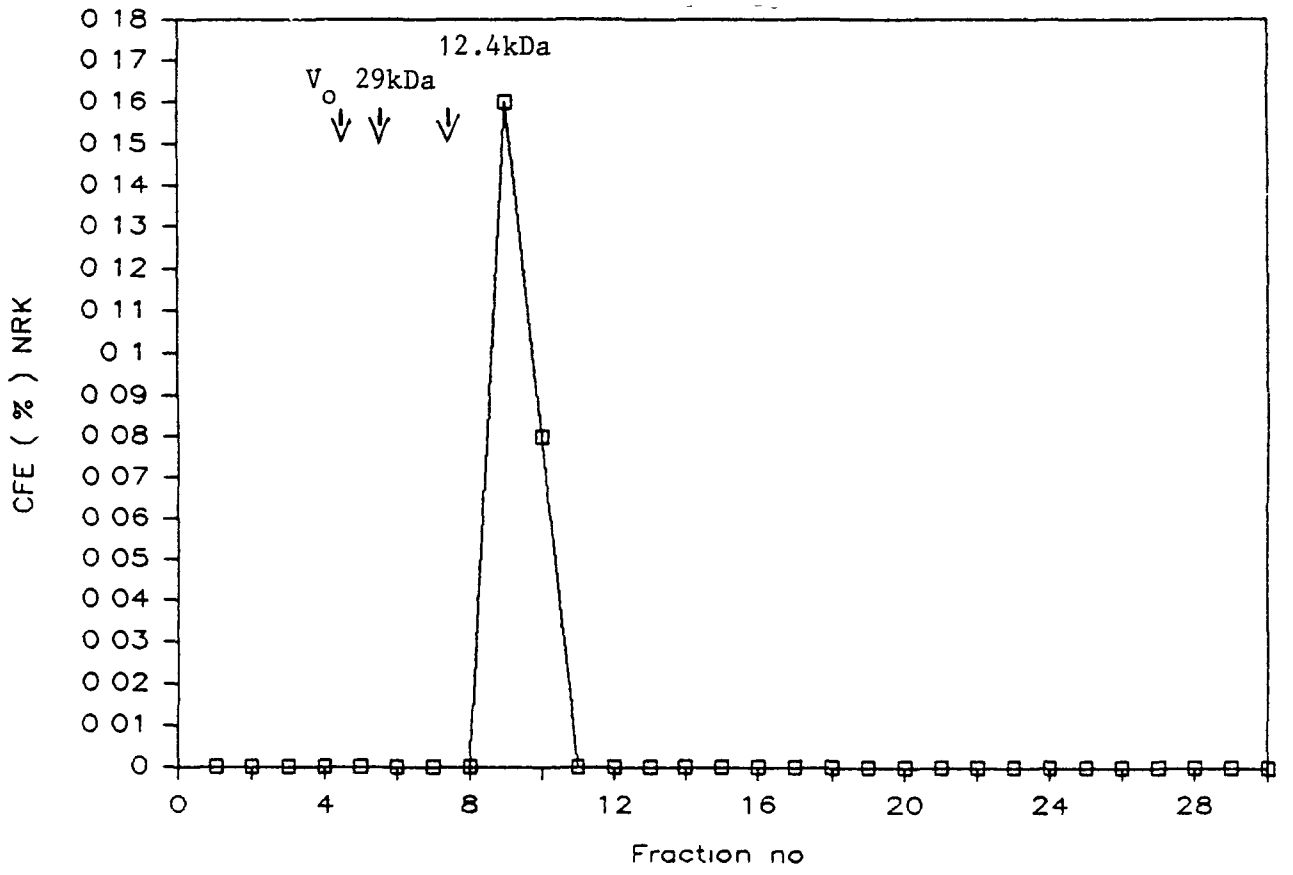


Fig. 3.16.4 Bio-Gel P-60 Chromatography of RPMI-2650 CM TGF Activity

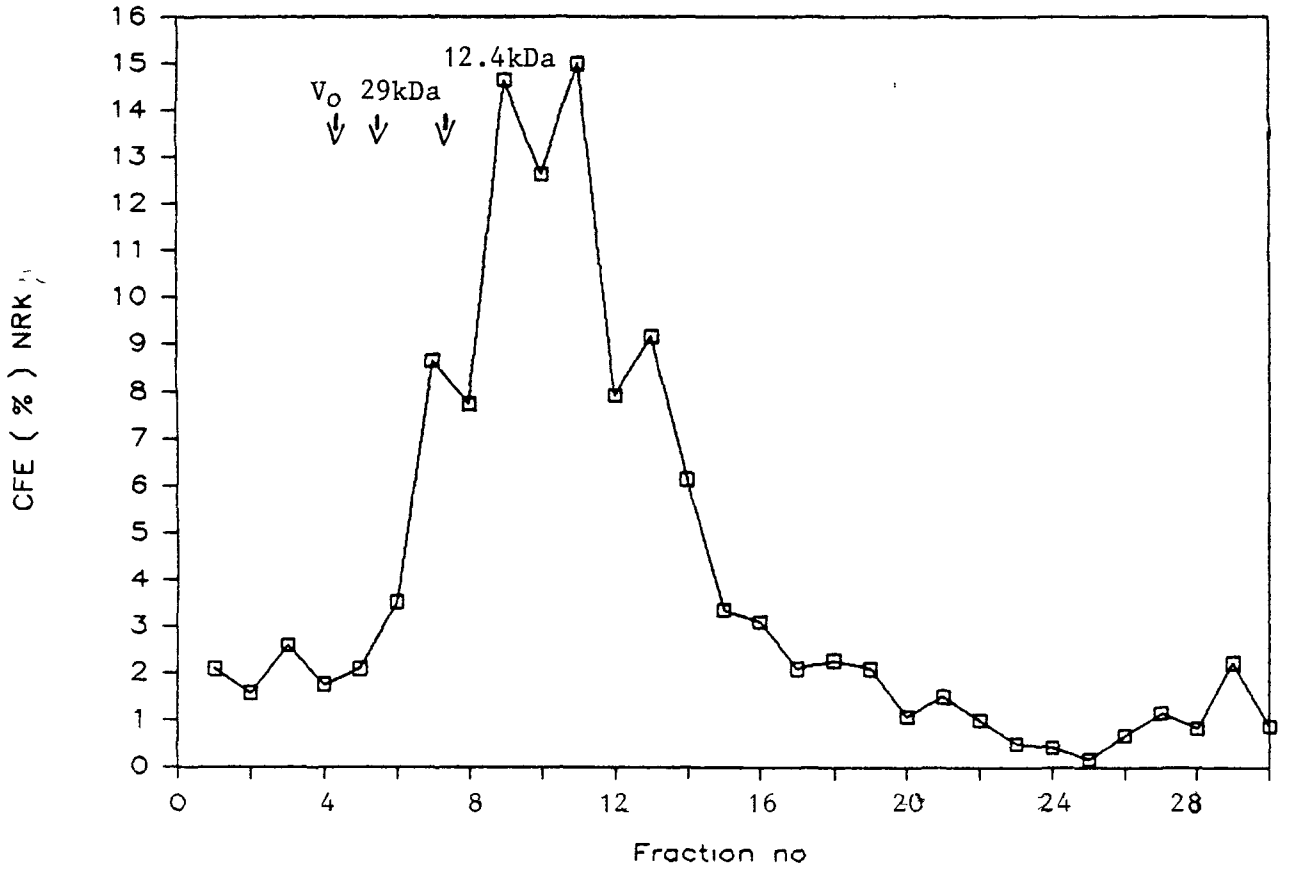


Fig. 3.16.5 Bio-Gel P-60 Chromatography of RPMI-2650 CM TGF + EGF Activity

3 17 1 Bio-Gel P-30 Chromatography of RPMI-2650 CM

A [550X] sample was prepared as described in Secion 2 18 and applied to a Bio-Gel P-30 column (2 2mg protein) 8ml fractions were collected and 0 8ml samples were assayed for protein content as described in Section 2 16 The protein profile is shown in Fig 3 17 1 The remaining fractions were lyophilized and reconstituted in 3 5mls growth medium and filter sterilized Various bioassay activity profiles were then determined The TGF profile of RPMI-2650 CM fractionated by Bio-Gel P-30 is shown in Fig 3 17 2 The TGF-β profile is shown in Fig 3 17 3 (-EGF), (+EGF) Fig 3 17 4 and the autocrine activity profile is shown in Fig 3 17 5

These results show that major peaks of growth activity elute around fraction [16] in these bioassays A second smaller peak of autostimulatory activity elutes at a lower molecular weight peak

Samples of each fraction (100µl of fractions reconstituted in 3 5ml volumes) were also tested in the ^{125}I-EGF radioreceptor assay but no activity was detected, possibly because the samples were insufficiently concentrated for this assay (in a later column run - 3 17 2 - this assay was successfully carried out)

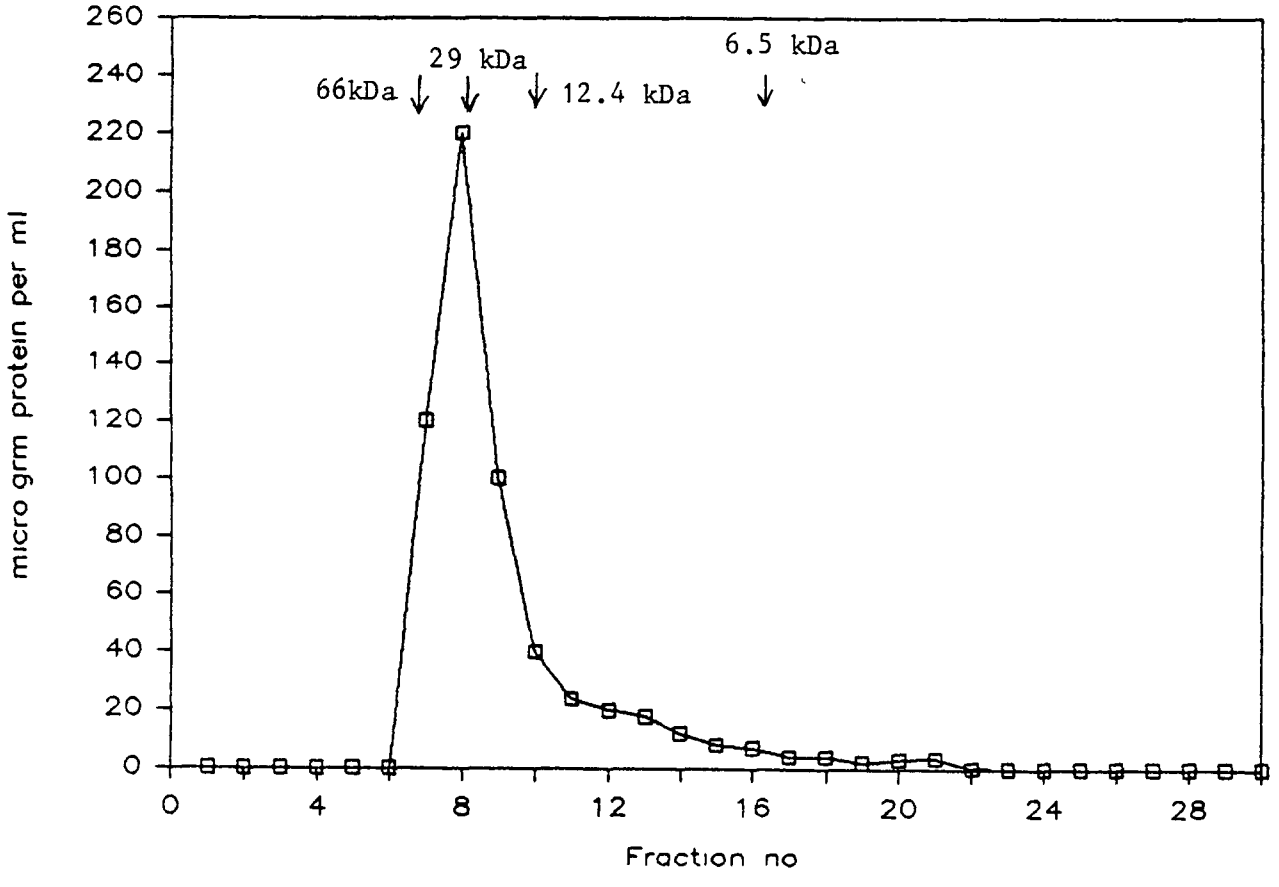


Fig. 3.17.1 Protein Profile of RPMI-2650 CM fractionated by Bio-Gel P-30 Chromatography

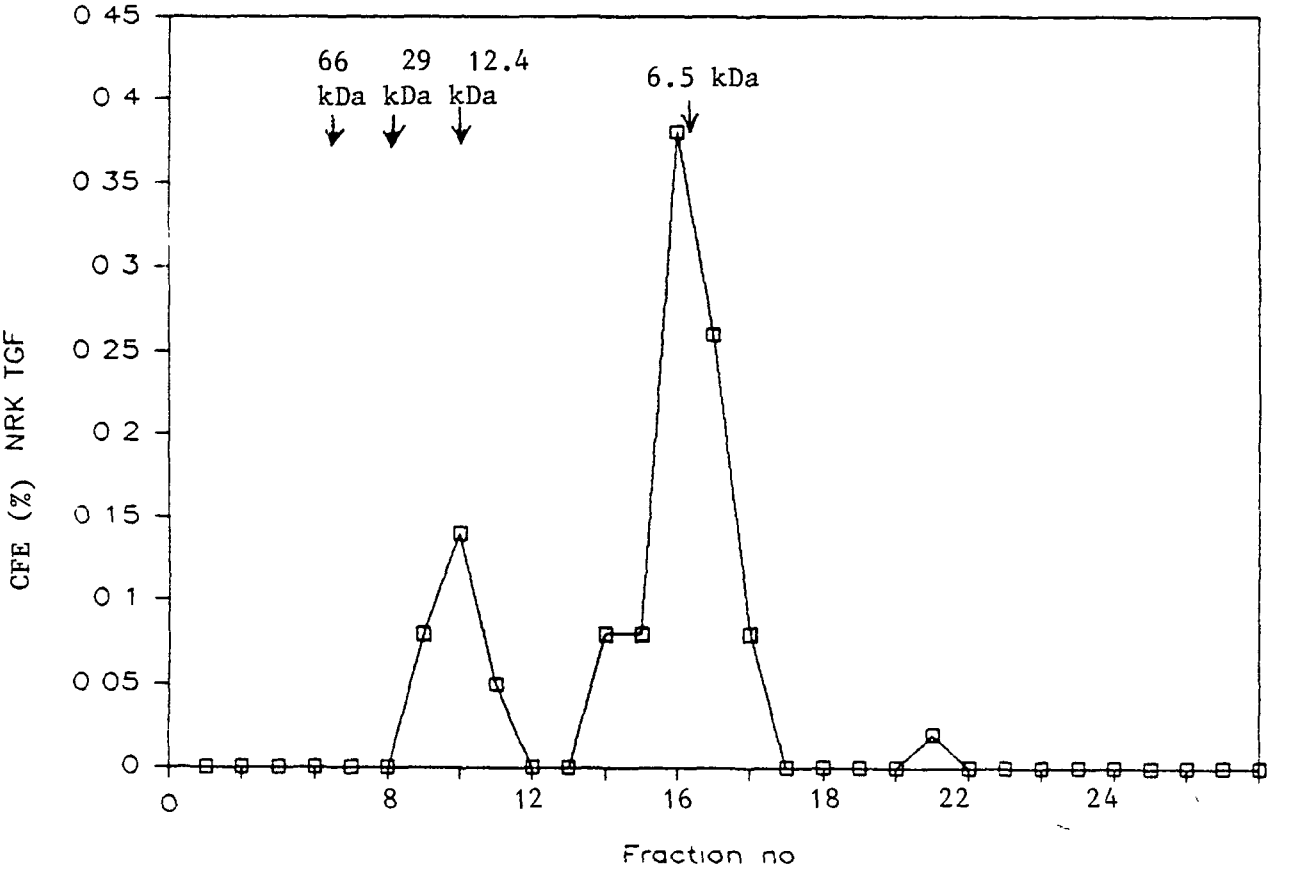


Fig. 3.17.2 TGF Activity of RPMI-2650 CM from Bio-Gel P-30 Chromatography

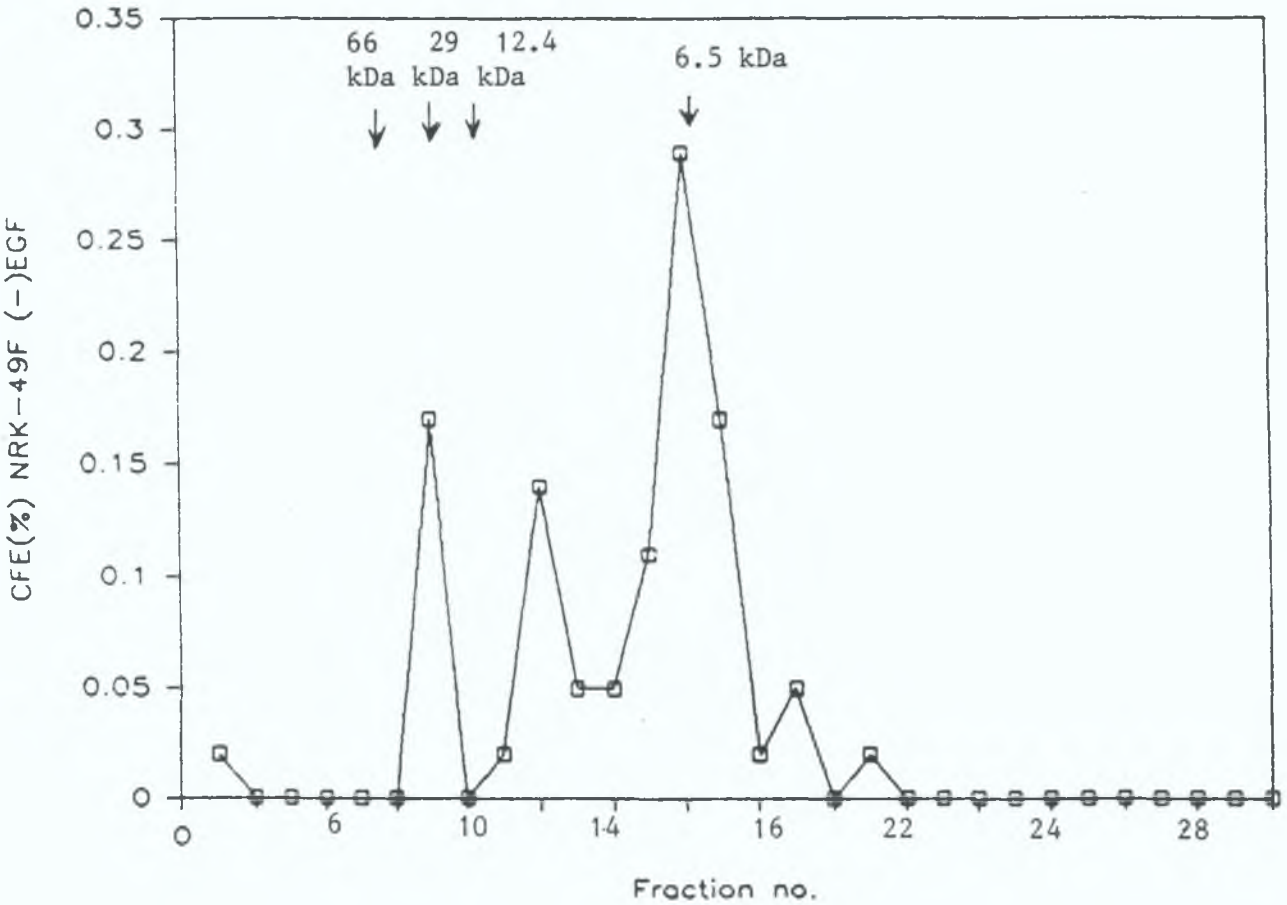


Fig. 3.17.3 TGF-β (-EGF) activity of RPMI-2650 CM from Bio-Gel P-30 Chromatography

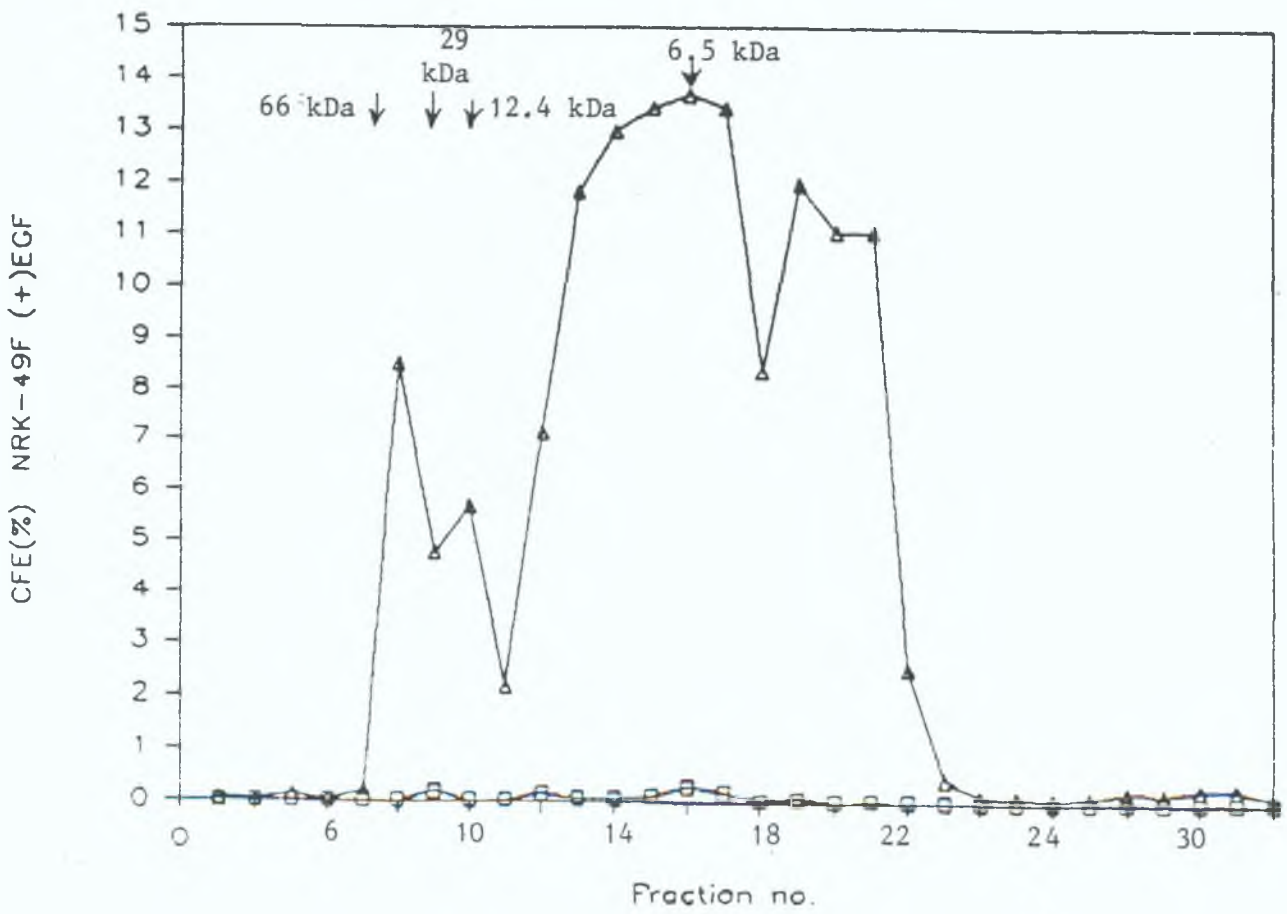


Fig. 3.17.4 TGF-β activity of RPMI-2650 CM from Bio-Gel P-30 Chromatography (NRK-49F-EGF activity of RPMI-2650 is also shown □)

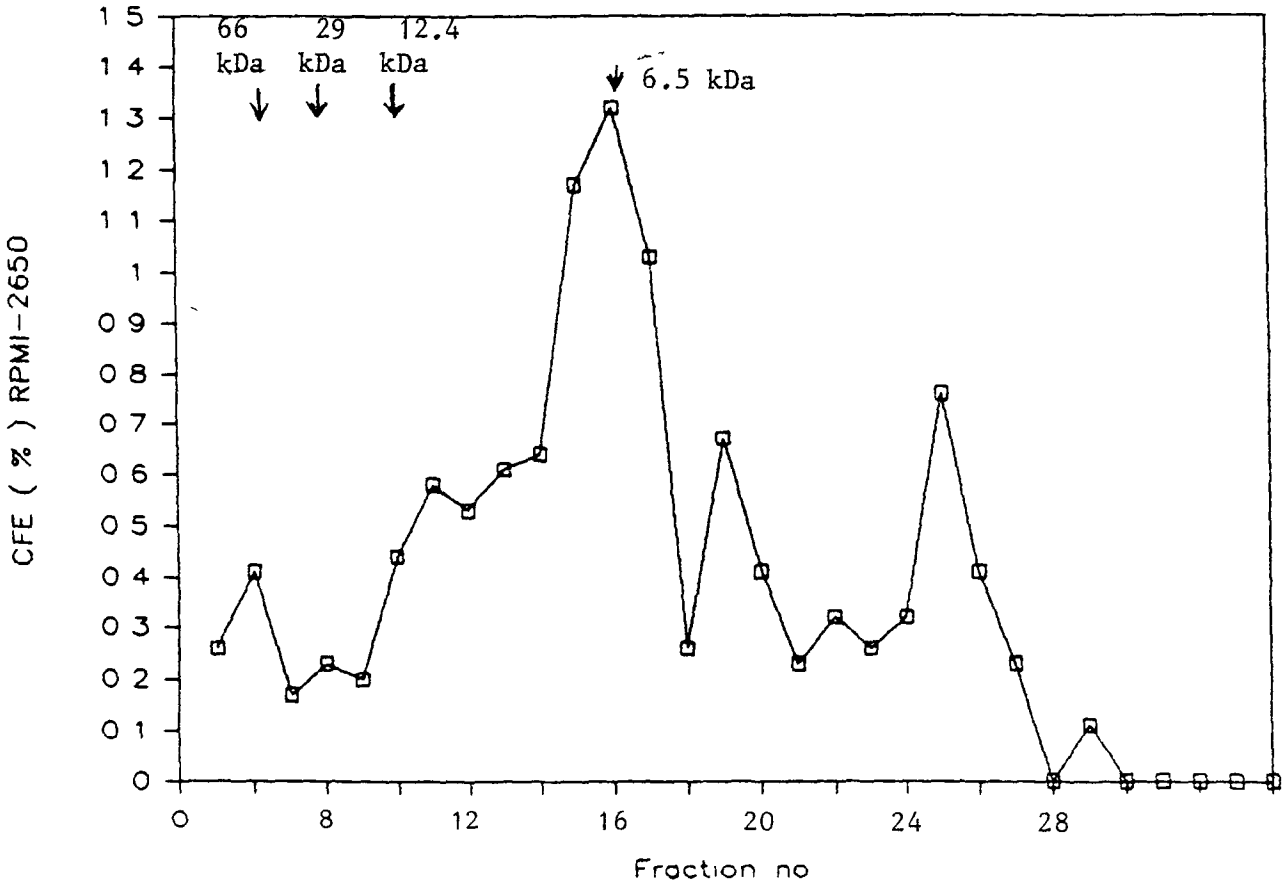


Fig. 3.17.5 Autocrine activity of RPMI-2650 CM from Bio-Gel P-30 Chromatography

3 17 2 ^{125}I-EGF inhibition and ^{3}H-Thymidine incorporation of Bio-Gel P-30 Chromatography of RPMI-2650 CM

A (600X) concentrated sample of RPMI-2650 CM was prepared as described in 3 18 1 and eluted from a Bio-Gel P-30 chromatography column 8ml fractions were collected 0 8ml samples were assayed for protein content as described in Section 2 16 The protein profile is shown in Fig 3 17 6 The remaining sample was lyophilized and reconstituted in 200µl growth medium 100µl samples were assayed for ^{125}I-EGF competition as described in Section 2 20 The remaining 100µl samples were brought to 1 3mls with growth medium, filter sterilized and assayed for autocrine activity and ^{3}H-Thymidine incorporation into NRK cells as described in 2 10 4 and 2 19 Fig 3 17 2 shows the ^{125}I-EGF inhibition profile of RPMI-1650 CM eluted by Bio-Gel P-30 chromatography and Fig 3 17 8 shows the ^{3}H-Thymidine incorporation profile into NRK cells by RPMI-2650 CM from Bio-Gel P-30 chromatography

The results in Fig 3 17 7 and Fig 3 17 8, show that RPMI-2650 CM contains an EGF like molecule which is also mitogenic for NRK cells in monolayer The autocrine assay set up at the same time as these assays did not work It would appear from these results that the TGF activity seen in Fig 3 17 2 is not necessarily due to TGF-α as was the general impression from that experiment, but to a separate molecule or molecules distinct from TGF-α Further purification was necessary to determine whether the TGF activity shown in Fig 3 17 2 was due to one or more molecules Further purification of RPMI-2650 CM was subsequently carried out by HPLC as in Section 3 18 1

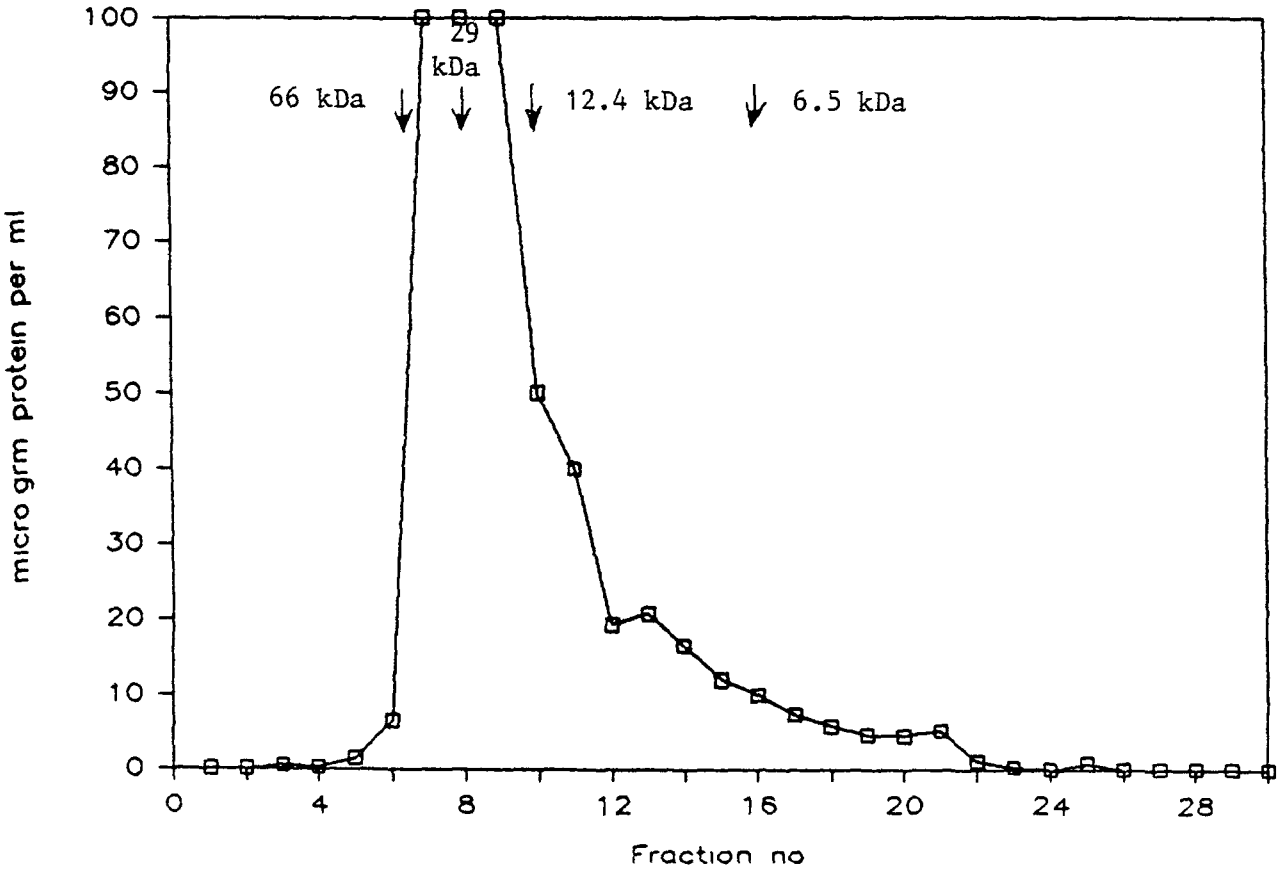


Fig. 3.17.6 Protein profile of RPMI-2650 CM from Bio-Gel P-30 Chromatography

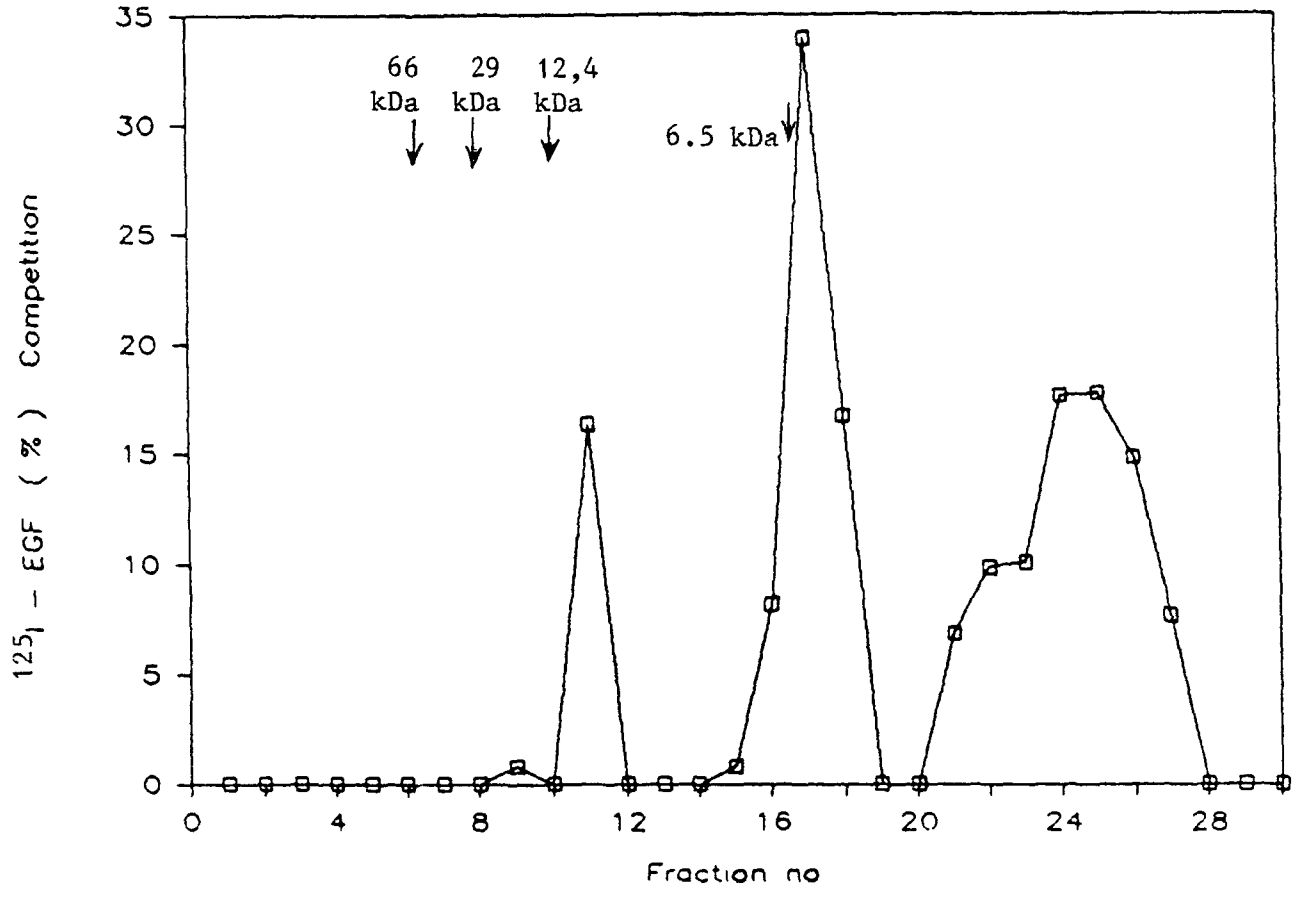


Fig. 3.17.7 ^{125}I EGF competing activity in RPMI-2650 CM from Bio-Gel P-30 Chromatography

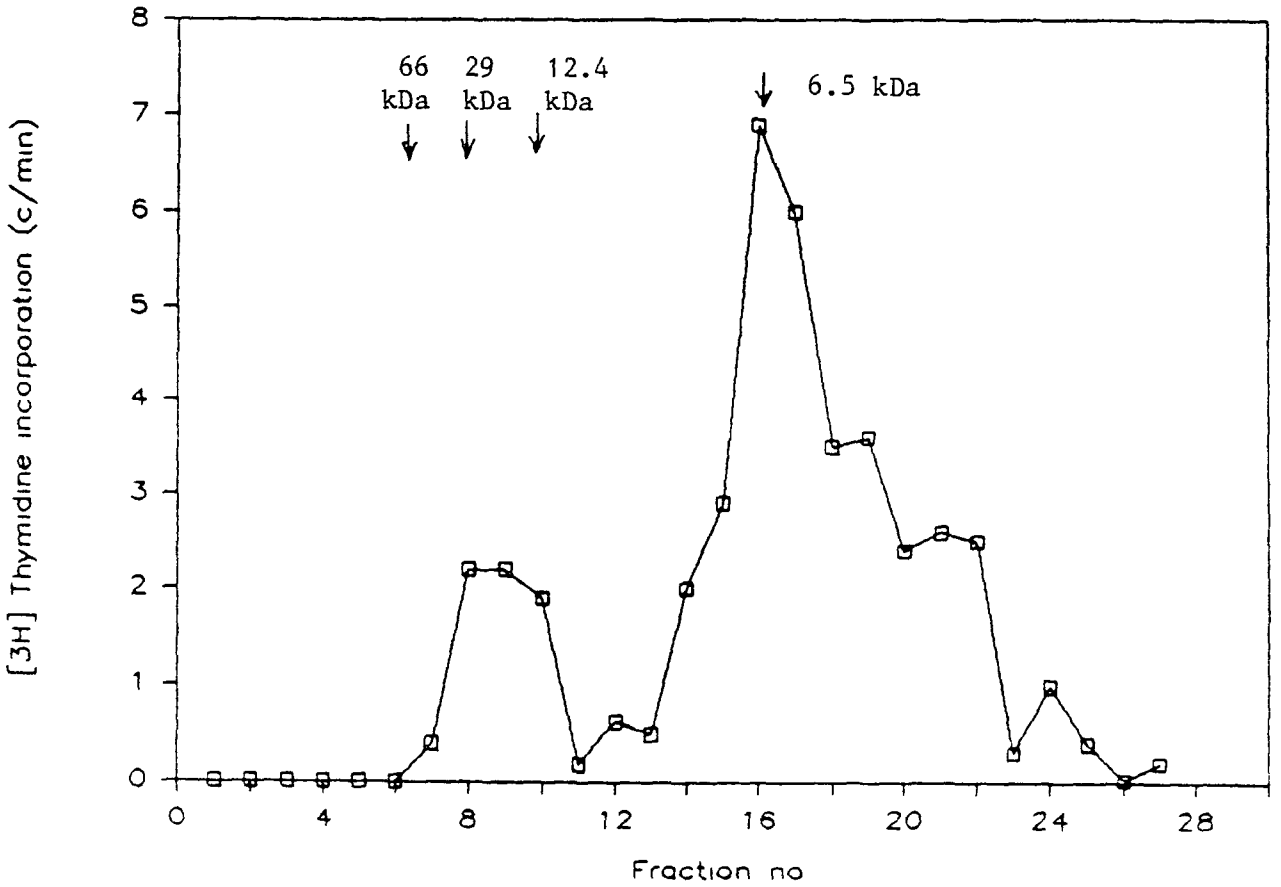


Fig. 3.17.8 ^{3}H Thymidine incorporation into NRK cells by RPMI-2650 CM from Bio-Gel P-30 Chromatography

3 18 1 Fractionation of ^{125}I-EGF competing activity from 663+N CM by HPLC

In order to check the feasability of HPLC separation of TGFs in RPMI-2650 CM, we first tested the system using CM from the high level TGF producer 663+N A 150ml concentrated sample of 663+N CM was prepared as described in Section 2 22 and applied to a C18 μBondapak column in 0 5ml of 0 1% TFA 1 6ml fractions were collected, lyophilized reconstituted in 500μl growth medium 100μl samples were assayed for ^{125}I-EGF competion activity No activity was detected A second run using a [300X] sample of pretreated CM, as described in Section 2 22, was dissolved in 1ml of 0 1% TFA This 1ml sample was run on the column 1 6ml fractions were lyophilized and reconstituted in 300μl of growth medium 100μl samples were assayed for ^{125}I-EGF competition activity A definite peak of activity was detected in fractions [16-20] as shown in Fig 3 18 1 Since only fractions 9-54 were assayed, the possibility that further activity would have been eluted outside these fractions cannot be excluded

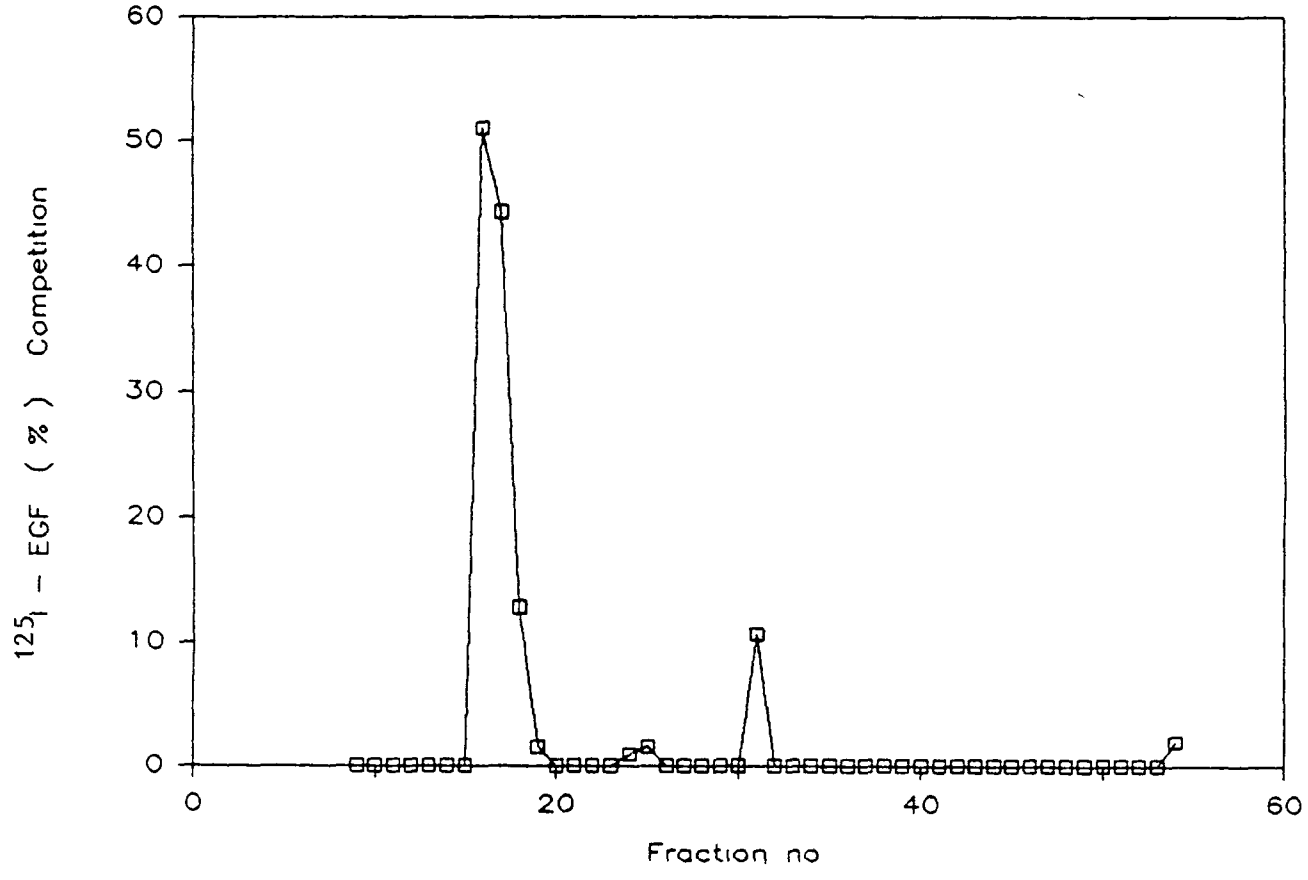


Fig. 3.18.1 ^{125}I-EGF competing activity of 663+N CM fractionated by HPLC

3 18 2 Fractionation of ^{125}I-EGF competing and TGF-β activities from RPMI-2650 CM by HPLC

Bio-Gel P-30 chromatography had separated the TGF activity (of RPMI-2650 CM) into two major peaks (Fig 3 17 2) A [700X] sample of RPMI-2650 CM was subsequently eluted on a Bio-Gel P-30 column and these two peaks were pooled as follows and applied to a C18 μBondapak column as described in 2 21

Pool I - High molecular weight fractions [8, 7, 10] at approximately 24mls This sample was lyophilized and reconstituted in 1ml 0 1% TFA and applied to the HPLC column for the first run

Pool II - Lower molecular weight fractions, [11-20] at approximately 80mls, were lyophilized and reconstituted in 1ml 0 1% TFA, was applied to the HPLC column in a second run

Up to 72 x 1 6 ml fractions were collected from each run These were lyophilized using a solvent trap Every third fraction was reconstituted in 200μl growth medium 100μl of this was assayed for ^{125}I-EGF competing activity The results from these assays are shown in Fig 3 18 2 and Fig 3 18 3

The remaining fractions were reconstituted in 1ml growth medium, filter sterilized and every alternative fraction was assayed for TGF-β or autocrine activity Fig 3 18 4 shows the results for the TGF-β assays No peak of activity was detected in the autocrine assay for either pool I or pool II

The failure to detect autocrine activity could be due to a number of factors

(1) Dilution on the HPLC below detectable limits
(2) Irreversible binding to the HPLC column
(3) Instability during the HPLC run
(4) Separation of two or more fractions on the HPLC, which need to act in combination in order to stimulate proliferation of RPMI-2650 cells

Explanations (2), (3) and (4) would imply non-identity of the RPMI-2650 autocrine activity with TGF-α or TGF-β , since the latter activities survived the HPLC procedure Explanation (1) seems unlikely, in the control RPMI-2650 conditioned media used, the TGF-β activity was 1 73 CFE units, and the autocrine activity was 0 11 CFE units This gave a ratio of approximately 17 0 1 (variations in this did occur) The highest peak of TGF-β activity from the HPLC gave a value of 1 36 units, on this basis, a peak of about 0 085 CFE units for autostimulatory activity would have been expected

The conclusion from the results are as follows

(a) Pool I from Bio-Gel P-30 chromatography of RPMI-2650 CM contains the majority of the TGF-β activity,

(b) Pool II contains the bulk of TGF-α activity, but also a significant amount of TGF-β activity

(c) No autocrine activity was detected in the HPLC fractions

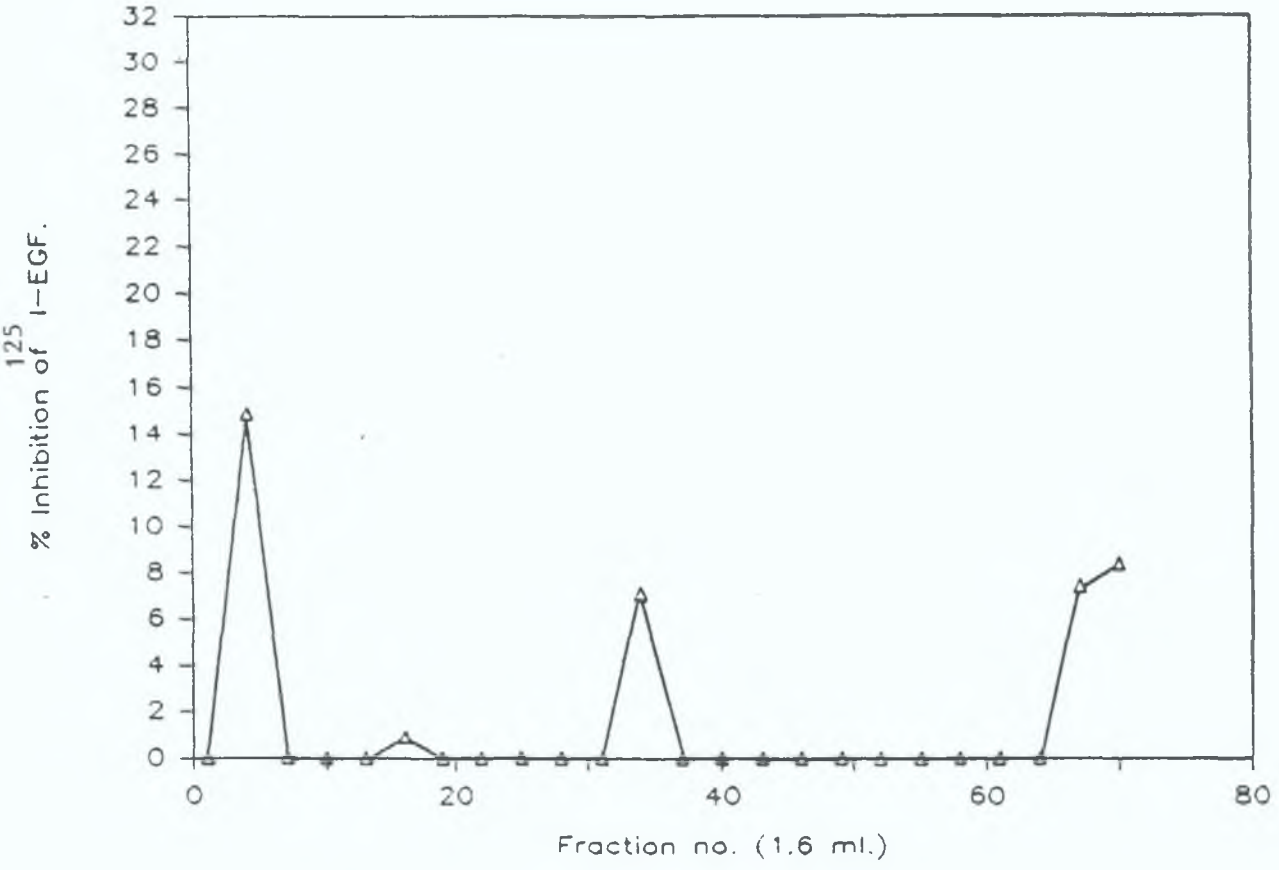


Fig. 3.18.2 ^{125}I-EGF % inhibition profile of RPMI-2650 Pool I CM from Bio-Gel P-30 fractionated by HPLC

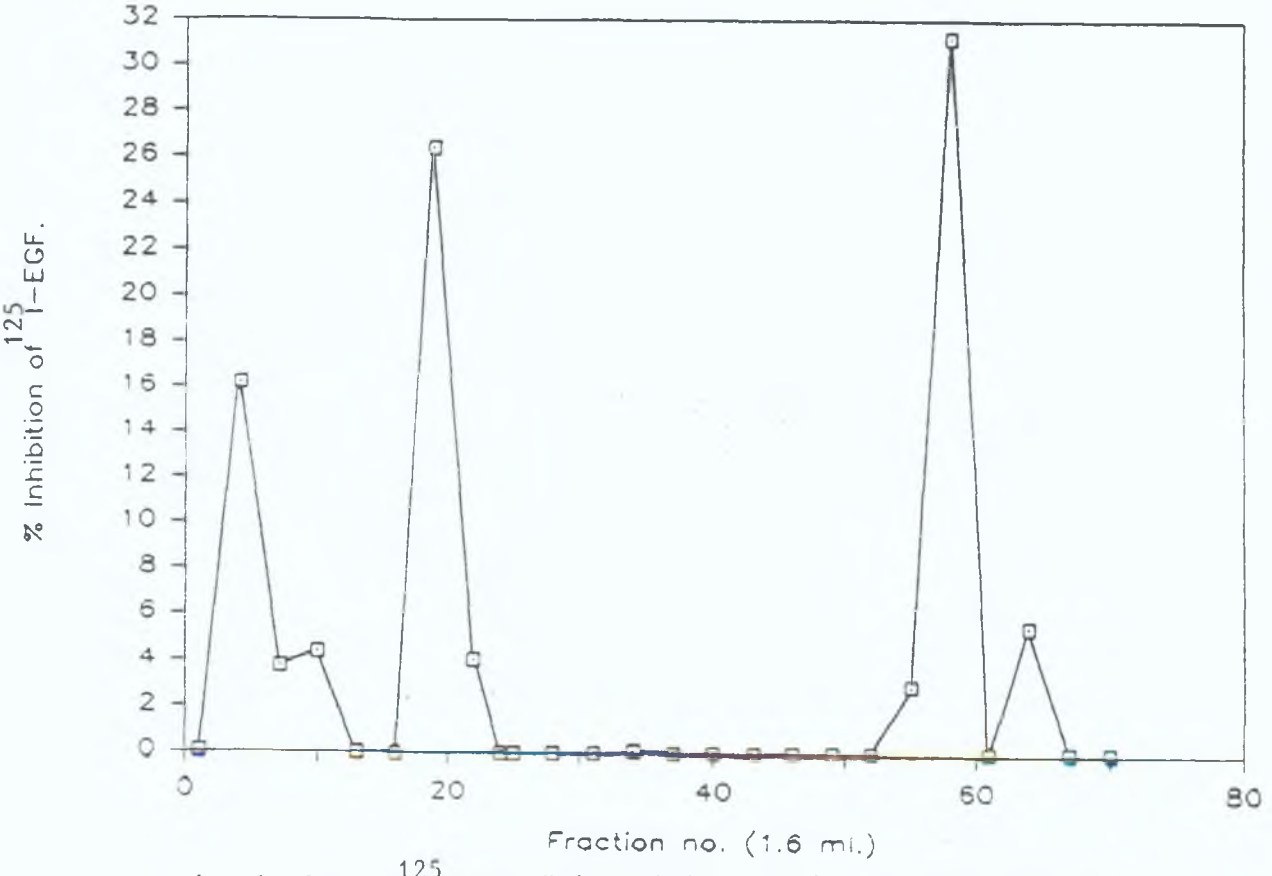


Fig. 3.18.3 ^{125}I-EGF % inhibition profile of RPMI-2650 Pool II CM from Bio-Gel P-30 fractionated by HPLC

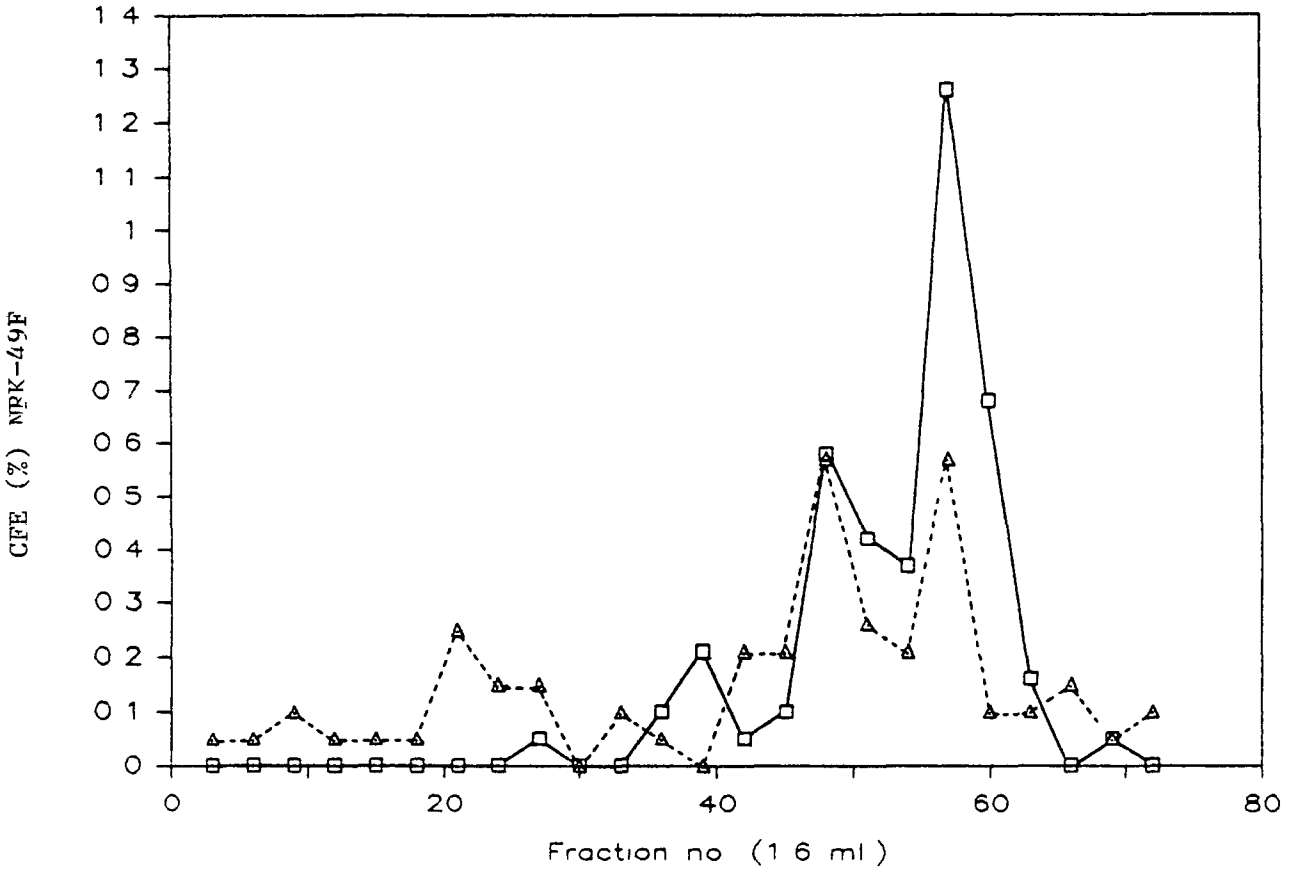


Fig. 3.18.4 TGF-β activity of RPMI-2650 CM from Bio-Gel P 30 Chromatography, fractionated by HPLC

RPMI-2650 CM from Bio-Gel P-30 chromatography

Pool I □ Pool II △

3 19 1 Ultrafiltration of RPMI-2650 CM

Batches of RPMI-2650 CM were collected from roller bottle cultures as described in Section 2 11 1. One large batch of CM was acid neutalized as described in Section 2 11 (on a scale-up basis) Both batches of CM non-acid treated and acid treated, were separated into 130ml lots and ultrafiltrered through a series of molecular weight cut-off membranes as described in Section 2 12 Samples from these ultrafiltered fractions were analysed for protein content as in Section 2 16.Fig 3 19 1 compares the protein content of each ultrafiltered fraction from the non-acid treated and the acid treated batches Table 3 19 1 shows the protein values

These results show that the retentates contain most of the protein while the filtrates have very little protein Both the non-acid treated and acid-treated samples have very similar profiles Some of the results are difficult to explain, in particular, the protein values in the 1000-5000 fraction are very high, while they are much lower in the < 5,000 fraction Some technical problem must have arisen in the preparation of one of these fractions

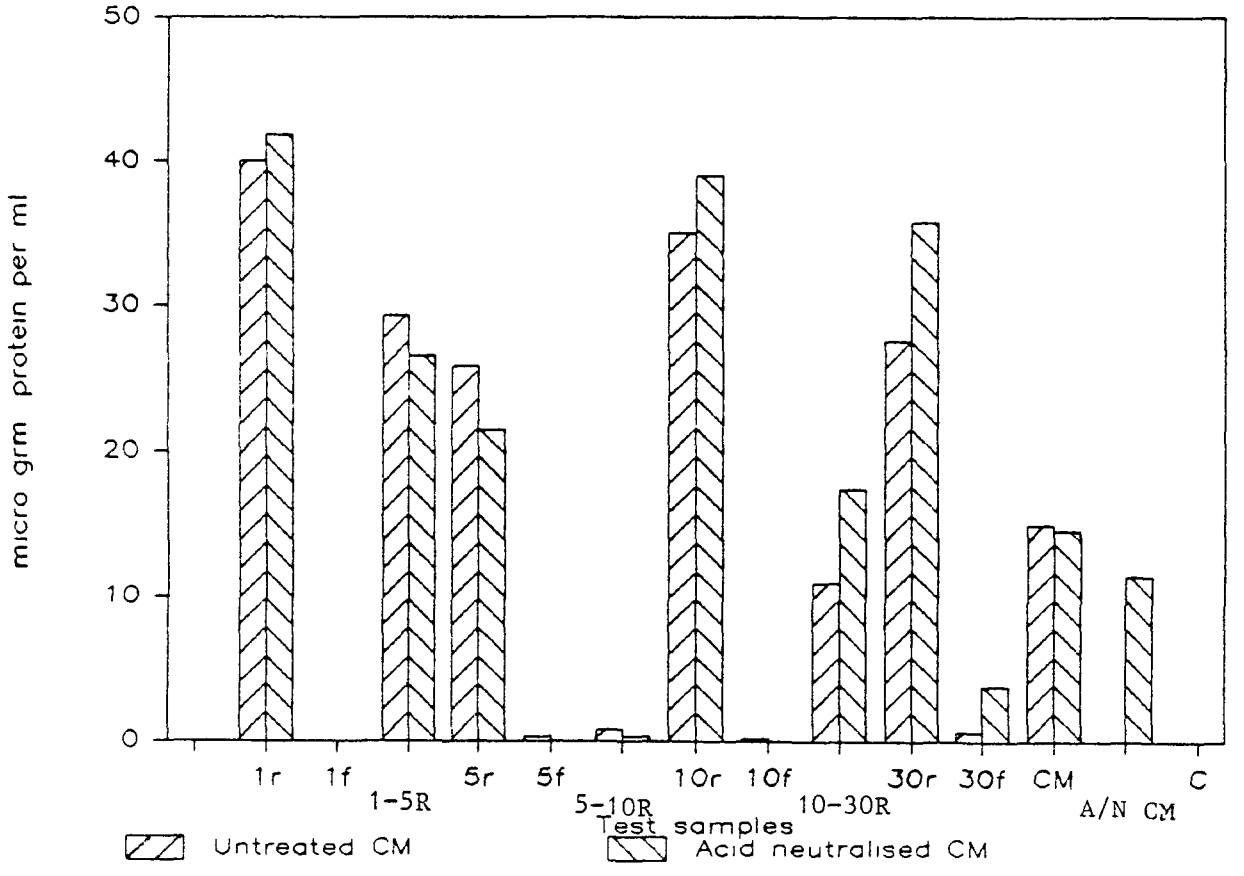


Fig. 3 19.1 Protein analysis of RPMI-2650 CM after ultrafilteration.

Table 3 19 1 Protein analysis of 2 RPMI-2650 CM batches, untreated and acid neutralized and concentrated by ultrafiltration

Test Sample *	µg protein per ml	
	untreated	acid neutralized
RPMI-2650 1,000 retentate	40 00	41 75
" 1,000 filtrate	0 01	0
" 1,000-5,000 retentate	29 30	26.50
" 5,000 retentate	25 75	21 50
" 5,000 filtrate	0 25	0
" 5,000-10,000 retentate	0 75	0 25
" 10,000 retentate	35 00	39 00
" 10,000 filtrate	0 12	0 01
" 10,000-30,000 retentate	11 00	17 50
" 30,000 retentate	27 50	35 75
" 30,000 filtrate	62	3 80
Control CM untreated	15 0(	14 56
Control CM acid neutralized	ND**	11 50
Control medium (MEM)	0	0

* All retentates were concentrated to [10X]

** ND - not done

3 19 2 TGF, TGF-β and autocrine activity of RPMI-2650 acid neutralized and ultrafiltered CM

The acid neutralized and ultrafiltered samples of RPMI-2650 CM as assayed for protein content in 3 19 1, were assayed for TGF, TGF-β and autocrine activity Fig 3 19 2 and Table 3 19 2 show the TGF activity of RPMI-2650 CM in the various ultrafiltered fractions The TGF activity in RPMI-2650 CM appears to be greater than 30kDa, with a small amount of activity escaping into the 30,000 filtrate Fig 3 19 3 and Table 3 19 3 show the autocrine activity of RPMI-2650 CM Some autocrine activity appears to be lost on acid neutralization as shown by the control untreated CM and the control acid neutralized CM However, the remaining autocrine activity is concentrated by ultrafiltration and appears to be greater than 30kDa See also Table 3 6 1 for autocrine activity of non-acid treated RPMI-2650 CM

Fig 3 19 4 and Table 3 19 4 show the TGF-β activity of RPMI-2650 CM after acid neutralization and ultrafiltration These results show that without EGF, RPMI-2650 CM contains very little NRK-49F stimulatory activity in the retentates In the presence of EGF however, the TGF-β activity of RPMI-2650 CM was strongly potentiated Acid neutralization of RPMI-2650 CM appears to stimulate TGF-β activity in RPMI-2650 CM as shown in the control acid neutralized and untreated CM samples

The overall conclusion from these experiments indicate the TGF, TGF-β like and autocrine activities of RPMI-2650 CM are relatively acid stable and of high molecular weight. The fact that they can be concentrated by ultrafiltration also establishes that the activity of CM in the autocrine assay is due to production of a stimulator rather than removal by the conditioning cells of inhibitory factors The loss of TGF activity through the 5,000 membrane in the TGF assay is puzzling since the 30,000 and 10,000 membranes appear to have retained most of this activity even though they have larger molecular weight cut-off values In the TGF-β assay, the reduction in activity between the 30,000 fraction to the 1,000 fraction in that order, may imply the interaction of two molecules One molecule greater than 30kDa is being inhibited or is in competition with EGF for the stimulation of RPMI-2650 CM TGF-β activity by a molecule which is increasingly found in the lower

molecular weight fractions Analysis of the retentate data in the RPMI-2650 autocrine assay in the student t test showed that the 1 kDa retentate was significantly different to the 30kDa retentate at the 5% significance level.

Table 3 19 2 TGF activity of RPMI-2650 CM, acid neutralized and ultrafiltered

Test Sample*	CFE (%) NRK**
RPMI-2650 CM 1R	15 54 ± 1.00
" 1F	0
" 1-5R	8 07 ± 0 75
" 5R	2 10 ± 0 56
" 5F	0.08 ± 0.00
" 5-10R	0
" 10R	20 89 ± 1 20
" 10F	0 08 ± 0 08
" 10-30R	9 23 ± 1 20
" 30R	16 15 ± 0 06
" 30F	0 41 ± 0 14
Control CM untreated	4 53 ± 0 70
Control CM acid neutralized	3 83 ± 0 50
Control medium (MEM)	0

* All retentates and filtrates are X(10^3) fractionation range and all retentates [10X] concentrates

** S E M (n = 3) of x 10^3 cells per 30mm plate

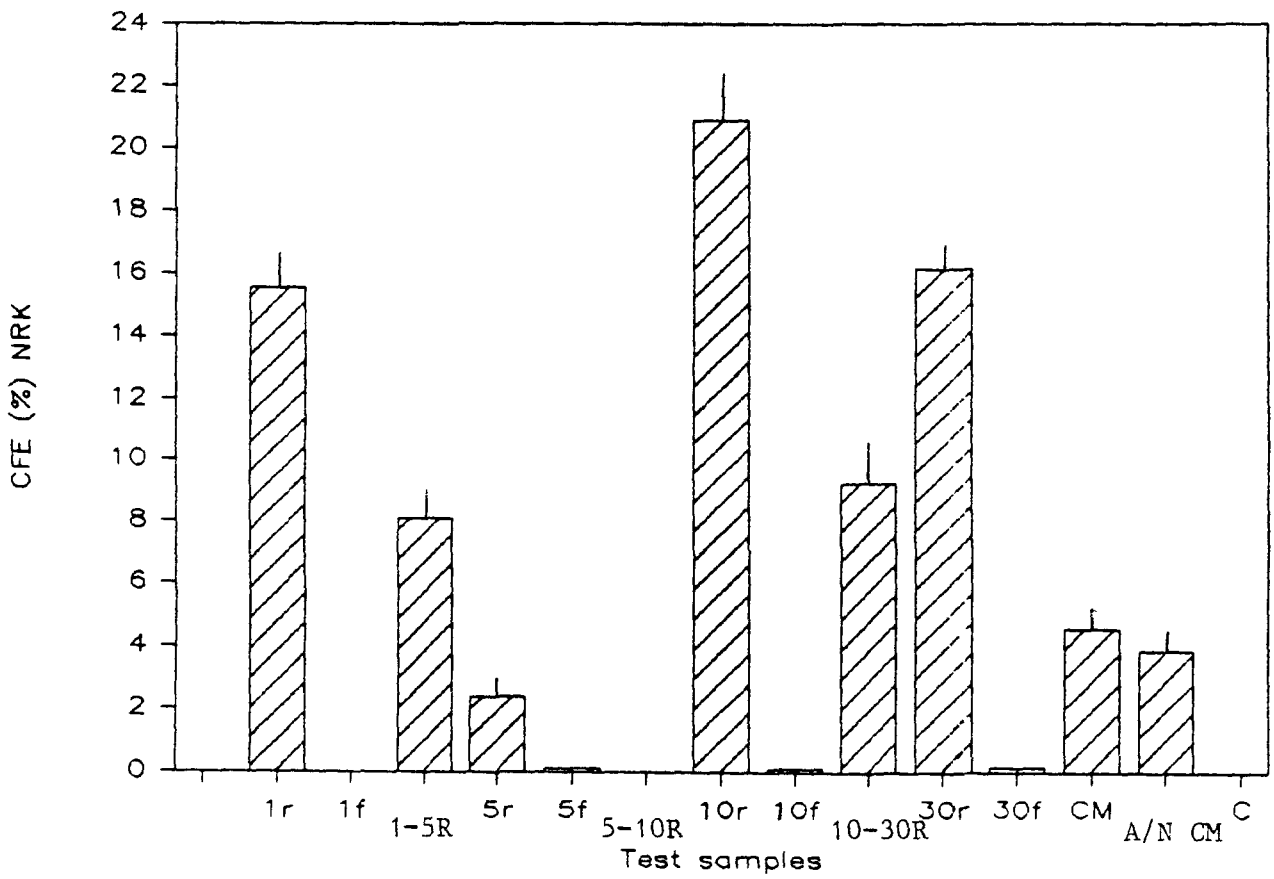


Fig. 3.19.2 TCF Activity of acid neutralized RPMI-2650 CM after ultrafiltration

Table 3 19 3 Autocrine activity of RPMI-2650 CM acid neutralized and ultrafiltered

Test Sample*	CFE (%) RPMI-2650**
RPMI-2650 CM 1R	0 44 ± 0 04
" 1F	0 26 ± 0 03
" 1-5R	0 28 ± 0 06
" 5R	0 53 ± 0 08
" 5F	0 18 ± 0 02
" 5-10R	0 11 ± 0 03
" 10R	0 60 ± 0 14
" 10F	0 18 ± 0 06
" 10-30R	0 28 ± 0 13
" 30R	0 69 ± 0 10
" 30F	0 24 ± 0 16
Control CM untreated	0 37 ± 0 05
Control CM acid neutralized	0 1 ± 0 01
Control medium (MEM)	0 06 ± 0.00

* All retentates and filtrates are X(10^3) fractionation range and all retentates are [10X] concentrates

** S E M (n = 3) of 1 8 x 10]4 cells per 30mm plate

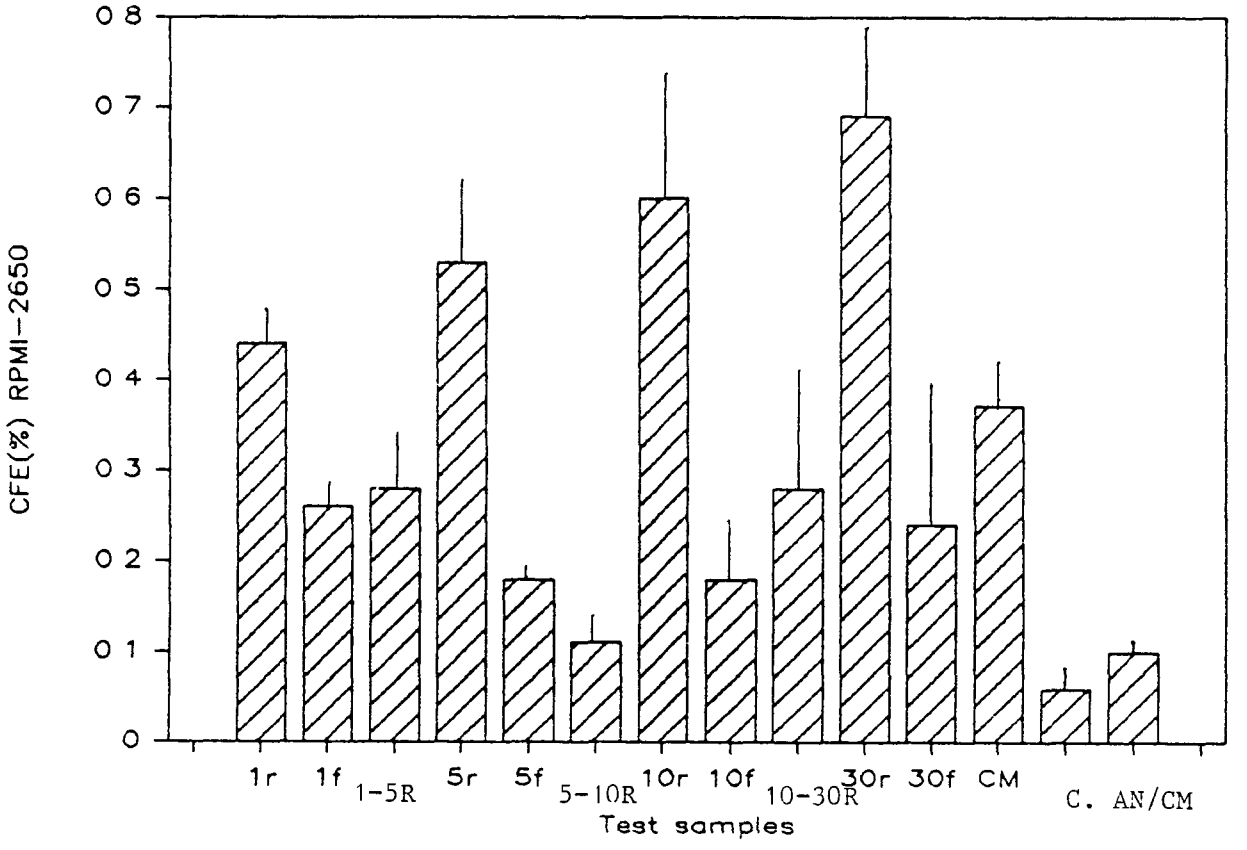


Fig. 3 19 3 Autocrine activity of acid neutralized RPMI-2650 CM after ultrafiltration

Table 3 19 4 TGF-β activity of RPMI-2650 CM acid neutralized and ultrafiltered.

Test Sample *	CFE % NRK-49F**	
	2ng ml^{-1} EGF	-EGF
RPMI-2650 CM 1R	3 15 ± 0.00	0 31 ± 0 12
1F	0 03 ± 0 01	0
1-5R	1 83 ± 0 15	0 13 ± 0 03
5R	3 09 ± 0 76	0 07 ± 0.00
5F	0 13 ± 0 03	0
5-10F	0 08 ± 0 02	0
10R	5 77 ± 0 45	0 17 ± 0 12
10F	0 12 ± 0.00	0
10-30R	0 34 ± 0 06	0 03 ± 0 02
30R	7 26 ± 0 43	0 15 ± 0 00
30F	0 13 ± 0 04	0
Control CM untreated	1 51 ± 0.00	0
Control Cm acid neutralized	2 73 ± 0 36	0 01 ± 0 01
Control medium (MEM)	0 13 ± 0 01	0

* All retentates and filtrates are X(10^3) fractionation range and retentates are [10X] concentrates

** ± S E M (n = 3)

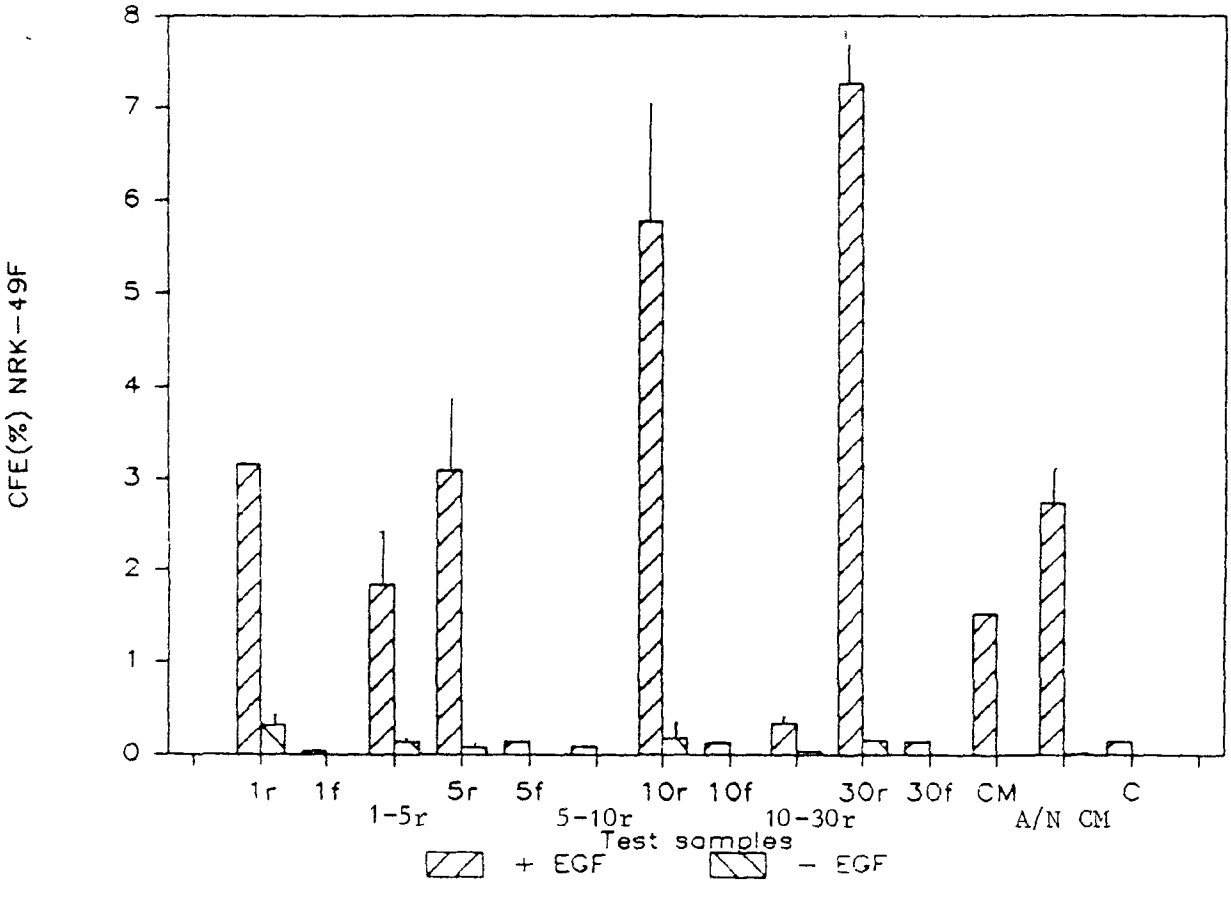


Fig 3.19.4 TGF-β activity of acid neutralized RPMI-2650 CM after ultrafiltration

3 20 Effect of Hormone treatment on EGF receptor levels in A431 and T47D cells

Progesterone, Dexamethasone and β-estradiol were incubated with T47D and A431 cells for 24 hours and examined in the ^{125}I-EGF radioreceptor assay as described in Section 2 21 Table 3 20 1 shows the result from three experiments These results show that the receptor levels for EGF on A431 and T47D cells are influenced by the presence of certain hormones ^{125}I-EGF binding by T47D cells is greatly influenced by progesterone while the binding by A431 cells is influenced by Dexamethasone Possible mechanisms and biological significance of these results are considered in the discussion

Table 3 20 1 Effect of hormone treatment on the EGF receptor on A431 and T47D cells

Test Sample		CPM ^{125}I-EGF binding			
		T47D	*	A431	*
Progesterone	Expt 1	286 34 ± 46.0	64 2	1012 6 ± 158 5	-13 3
	2	260 75 ± 26.0	92 1	1009 7 ± 111 9	4 6
	3	191 70 ± 44.0	24 8	ND	-
Dexamethasone	Expt 1	200 00 ± 23 9	14 7	1709 0 ± 88 8	46 4
	2	104 00 ± 35 6	-23 4	1476 ± 109 0	53 0
	3	170 20 ± 9 5	10 8	2317 8 ± 54 6	42 4
β-Eastradiol	Expt 1	151 30 ± 25.0	-13	1058 3 ± 191 0	-9 4
	2	191 70 ± 40 9	41 2	1040 7 ± 197 1	7 8
	3	168 50 ± 28.0	9 7	1593 2 ± 34 4	-2 3
No Hormone	Expt 1	174 30 ± 52 0	0	1167 0 ± 73 4	0
	2	135 70 ± 11 9	0	964 7 ± 38 4	0
	3	153 60 ± 47 6	0	1627 0 ± 117 0	0

* Percentage change over control - no hormone treatment

3 21 TGF Activity in normal and tumor bearing rats

In spite of their suggestive nomenclature, little definitive data is available on the connection between transforming growth factors and cancer In collaboration with Dr Veronique Preat of the University of Louvain (Belgium)*, who provided the serum samples, we examined TGF serum levels in normal, tumour bearing, and partially hepatectomised rats, available as part of a programme on liver chemical carcinogenesis Samples of lyophilized serum from rats were reconstituted in 1ml ultrapure water, filter sterilized, diluted to 50%, 25%, 12 5% and 10% and assayed for TGF activity The results are shown in Fig 3 21 1 Results of colony size distribution in the 25% dilution samples are shown in Table 3 21 1

These results show that rat serum contains TGF activity which is sensitive to dilution The cancer and nodule samples contain a lot of TGF activity However, size distribution analysis of colonies in these samples in the TGF assay show that the tumour sample contains a higher percentage of large colonies than the nodule sample The cancer sample, therefore, contains a more potent growth stimulatory activity than any of the other samples The partial hepatectomy sample has a reduced level of TGF activity indicating that the high levels seen in serum from pre-malignant and malignant livers are unlikely to be merely proliferation related Control serum showed little activity and all colonies were small as shown in the size distribution information Control medium showed no activity

* Laboratory de Biochimie, Toxicologique et Cancerologique, Universite Catholique de Louvain, Bruxelles, Belgium

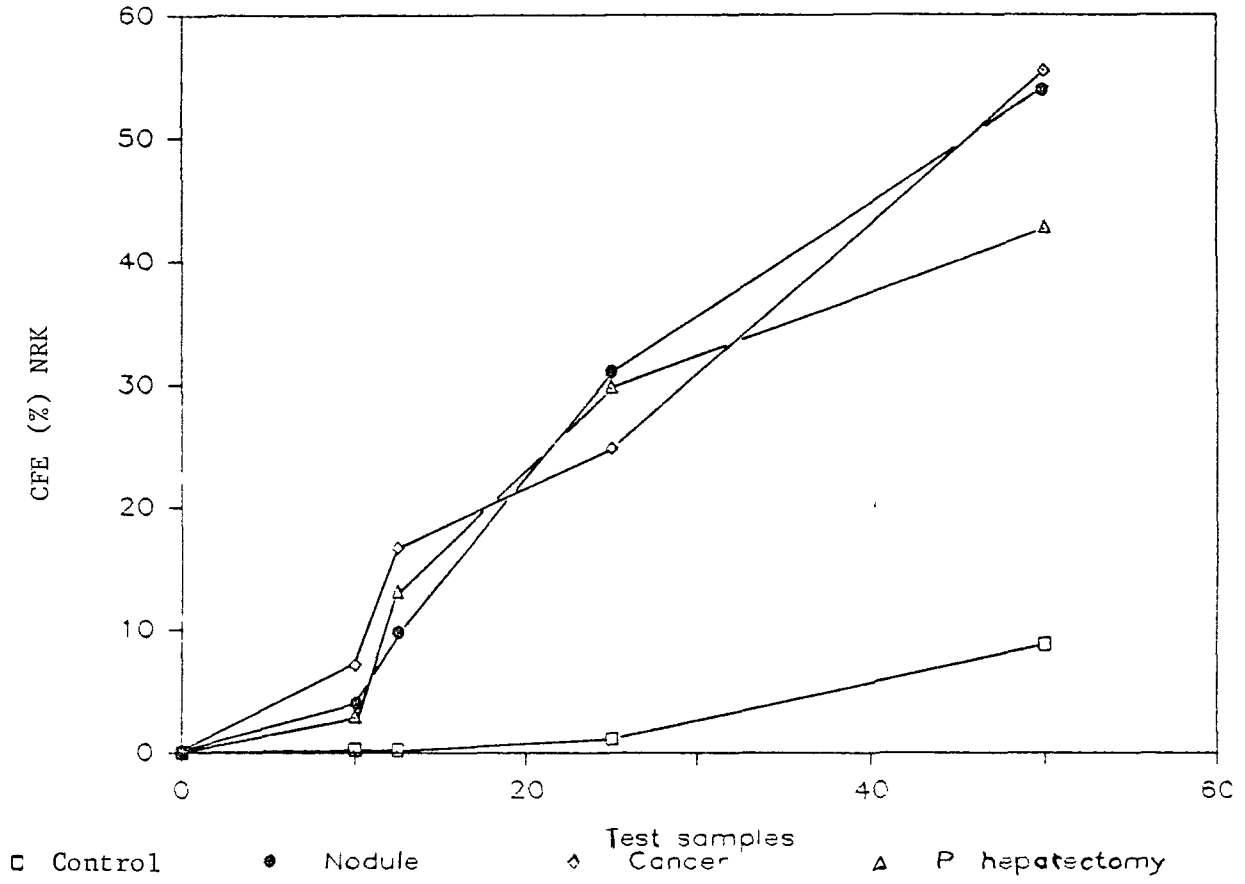


Fig. 3.21 1 Dilution effect of rat serum samples in the TGF assay

Table 3 21 1 Effect of rat serum samples on colony size distribution in the TGF assay

Test Sample	Diameter of NRK colonies (in μm)*					
	120-124	240-365	365-490	490-610	610-730	>730
Control	100%	-	-	-	-	-
Nodule	91 1	6 6	2 2	-	-	-
Cancer	45 7	25 7	11 4	11 4	4 2	1 4
Partial hepatectomy	43 2	31 3	25 3	-	-	-

* Results expressed as % of total number of colonies counted in 5 fields of view

3 22 Effect of pure growth factors and RPMI-2650 CM in the TGF, TGF-β and autocrine assays

Samples of known growth factors were prepard as described in Section 2 23 All growth factors including a concentrated sample of RPMI-2650 CM from Bio-Gel P-30 were prepared with a carrier protein (B S A 1mg%) Preliminary analysis of various concentrations of B S A in the TGF and TGF-β assays showed no effect at this concentration

The purified sample of RPMI-2650 CM assayed with the pure growth factors was purified by Bio-Gel P-30 chromatography and was concentrated by 800 times from the original CM Fractions [10-18] were pooled, lyophilized and reconstituted in 5mls growth medium

Table 3 22 1 and Table 3 22 2 show the results of these growth factors in the TGF, TGF-β and autocrine assays

In the TGF assay, TGF is active on its own (presumably in co-operation with TGF-β present in the serum in the assay system) TGF-β alone is inactive At the concentrations used, TGF-α does not show a linear dose response curve EGF is active in the TGF assay and FGF also shows a slight stimulatory effect RPMI-2650 CM also shows a dilution effect The results from the combined growth factors show that RPMI-2650 CM with TGF or EGF shows a stimulatory effect significantly greater than most of the other combined growth factors An interesting finding was the lack of extra stimulation when TGF-β and RPMI-2650 and RPMI-2650 CM were combined, however, TGF-α , TGF-β plus RPMI-2650 CM were stimulatory to approximately the same level as TGF-α plus RPMI-2650 CM

The TGF-β assay shows an ihnhibitory effect with increasing concentrations of TGF-α As expected, TGF-β is stimulatory in this assay and RPMI-2650 CM is very active in this assay, supporting our earlier results which suggest tht RPMI-2650 cells secrete a TGF-β like factor

The autocrine assay shows a saturation stimulation effect with TGF- and TGF-α All growth factors appear to be stimulatory for RPMI-2650 cells including RPMI-2650 CM It would appear from these results that RPMI-2650 cells are extremely sensitive to high concentrations of growth factors which cause inhibition as shown by the effect caused by the combined effect of TGF-α and TGF-β The addition of RPMI-2650 CM to these growth factors inhibits the growth of RPMI-2650 cells further

It would appear, therefore, from these results that RPMI-2650 CM contains TGF-β and a lesser amount of TGF-α From the TGF-β assay, it was estimated that the original sample of RPMI-2650 contained 2 5 pM TGF-β High levels of growth factors in the autocrine assay are inhibitory The stimulatory effect of FGF in this assay is interesting The possibility that the B S A included with the growth factors during preparation contains low levels of contaminating growth factors, which could act in co-operation with the added growth factors, cannot be excluded

TABLE 3.22 1 Comparison of known growth factors and RPMI-2650 CM in the TGF, TGF-β and autocrine assays

Test Sample	CFE (%) NRK* Colony diameter size > 120μm	CFE (%) NRK* Colony diameter size > 50μm	CFE (%) NRK-49F	CFE (%) RPMI-2650
TGF-α 50ng ml^{-1}	5 57 ± 0.22	14.82 ± 2.67	0.059 ± 0.006	0.25 ± 0.04
TGF-α 5ng ml^{-1}	3.38 ± 0.62	15.75 ± 1.45	0.109 ± 0.02	0.29 ± 0.03
TGF-α 0.5ng ml^{-1}	2 5 ± 0	11.75 ± 1.2	0.172 ± 0.02	0.33 ± 0.09
TGF-β 2ng ml^{-1}	0	0	1.46 ± 0.79	0.13 ± 0.05
TGF-β 0 2ng ml^{-1}	0	0	0.197 ± 0.028	0.21 ± 0.02
TGF-β 0 02ng ml^{-1}	0	0	0.105 ± 0.02	0.19 ± 0.08
EGF 2ng ml^{-1}	3 04 ± 0.44	15.1 ± 1.55	0.08 ± 0.01	0.25 ± 0.05
PDGF 1ng ml^{-1}	C**	C	0.084 ± 0.01	0.27 ± 0.03
Bombesin 10nM (16 2ng ml^{-1})	0	0	0.096 ± 0.026	0.23 ± 0.02
FGF 100ng ml^{-1}	0.4 ± 0.08	1.76 ± 0.78	0 109 ± 0.012	0.35 ± 0.04
FGF 10ng ml^{-1}	0	0	0.109 ± 0.016	0.19 ± 0.01
RPMI-2650 CM [X]	3 67 ± 0.29	11 58 ± 1.45	6.116 ± 0.19	0.13 ± 0.05
RPMI-2650 CM [$\frac{1}{5}$ X]	0	1.37 ± 0.44	3.023 ± 0.324	0.078± 0.03
RPMI-2650 CM [$\frac{1}{10}$ X]	0	0 29 ± 0.29	1.89 ± 0.271	0.091± 0.01
Control Medium	0	0	0.067 ± 0.015	0.078 ± 0.02

* ± S E M (n = 3) of 6 x 10^3 NRK cells, 3 x 10^4 RPMI-2650 cells and 2 x 10^4 NRK-49F cells per 30mm plate

** (Contaminated

TABLE 3 22 2 . Comparison of known growth factors and RPMI-2650 CM in the TGF, TGF-β and autocrine assay

Test Sample *	CFE (%) NRK** Colony diameter size		CFE (%) NRK-49F**	CFE (%) RPMI-2650**
	> 120μm	> 50μm		
PDGF/EGF/TGF-β	2 69 ± 0.34	13.24 ± 3.07	0.206 ± 0.016	0.019 ± 0
PDGF/FGF/TGF-α	4 02 ± 0.51	14.72 ± 0.88	0.097 ± 0.021	0.012 ± 0.04
PDGF/EGF/RPMI-2650 CM	5 95 ± 0.72	20.28 ± 3.28	2.195 ± 0 159	0.088 ± 0.014
TGF-α/TGF-β	3.61 ± 0.99	13.25 ± 1.79	0 122 ± 0.026	0.13 ± 0.04
TGF-α/RPMI-2650 CM	9.86 ± 0.89	23.85 ± 0.59	2.037 ± 0.176	0.052 ± 0.02
TGF-β/RPMI-2650 CM	0 04 ± 0.08	1.37 ± 0 44	2.25 ± 0.27	0.029 ± 0.01
TGF-α/TGF-β/RPMI-2650 CM	8 9 ± 1.77	24.14 ± 0.14	2.169 ± 0.064	0.025 ± 0.01
EGF/TGF-β	3.53 ± 0.21	14.34 ± 1.37	0.105 ± 0.016	0.046 ± 0.01
Control Medium	0	0	0.067 ± 0.015	0 078 ± 0.02

* PDGF 1ng ml^{-1}, EGF 2ng ml^{-1}, TGF-α 5ng ml^{-1}, TGF-β 0.2ng ml^{-1}; RPMI-2650 CM [$\frac{1}{5}$]

** ± S E M (n = 3) of 6 x 10^3 NRK cells, 3 x 10^4 RPMI-2650 cells and 2 x 10^4 NRK-49F cells per 30mm plate

SECTION 4

DISCUSSION

4 DISCUSSION

Current research in the area of growth factors is providing new insights into the control of animal cell growth The malfunction of cellular control mechanisms can lead to different types of disease Tumour cell growth is of particular interest due to the high incidence of death to young and old from the various forms of cancer The development of *in vitro* methods to establish and grow tumour cells on a routine basis is an important area of cancer research, but for many tumour cell types it is not very successful Tumours contain a heterogenous population of normal and malignant cells (Hart *et al* , 1981) and a small population of stem cells may be responsible for tumour growth Because of the presence of mesodermal tissue which grows profusely it is difficult to routinely culture tumour cell lines which could be used to determine chematherapeutic measures for individual patients and to study a range of biochemical immunological and other parameters which may be important in tumour growth and progression

The failure of tumour cells, which grow so readily *in vitro*, to grow progressively in culture, could be due to many factors including lack of a perfusion system or absence of cellular interactions Another possibility, discussed in more detail in Section 1 14, is that tumour cell growth requires high local concentrations of locally produced growth factors The results presented here relate to the investigation of production of the autocrine and other growth factors by the human carcinoma line RPMI-2650 (Moore and Sandberg, 1963) These cells originated in a pleural effusion from a patient with carcinoma of the nasal septum Extensive growth of human cells in culture generally leads to changes in the cells karyotype and after many passages, the cells may not be representative of the cell population in the original tumour RPMI-2650 cells are however, available at a low passage level which is important if experimental results from this cell line are to be relevant for comparison to tumour cell growth This cell line is quasi diploid and does not have the extensive karyotypic abnormalities of many widely used human cell lines like Hela

The aberations in control mechanisms which are responsible for aberrant growth of tumour cells are not understood fully How do tumour cells escape from normal control systems? Why are some cells responsive to certain growth regulators while others are not? How many mechanisms are involved? These are some of the questions often asked of how cancer occurs and continues to progress and becomes malignant or benign

Preliminary experiments were set up with serially diluted RPMI-2650 cells in monolayer It was found that at low cell concentrations no colonies formed, but as the concentration of cells plated increased the number of colonies formed increased exponentially The concentration-dependent nature of RPMI-2650 cells at low concentrations was examined in a double layer agar assay system (see Section 2 10 1) A low concentration of RPMI-2650 cells suspended in a semi-solid agar matrix supplemented with growth medium was set up with increasing concentrations of RPMI-2650 cells physically separated from these cells as a "feeder" monolayer, on the base of the tissue culture dish The interesting finding from this experiment was that the cells in the feeder layer stimulated those in the upper layer in a concentration dependent manner However, at the higher feeder concentrations the amount of stimulation decreased This could have been due to nutrient depletion or build up of waste products, since these cultures were part of a closed system, which was capable of supplying a limited amount of nutrients Alternatively, a cell-produced auto-inhibitor could be involved

The work described here concentrated on an explanation of the autostimulatory effect in RPMI-2650 cells Medium was collected from cultures of RPMI-2650 cells ("conditioned medium", CM) and was incorporated with RPMI-2650 cells in the upper layer of the double layer assay This medium was found to stimulate RPMI-2650 cells This finding proved that the physical presence of feeder cells was not necessary to stimulate cell growth It also opened the way to biochemical investigation of the CM effect The samples of CM tested in the assay indicated that it was altered in some fundamental way by the growth of RPMI-2650 cells The possibilities that removal of an inhibitor or production of an autocrine growth factor were the reasons for this stimulatory effect were examined However, before this could be achieved, the RPMI-2650 autocrine assay had to be developed to a reliable stage

Various parameters of the RPMI-2650 autocrine assay were examined The sensitivity of the assay varied from experiment to experiment but the activity profiles were generally the same One of the major problems in the assay was a high background At first all CM samples were collected in medium with 10% foetal calf serum It was shown, however, that 5% foetal calf serum, while halving the cost of this expensive medium supplement, reduced the cell growth yields by only 10% All cell lines were cultured with 5% foetal calf serum, while CM was prepared in medium without foetal calf serum which facilitates later purification We found that concentrated (ultrafiltered) CM prepared without foetal calf serum stimulated RPMI-2650 cells to grow This confirmed that cell-derived rather than serum-derived factors were being examined Other parameters like indicator cell concentration and type of collection medium for CM were also examined To allow for variation in sensitivity in the assay, a cell concentration well above the cut-off point was taken and used in most of the assays RPMI-2650 CM was prepared in the two media types, SLM and MEM MEM is a basic medium while SLM contains extra vitamins and amino acids and a carbon source in the form of sodium pyruvate which unlike the glucose in MEM is shunted metabolically towards the TCA cycle rather than to lactic acid production (lactic acid is responsible for lowering the pH of the medium which may prevent cell growth) While SLM is superior for growth of RPMI-2650 cells, the background in SLM was usually much higher and may have masked autocrine activity The semi-solid matrix in the assay (agar) was also examined and compared with agarose Background CFE was greater in agarose than in agar The assay could have been alternatively adjusted (cell number, serum concentration) to reduce the agarose background CFE as agarose is a purer form of agar Agar may contain toxic impurities at a greater concentration than in agarose It was found that in many of the agarose assays, RPMI-2650 colonies disintegrated and became difficult to count The reason for this is not understood

We found that the autocrine assay was very sensitive to assay conditions Careful pretreatment was essential if the assay was to be successful Indicator RPMI-2650 cells taken from exponential log phase cultures gave good results while cells from confluent cultures often gave less predictable results It was also found that while in culture, cell lines sometimes became progressively

granular and growth slowed down Work with these cells generally gave no results or very poor results Analysis of medium samples from these cultures in a modified mycoplasma test developed in our laboratory showed that such cells were often contaminated with mycoplasma Despite regular screening for mycoplasma the problem continued to arise occasionally

Other experimental variables such as the presence of high levels of foetal calf serum in the assay (and experimental error) may be responsible for the variability often found in this assay Rizzino, (1984) has shown that a serum-free agar assay has proved very useful in detecting growth factors Future work with the autocrine assay would benefit greatly from the development of a serum-free based method

Further analysis of the autocrine activity of RPMI-2650 cells was difficult due to the small amounts of growth factor secreted into the medium Large scale cell culture was the next step However the presence of autocrine growth factors in other cell lines led us to examine the relationship between our autocrine growth factor and those in the literature Halper and Moses, (1983) showed the presence of an autocrine factor in CM from SW-13 cells, derived from a human adenocarcinoma of the adrenal cortex This autocrine factor was found to separate from other known growth factors by HPLC An autocrine factor for human small cell lung cancers (Bombesin) is also known (Carney et al , 1970) The most widely known but not well understood transforming growth factors also function in an autocrine manner (Sporn and Todaro, 1980, Sporn and Roberts, 1985b) The autocrine hypothesis as discussed in the Introduction has become a concept in the link between growth factors and cancer research Transforming growth factors have been found in many tissue types both normal and tumour, in many cell types in culture and in body fluids (Childs et al , 1982, Roberts et al , 1983, Sherwin et al , 1983 Cooper et al , 1984, Hamburger et al , 1985, Zwiebel et al , 1986) We looked at the possibility that RPMI-2650 CM contained a transforming growth factor

Transforming growth factors (TGFs) are a family of growth factors which are generally identified by their ability to induce normal fibroblasts to grow in a semi-solid matrix (De Larco and Todaro, 1978) The ability of TGFs to render cells anchorage-independent

is not absolutely unique to TGFs, however it is used widely as a basic detection assay (anchorage-independent growth is a function of the total growth factor concentration in the medium (Kaplan and Ozanne, 1983)

Anchorage independent assays were examined for their ability to detect TGFs A positive TGF control was prepared from a virus transformed cell line, 663+N This cell line is a myeloproliferative sarcoma virus (MPSV) transformed NRK line which was known to produce TGFs It was kindly supplied by Dr Ian Pragnall, Beatson Institute for Cancer Research, Glasgow (Koury and Pragnall, 1982) Pilot studies on optimised production of TGFs was undertaken with this line

Three normal fibroblast cell lines were examined as indicators in the TGF assay NRK cells appeared to be the most sensitive NRK-49F and AKR-2B cells showed very little response NRK cells differed from NRK-49F and AKR-2B cells in that besides responding with a higher CFE in the presence of TGF, the colonies were much larger and easy to count (De Larco et al , 1981, Nickell et al , 1983, Richmond et al, 1985, Fernandez-Pol et al , 1986) Once a suitable indicator cell line was located we set about optimizing the assay (Dooley and Clynes, 1986) The assay was first set up with 1 2 x 10^3 cells per plate A change of foetal calf serum batch caused some difficulties since then the assay system was optimized using one particular batch of foetal calf serum Foetal calf serum variation meant that the optimum conditions with one batch were different for another batch We subsequently selected an alternative batch of foetal calf serum which was used in all other growth factor work

A range of NRK indicator cell concentrations were examined in the TGF assay, we found that below 4 x 10^3 cells per dish, NRK cells responded in a random manner At 6 x 10^3 cells per dish a low background or no background CFE was observed, The importance of borderline background was important for analysis of many batches of CM with low levels of TGF activity (RPMI-2650 CM) Our positive control, 63+N CM, contained a lot of TGF activity and became inhibitory at high concentrations We found that RPMI-2650 CM contained low levels of TGF activity Samples of RPMI-2650 CM

which were concentrated by ultrafiltration showed increased TGF activity with increased concentration

The passage number of NRK cells were examined for their response to TGF factors as the indicator cell line aged We found that as these cells aged, they responded less efficiently to TGF factors In an effort to standardize the assay, NRK cells were regularly thawed from liquid nitrogen stocks and strictly used between passages 17-22 Once the cells aged beyond this passage level they were discarded

The basic anchorage independent matrix in the assay was also examined It was found here again that like RPMI-2650, NRK cell growth was greater in agarose than in agar and the colonies which formed in agarose became very diffuse A low background was more useful so agar was chosen as the anchorage independent support in this assay Agarose may have a more important role to play in clonogenic assays where cells from human tumours are grown in a semi-solid nutrient media and used as models for examining the biology of human tumours and the response of patients to drug therapy In these assays, encouragement of clonal growth rather than low background is required (Hayes et al , 1985) Enderson et al , (1985), showed that the conditions employed in setting up soft agar assays greatly influenced the sensitivity of primary human clonogenic assays

The production of TGF activity by two passsage levels of 663+N cells was examined over a period of time It was found that production of TGF activity increased for up to three days in culture The limitation of the collection method was that 663+N cells continued to grow and secrete growth factors for this period of time in a serum free medium but due to basic nutrient depletion or accumulation of waste products and pH change the cells were unable to continue growing These results also showed that 663+N cells appeared to secrete more TGF activity as they aged (increasing passage number) This occurrence may be explained by adaption of cells to growth in culture It is known that in vivo tumours consist of a heterogenous population of cells (Hart and Fiddler, 1981), subpopulations of cell lines may arise in culture and faster growing cells may overgrow the other cells in culture

Preliminary experiments showed that while 663+N cells secrete a lot of TGF activity, these growth factors were inefficient in stimulating RPMI-2650 cells RPMI-2650 cells differ from NRK and 663+N cells in that RPMI-2650 cells are epithelial and NRK and 663+N cells are fibroblastic cells The essential control mechanisms of these cells are basically different It seemed however, that RPMI-265 cells were producing some growth factors similar to 663+N growth factors, since CM from both these cell types were active in the TGF assay Preliminary purification by ultrafiltration of CM from these two cell lines showed that 663+N cells were producing TGF activity which was predominantly in the 5-30 kDa range and activity from RPMI-2650 was predominantly in the greater than 30 kDa range However, it also seemed likely that each cell line had a small amount of activity in the predominant molecular weight range of the growth factor of the other cell line The two known TGFs have molecular weights of 6 kDa and 25 kDa The possibility that RPMI-2650 cells were producing predominantly the 25 kDa molecule (TGF-β) and 663+N cells were producing predominantly the 6 kDa molecule (TGF-α) seemed likely (Derynck et al , 1985, Roberts et al , 1985, Hanauske et al , 1987) Given that TGF-α and TGF-β may interact in solution, and that the fractionation cut-off limit of ultrafiltration membranes is very approximate and depends on shape as well as size, this seems to be a reasonable working hypothesis

RPMI-2650 CM was examined for dilution effects in a number of assays The response of RPMI-2650 CM to dilution was important if the growth factor activity were to be detected following purification procedures like column chromatography Samples of RPMI-2650 CM were concentrated by ultrafiltration and serial dilutions tested in the autocrine assay RPMI-2650 cells responded in a linear manner to dilution of CM from RPMI-2650 cells This pattern emerged repeatedly and also that the cells responded to a narrow range of concentrations On dilution RPMI-2650 cells responded to the CM and at higher concentrations saturation occurred

All other dilution curves involving 663+N CM in the TGF assay and RPMI-2650 CM in the TGF and TGF-β assay showed a linear relationship at low dilution, with a saturation effect at high concentration Sufficient data were not available to generate and

analyse the exact shape of the curves However, there was some suggestion of a sigmoidal shape in some cases, this was particularly pronounced in the case of RPMI-2650 CM in the TGF assay, but not for 663+N in the same assay A sigmoidal effect could suggest the cooperative action of 2 factors, one of them being present at limiting concentrations TGF-α and TGF-β are known to act in combination in stimulation of NRK cell growth in the TGF assay (Anzano et al , 1983) From the results presented here, it is evident that 663+N produced considerably more (perhaps by a factor of 20 times) TGF- than RPMI-2650, and the sigmoidal curve for the latter may indicate dilution of TGF-α below a critical level in the presence of excess TGF-β The limited data presented here do not allow fuller speculation on this or other possibilities (such as inhibitory molecules or effects on receptor activity)

Concentration of CM from RPMI-2650 and 663+N cells by ultrafiltration caused a large increase in TGF, TGF-β, ^{125}I-EGF binding and autocrine activity, showing that both of these cell types were secreting growth factors into their extracellular environment This was an important finding which dismissed the possibility that removal of inhibitors was responsible for growth stimulation by conditioned medium Initial experiments in whch RPMI-2650 and 663+N CM were concentrated by ultrafiltration showed that RPMI-2650 TGF activity was predominantly in the 30 kDa range while 663+N growth factor activity was predominantly in the greater than 5 kDa retentate, but not in the greater than 30 kDa retentate, indicating a TGF size range of 5-30 kDa for the 663+N TGF factor

Results of the autocrine activity of ultrafiltered RPMI-2650 CM were difficult to interpret However, acetic acid treatment and neutralization, although lowering the autocrine activity in the unconcentrated CM, allowed successful ultrafiltration of autocrine activity Acid treatment results in some loss of protein, and may remove competing factors or activate other growth factors (Lawrence et al , 1984, Pircher et al , 1984)

These results suggest that there are at least two components in the autocrine activity of RPMI-2650 cells One of these components is of low molecular weight (as seen in the activity of the less than 1 kDa retentate) and one of high molecular weight (greater than 30

kDa) There is some indication also from the results of an inhibitory activity in the greater than 1 kDa retentate There is an indication that this may be a TGF-α-like factor since CM from 663+N cells which contains a substantial amount of TGF-α, is also slightly inhibitory in the RPMI-2650 autocrine assay Further work would be necessary to establish this The fact that the retentate activities are in the order 30 kDa > 10 kDa > 5 kDa > 1 kDa is not readily explicable

Ultrafiltered RPMI-2650 CM tested in TGF assay showed enhancement of activity in all retentates, whether acid-neutralized or untreated The 5 kDa retentate gave considerably lower activity than the 1 kDa, 10 kDa or 30 kDa retentates The substantial loss of TGF activity in the 5 kDa retentate was a recurring feature of this ultrafiltration membrane (or of the batch used) We came to the conclusion that this membrane was faulty since the activity lost through this membrane was again concentrated in the 1-5 kDa retentate This batch of membranes may have been "leaky" to protein, as evidenced by the high protein level in the 5 kDa filtrate when subsequently concentrated over a 1 kDa cut-off membrane

In the TGF-β assay, acid-neutralization of RPMI-2650 CM was shown to stimulate TGF-β activity The presence of EGF in this assay potentiates TGF-β like activity in RPMI-2650 CM which is retained in the 30 kDa retentate TGF-β activity is significantly decreased in the 2 kDa retentate and this may be caused by an inhibitor of TGF-β activity in this retentate The loss of TGF-β in the 5 kDa retentate may as mentioned above, be a feature of this batch of membranes

Purification of RPMI-2650 CM was hampered by the low levels of growth factors secreted into conditioned growth medium Cells were routinely grown in 25cm^2 flasks which yielded 30mls of 1-day CM over a 3 day period Ths meant a very long preparative time and/or flask number before up to 500mls of CM could be obtained In general, large scale animal cell culture methods are an important source of substantial amounts of complex proteins (including antibodies and enzymes) produced by mammalian cells which cannot be produced in bacteria because of their inability to correctly process or modify these molecules (Arathoon and Birch, 1986)

We examined a number of large scale methods of cell culture and compared the differences in handling and CM production The methods examined were roller bottle, microcarrier and suspension culture We found that microcarriers, which are ideal for growth of anchorage dependent cells, since the growth of these cells on dextran beads increases the ratio of surface area to volume of medium as compared to monolayer cultures (e g roller bottle systems), are somewhat cumbersome in practice Roller bottles were easy to handle and gave a reasonable yield of CM (300mls of 1-day CM in 3 days) This method was used for CM production Suspension culture systems were found to be very suitable for culturing RPMI-2650 cells While the subculture of these systems was simple, suspension cultures tended to require more handling than roller bottle cultures In this system RPMI-2650 cells were grown in suspension and grew into clumps after a few days in culture This culture method yielded 300mls of 1-day CM in 3 days from a 100ml culture or 600mls of 1-day CM in 3 days from a 200ml culture On a comparative basis a $25cm^2$ flask could yield up to 4×10^6 RPMI-2650 cells A roller bottle could yield over 10^8 RPMI-2650 cells (670 cm^2) and a 100ml suspension culture could yield 2×10^8 RPMI-2650 cells This makes suspension cultures more economical in terms of medium costs and space

TGF and autocrine activity in RPMI-2650 CM appeared to be relatively stable to acid treatment Concentration by ultrafiltration followed by dialysis and lyophilization did not appear to decrease activity An early experiment showed that a lot of TGF activity was lost after CM samples were filter sterilized using an ordinary millipore filter (Millex GS), so a special low protein binding millipore filter was used in all subsequent experiments

The major purification step attempted, was the use of gel filtration in acetic acid which has been shown (De Larco and Todaro, 1980) to separate TGFs from the bulk of protein in CM, thus providing a purer TGF preparation for subsequent assays or for futher purification by HPLC Initial runs were undertaken using Bio-Gel P-60, because of its wide fractionation range (3-60 kDa) CM from 663+N, a high-level TGF producer, was fractionated initially Analysis of the resulting fractions showed that the TGF activity in this CM was separated from the bulk of protein into one

major and one small peak These results were similar to those of De Larco and Todaro (1980) who partially purified sarcoma growth factors from Moloney MSV-transformed 3T3 cells

A sample of RPMI-2650 CM was then fractionated by Bio-Gel P-60 and showed a small trace of TGF activity When EGF was incorporated into the assay a broad peak of activity resulted Since the initial runs with Bio-Gel P-60 indicated that the TGFs size range was 5-30 kDa, subsequent work was with Bio-Gel P-30 which had a fractionating range of 2 5-40 kDa Samples of RPMI-2650 CM fractionated by this column were assayed for TGF, TGF-β, autocrine, ^{125}I-EGF binding inhibition and ^{3}H-thymidine incorporation These reults showed that RPMI-2650 CM contained growth factors which were active in all these assays We found that the TGF activity consisted of two peaks of activity A small peak of activity in the 20-30 kDa range and a second larger peak in the smaller molecular weight range 5-15 kDa The TGF-β assay also showed two peaks A minor peak in the high molecular weight area and a very broad peak in the lower molecular weight range approximately similar to those of the TGF assay peaks The autocrine assay showed one definite peak in the lower size range 5-15 kDa Both the ^{125}I-EGF radioreceptor and the ^{3}H-thymidine assays showed one main peak of activity in the low molecular weight range

This work led the way to further purification of RPMI-2650 CM, by removing bulk protein from the active protein molecules and presented a relatively pure sample of growth factors for HPLC, and which we were to subsequently assay in comparison to other known growth factors Accurate molecular weight determination of these active molecules was difficult due to the broad bands of activity which resulted Smaller fraction sizes might be advisable for future work

Fractionation of the RPMI-2650 growth factor activity by HPLC was the next step This system was set up with 663+N CM as a positive TGF-α control, and using a method described by Anzano et al , (1983) We fractionated 663+N CM and lyophilized the fractions before analysing them in the ^{125}I-EGF radioreceptor assay The results from this experiment showed that the ^{125}I-EGF competition binding activity in 663+N CM eluted at about 28-30% acetonitrile

Anzano et al , found that their ^{125}I-EGF competing activity eluted at 31-32% acetonitrile

Based on these findings, we prepared a batch of RPMI-2650 CM and partially purified it by Bio-Gel P-30 We lyophilized two pools of fractions which corresponded to the main peaks of activity detected in the TGF assay of a preliminary Bio-Gel P-30 run These pools of activity were eluted separately from the HPLC column Every third fraction from each run was then assayed in the ^{125}I-EGF radioreceptor assay, the autocrine assay and in the TGF-β assay Activity was detected in the ^{125}I-EGF radioreceptor assay and in the TGF-β assay but not in the autocrine assay

Pool I which contained the larger molecular weight molecules from the Bio-Gel P-30 run showed very little ^{125}I-EGF competitive activity Pool II, however, showed two small peaks, one of which eluted around 28-30% acetonitrile and the second major peak eluted around 58-60% acetonitrile The results from the TGF-β assay showed two main peaks of activity in Pool I which were eluting around 38-40% acetonitrile, with a range of 58-80% acetonitrile for the second peak Pool II showed a small early peak which eluted around 28-30% acetonitrile and two other peaks in the same position as the two peaks eluted in Pool I at 38-40% acetonitrile and 50-80% acetonitrile

These results showed that Pool I contained no significant levels of ^{125}I-EGF activity but contained most of the TGF-β like activity, while pool II contained ^{125}I-EGF activity and some TGF-β like activity The TGF-β like peaks of activity from RPMI-2650 CM which showed up after HPLC are similar in position to those detected by Anzano et al , (1983) using CM from virally transformed cells

These results would inidcate that the high molecular weight Pool I samples, contained a TGF-β like molecule The lower molecular weight Pool II, contained all the TGF-α like activity but also contained a substantial level of TGF-β like activity This finding shows that gel filtration chromatography does not separate TGF-α and TGF-β like molecules effectively, whereas HPLC purification does, in agreement with other published work (Anzano et al , 1985)

Loss of autocrine activity in the HPLC fractions could be due to a number of factors Since control samples of CM did show activity, it is possible that the autocrine factor may have been unstable during the run or was irreversibly bound to the column matrix If this was the case, then the autocrine activity of RPMI-2650 is dissimilar to other known TGFs The possibility of a third distinct group of growth factors separate from the known growth factors has been discussed by Halper and Moses (1983) and Richmond et al , (1985) Another possibility may be separation of two molecules which must act in concert to stimulate activity

The discrepancy between the identity of the TGF-β like molecule found in Pool II (lower sized factors) and its proposed molecular weight has been discussed by Massague (1983) who found that a high molecular weight molecule interacted with the chromatography matrix of Bio-Gel P-60 and, therefore, eluted at a later fraction However, it is also possible that the TGF-β-like molecule secreted by RPMI-2650 cells may interact with the TGF-αlike molecule since they co-elute

The effect of known purified growth factors was examined and compared to a partially purified sample of the active fractions of RPMI-2650 CM eluted from Bio-Gel P-30 in the TGF, TGF-β and autocrine assays The TGF assay showed that TGF-α and EGF were stimulatory In the case of TGF-α, where different concentrations were examined, the response was dose dependent The effect of combined growth factors showed that the TGF activity of RPMI-2650 CM was potentiated by the addition of TGF-αor EGF No potentiation was observed on addition of TGF-β These results suggest that RPMI-2650 CM contains a TGF-β like molecule In the TGF assay NRK indicator cells will grow when EGF or TGF-α are present but growth is limited The presence of TGF-β stimulates the formation of large NRK colonies (Brown and Blakely, 1984) The lack of increased stimulation found when TGF-α and TGF-β were added is difficult to explain Growth stimulation was expected It is likely that our range of TGF-β concentrations was too dilute to be detected in this assay Sipes et al , (1985) found that 5ng ml^{-1} TGF-β with 5ng ml^{-1} TGF-α showed stimulation in the TGF assay We used a 25 times less concentrated sample TGF-β is present in the serum in the TGF assay, and the amount added here (0 2ng ml^{-1}) may be insufficient to cause additional stimulation

RPMI-2650 CM would add TGF-α and TGF-β to the system so that stimulation could be expected

The TGF-β assay showed stimulation by TGF-α and RPMI-2650 CM The stimulatory effect by RPMI-2650 CM was far greater than that by TGF-β We estimated from this data that our original RPMI-2650 contained 2 5pM TGF-β TGF-α caused increased activity with a decrease in concentration This was not surprising since it was previously shown that the TGF-α activity in a sample of 663+N CM , high in TGF-α like growth factor, was inhibited by the presence of EGF in the TGF-β assay TGF-α and EGF are very similar growth factors and compete equally for binding to the EGF receptor (Lee et al , 1985) This situation appears to have arisen here EGF is an inherent additive to the TGF-β assay, and the negative response observed at high concentrations of TGF-α may be caused by down-regulation of EGF receptor binding sites in the presence of excess EGF-like molecules (Cuatrecasas, 1982) The combined growth factors showed no obvious changes in growth The response of cells in culture is very obviously a function of the growth factors present (van Zoelen et al , 1986)

The results from the autocrine assay showed that all the purified growth factors stimulated the growth of RPMI-2650 cells Dilution effects were not obvious with TGF-α or TGF-β The most significant growth effect was observed for 100ng ml^{-1} FGF (fibroblast growth factor) which was an interesting finding FGF, like TGF-α, EGF and TGF-β (Lee et al , 1985, Roberts et al , 1986) is a stimulator of angiogenesis and is involved in wound healing, tissue repair and also in promoting vascular tissue growth (Baird et al , 1985) FGF has also been reported to stimulate anchorage-independent growth of AKR-2B and NR-6 cells (Rizzino and Ruff, 1986) Almost all combinations of tne purified growth factors tested appear to be inhibitory for RPMI-2650 cell growth We had shown previously that RPMI-2650 cells responded to a very narrow growth factor concentration range

These results show that of all the combined growth factors, TGF-α + TGF-β, and TGF-α + EGF are less inhibitory than other growth factor combinations than RPMI-2650 CM These results would suggest that RPMI-2650 cells are very sensitive to the overall growth

factor content in the medium and are inhibited by high concentrations of growth factors

The fact that the assay systems upon which the TGF, TGF-β and autocrine assays are based, include a high serum concentration (8 6%), may complicate the results and their interpretation The cells are normally grown in medium supplemented with 5% foetal calf serum, and transfer to a higher serum concentration may introduce a stress factor It has been shown that serum contains transforming growth factors (Childs et al , 1982) and that the transformation of NRK cells is caused by the interaction of three growth factors TGF-α, TGF-β and PDGF (platelet-derived growth factor) (Assoian et al , 1984) which have been isolated from human platelets Since serum contains high levels of PDGF (released from platelets during clotting) it is possible for this assay to detect the presence of added TGF-α and TGF-β

In the growth factor assays which we set up, BSA (1mg/100ml) was also included as a carrier protein for the purified growth factors (and RPMI-2650 partially purified CM) Although this had no effect when added as a control in the various assays, it is possible that BSA may contain trace levels of growth factors or inhibitors which on their own show no marked effect but could be activated in the presence of high concentrations of specific growth factors

As a possible means of explaining the inhibitory effect of high concentrations of TGF-α on RPMI-2650 cells, we examined the EGF receptor content of these cells relative to NRK cells and to A431 cells which are known to have an unusually high number of EGF receptor binding sites (Haigler et al , 1978) NRK cells bound up to 18 times more ^{125}I-EGF than RPMI-2650 cells and A431 cells bound about 3 times more ^{125}I-EGF than NRK cells The difference in the ability to bind ^{125}I-EGF may relate to ability to respond to TGF-α The response of the cells to TGF-α is however, influenced by other growth factors, like TGF-β which has a biphasic effect on EGF receptors in NRK cells (monolayer) (Assoian, 1985) Low affinity EGF binding sites increase in number, whereas the high affinity sites undergo a transient decrease in affinity followed by a prolonged increase in number This alteration in the affinity of EGF receptors causes the cells to respond differently to the same amount of TGF-α in the presence of TGF-β The response of

RPMI-2650 cells and NRK cells is most likely modulated through a complex interaction of growth factors and their receptors in our assay system

We examined the relationship between EGF receptor levels and hormones using A431 cells and a human breast cancer cell line T47D We found that A431 cells had the capacity to bind up to 8 times more ^{125}I-EGF than T47D cells and that the receptor binding capacity was increased in these cells by different hormones Dexamethasone caused a significant increase in the capacity to bind ^{125}I-EGF in A431 cells and progesterone caused a significant increase in the capacity to bind ^{125}I-EGF in T47D cells Growth factors or hormones can influence the EGF receptor binding ability in different cell types (Murphy et al , 1985, Newman et al , 1986) We also prepared hormone treated and control cultures for analysis of EGF receptor mRNA levels and types by Dr D Headon, University College, Galway, but final results are not yet available It would be of interest to determine whether or not hormonal control is at a transcriptional or other level

We also carried out some collaborative work with Dr V Preat, in the University of Louvain in Belgium Various samples of rat sera were examined for their ability to stimulate NRK colony formation in the TGF assay We found that samples of serum from rats with carcinogen-induced malignant hepatomas caused a massive stimulation of NRK colony growth while serum from rats bearing pre-malignant liver nodules gave a lesser, but still high activity The non-treated controls gave background levels of activity Samples of serum from partially hepatectomised rats stimulated NRK colony formation but not to the extent seen in the tumour and nodule samples These results show that it may be possible to use assays like the TGF assay to detect raised levels of growth factors in serum samples from patients with cancer It may be possible to monitor the levels of growth factor activity before and after chemotherapy The isolation of increased levels of a high molecular weight TGF-α from the urine of patients with disseminated adenocarcinoma of breast has now been shown by Stromberg et al (1987) They suggest that their urinary TGF-α assay may in some cases assist mammography as a useful method for early detection of breast cancer

The purpose of the work described here was to determine the nature of the cut-off effect in RPMI-2650 cells The data presented established the involvement of autocrine factor(s) Analysis of RPMI-2650 conditioned medium led us to the conclusion that RPMI-2650 cells secrete both TGF-α-like and TGF-β-like growth factors into their exracellular environment Whether the autocrine growth activity in RPMI-2650 cells is due to TGF-α or TGF-β or other known growth factors or to a novel autocrine growth factor is still uncertain HPLC analysis of a partially purified sample of RPMI-2650 autocrine activity showed complete loss of autocrine activity, while both the TGF-α-like and TGF-β-like growth factors survived this purification step like other known TGFs (Halper and Moses, 1983) There is a possibility that the autocrine factor was diluted below its active level, since it is particularly sensitive to dilution however, given the usual ratio of TGF-β to autocrine activities in CM and the amount of TGF-α eluted from HPLC, this explanation is unlikely Possible explanations for the loss of RPMI-2650 autocrine activity on HPLC (including instability of separation of two components) have been discussed earlier In the ultrafiltration experiments it was shown that a large autocrine factor > 30 kDa and an acid-activated autocrine factor < 1 kDa are present in RPMI-2650 CM

The ultrafiltered retentates contain the concentrated factors of higher molecular weight than that of the cut-off filtration membrane used, but it is important to remember that the non-concentrated factors of lower molecular weight than the cut-off membrane used are also present The 30 kDa retentate, for instance, may therefore contain both growth factors in a different ratio from that in the original CM, while the 1,000 filtrate may contain the smaller of the autocrine factors on its own

In the retentates used for gel filtration, the lower molecular weight form would be present at a much lesser concentration and is therefore, not detected after column chromatography or HPLC The data presented here do not allow us to say with certainty that the activity in the 1,000 filtrate is due to a secreted factor, as opposed to removal of toxic or inhibitory substances by the conditioning cells The autocrine activity in the larger molecular weight range could possibly be caused by the interaction of specific concentrations of TGF-α-like and TGF-β-like growth factors

which could act in concert to stimulate RPMI-2650 growth The combined effect of TGF-α and TGF-β is an accepted mechanism of autocrine activity in NRK cells (Anzano et al , 1983)

It was hoped that an examination of pure growth factors in the autocrine assay would help to determine the identity of the autocrine factors The effect of the purified known growth factors may, however, have been influenced by the presence of foetal calf serum in the assay which provided low levels of various mitogens The inhibitory effect seen with the combined growth factors is difficult to explain, but may be due to the presence of excess levels of growth factors Inhibitory effects have been observed with many growth factors (Feng et al , 1986) In different systems, TGF-β may act as an inhibitor, a stimulator and a potent inducer of differentiation (Roberts et al , 1985, Like and Massague, 1986, Massague, 1987) Since the autocrine assay developed for RPMI-2650 involved anchorage-independent growth in agar, the autocrine activity detected could relate to growth stimulation per se, or to specific stimulation of anchorage-independent growth A possible link between the accumulation of an abundant extracellular matrix and anchorage-independent growth has been suggested by Ignot and Massague (1986) A mitogenic effect of TGF-β may be due to the increase of EGF receptor number or affinity (Assoian, 1985) RPMI-2650 cells contain low numbers of EGF receptors, so that an autocrine mechanism involving TGF-α alone seems unlikely

In order to establish the identity of the autocrine growth factor(s) produced by RPMI-2650 cells, further purification and characterization work is necessary High level producer clones, if available, would be helpful An important step would be scale-up of suspension or microcarrier culture of the cells, to provide a more concentrated sample for HPLC analysis HPLC involving a more concentrated sample should resolve the question of whether the disappearance of autocrine activity on HPLC reported here was due to dilution, inactivation or separation of synergistic factors Collecting smaller fraction volumes might also enhance resolution in gel filtration, and thus assist in discrimination between TGFs and autocrine factors A repeat of the experiment using known purified growth factors (especially FGF) combined with a range of stability tests (e,g heat, pH, sulphydryl reducing agents etc)

should narrow the range of possible candidate growth factors However, it may be difficult to devise a method for concentration of the hypothesised low moleclar weight autorcrine factor, thus making purification difficult Our studies showed that RPMI-2650 cells possess low levels of EGF receptors on their surfaces Binding studies with other iodinated purified growth factors (e g TGF-β) (Frolik et al , 1984, Fanger et al , 1986) and quantative determination of receptor numbers and affinity by Scatchard analysis would be valuable in assessing possible autocrine mechanisms Growth blockage by treatment with antibodies to a specific growth factor receptor would provide the best evidence for involvement of that growth factor in growth control in the cell system being examined This has been used by Cuttitta et al , (1985) to confirm an autocrine role for bombesin in human small cell lung cancer cells It must be remembered, however, that growth factors may act in stimulatory or inhibitory fashion depending on their concentration and on combinations of other growth factors present Development of a serum-free autocrine assay might simplify interpretation of the results Given the day-to-day variation in results in the autocrine assay, use of a standard active CM in a range of experiments would be useful, and would allow quantitative data on yield and purification factor to be recorded

Further research along the lines suggested would appear to be worth considering Only by dissecting the molecular details of growth control in a range of human tumour lines, will the mechanisms by which cells grow and differentiate in normal developing and adult tissues and in malignant cells in vivo become clear Such knowledge is needed in order to develop the rational therapy for cancer which is urgently needed

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SECTION 5

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ABBREVIATIONS

A gm	agar growth medium
A T C C	American Tissue Culture Collection
B S A	Bovine serum albumin
C F E	Colony forming efficiency
C M	Conditioned medium
C P M	Counts per minute
D M E	Dulbeccos Modified Eagles Medium
E D T A	Ethylene diamino tetraacetic acid
E G F	Epidermal growth factor
F C S	Foetal calf serum
F G F	Fibroblast growth factor
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl) + piperazineethansulphonic acid
HPLC	High performance liquid chromatography
^{125}I-EGF	Iodine labelled Epidermal growth factor
MEM	Minimum essential medium
n	Number of replicates
NaOH	Sodium hydroxide
NEAA	Non-essential amino acids

NGF	Nerve growth factor
NRK	Normal rat kidney cells
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
P/S	Penicillin/streptomycin
r p m	revolutions per minute
S E M	Standard error of the mean
TGF	Transforming growth factor
Ve	Column elution volume
Vo	Column void volume