# Serum-free Cell Growth

# and

# Differentiation Studies

# in

# Cultured Cell Lines

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# SERUM-FREE CELL GROWTH AND DIFFERENTIATION STUDIES IN CULTURED CELL LINES

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1997

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Paula Meleady Date: 25/7/97

# ABSTRACT

The thesis is divided into two main sections investigating growth and differentiation in cultured cell lines. Investigations were carried out into the establishment of serum-free media (SFM) for the long-term subcultivation of the industrially important cell line, CHO-K1. When the cell line was cultured in a high calcium containing medium, cellular growth was found to be inhibited after 4 to 6 passages on average. However, by growing the cells in a low calcium medium, continuous cultivation was possible. It appears that the calcium concentration in the SFM is critical to this long-term growth. The SFM developed were also capable of supporting growth of CHO-K1 in suspension culture with Pluronic F68 supplementation and in microcarrier culture.

Serum-free media was also developed for the growth and sub-cultivation of a human lung cancer cell line, DLKP. This cell line contains three morphologically distinct sub-populations. The growth pattern of the three clones differed in serum-free medium with only one of them, DLKP-I, capable of proliferating in the above medium. However, the addition of fibronectin to the SFM permitted growth of the other two clones. All sub-populations could be subcultured indefinitely in SFM. DLKP-A, a multiple drug resistant variant of the parental DLKP cell line, was also successfully grown and subcultured in SFM.

Differentiation studies were also carried out on DLKP. This cell line was originally histologically diagnosed as a 'poorly differentiated carcinoma of the lung'. Treatment of the cells with the differentiation-inducing agent, 5-bromodeoxyuridine (BrdU) resulted in the induction of $\alpha_1\beta_1$ integrin and upregulation of $\alpha_2\beta_1$ integrin expression. Associated with this change in integrin expression was a functional change in the properties of the cells themselves with BrdU treatment. The cells developed different adhesive properties showing more rapid attachment to the extracellular matrix proteins, collagen, laminin and fibronectin, and to basement membrane compared to untreated cells. Addition of an anti-integrin blocking antibody to BrdU-treated cells reversed the changes in adhesive properties of the cells.

BrdU treatment of DLKP cells also induced the expression of a number of epithelial antigens, including epithelial specific antigen (a cellular adhesion molecule) and keratin 19 filaments. A significant upregulation of keratin 19 protein expression was also observed in the human lung adenocarcinoma cell line, A549, after BrdU treatment. Preliminary RT-PCR analysis has shown that the effect of BrdU on keratin 19 mRNA expression appears to be at the post-transcriptional/ translational level.

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# ABBREVIATIONS

| | | |
|---|---|---|
| Ab | - | Antibody |
| Acetyl CoA | - | Acetyl Coenzyme A |
| ATCC | - | American Tissue Culture Collection |
| BrdU | - | Bromodeoxyuridine |
| BSA | - | Bovine Serum Albumin |
| cDNA | - | complementary Deoxyribonucleic Acid |
| CEA | - | Carcinoembryonic antigen |
| CHEF | - | Chinese Hamster Embryo Fibroblasts |
| CHO | - | Chinese Hamster Ovary |
| CM | - | Conditioned medium |
| CVDE | - | Crystal Violet Dye Elution |
| Da | - | Daltons |
| Db-cAMP | - | Dibutryl-Cyclic AMP |
| DEPC | | Diethyl Pyrocarbonate |
| dhfr | - | Dihydrofolate Reductase |
| DMEM | - | Dulbecco's Minimum Essential Medium |
| DMF | - | Dimethylformamide |
| DMSO | - | Dimethyl sulfoxide |
| DNase | - | Deoxyribonuclease |
| ECM | - | Extracellular matrix |
| EDTA | - | Ethylenediaminetetraacetic acid |
| EGF-R | - | Epidermal Growth Factor Receptor |
| EGP | - | Epithelial Glycoprotein |
| EHS | - | Engelberth Holm Sarcoma |
| ELISA | - | Enzyme Linked Immuno Sorbent Assay |
| ESA | - | Epithelial Specific Antigen |
| FAK | - | Focal Adhesion Kinase |
| FBS | - | Fetal Bovine Serum |
| FCS | - | Fetal Calf Serum |
| FGF | - | Fibroblast Growth Factor |
| H SFM | - | Ham's F12 Serum Free Medium |
| H/D SFM | - | Ham's F12:DMEM (1:1) Serum Free Medium |
| H/M SFM | - | Ham's F12:MEM.S (1:1) Serum Free Medium |
| HB | - | Heparin Binding |
| HDL | - | High Density Lipoprotein |
| HEPES | - | N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] |

| | | |
|---|---|---|
| $IC_{50}$ | - | Inhibitory Concentration 50% |
| ICAM | - | Intercellular Adhesion Molecule |
| ICE | - | Interleukin 1β-Converting Enzyme |
| Ig | - | Immunoglobulin |
| IGF-1 | - | Insulin-like Growth Factor - 1 |
| IL-6 | - | Interleukin-6 |
| IL-8 | - | Interleukin-8 |
| K | - | Keratin |
| LDL | - | Low Density Lipoprotein |
| MAP-kinase | - | Microtubule Associated Protein Kinase |
| MC | - | Methylcellulose |
| MDCK | - | Madin-Darby Canine Kidney |
| MDR | - | Multiple Drug Resistence |
| MEM | - | Minimum Essential Medium |
| min | - | Minute(s) |
| MMLV-RT | - | Moloney Murine Leukemia Virus-Reverse Transcriptase |
| MMP | - | Metalloproteinase |
| mOsm/kg | - | Milli - Osmoles per Kilogram |
| mRNA | - | Messenger RNA |
| NCAM | - | Neural Cell Adhesion Molecule |
| NCTCC | - | National Cell & Tissue Culture Centre |
| NE | - | Neuroendocrine |
| NEAA | - | Non-Essential Amino Acids |
| NGF | - | Nerve Growth Factor |
| NRK | - | Normal Rat Kidney |
| NSCLC | - | Non-Small Cell Lung Carcinoma |
| NSCLC-NE | - | Non-Small Cell Lung Carcinoma - Neuroendocrine |
| P | - | Passage |
| PBS A | - | Phosphate Buffered Saline A |
| PDGF | - | Platelet Derived Growth Factor |
| PEG | - | Polyethylene Glycol |
| PF68 | - | Pluronic F68 |
| $PIP_2$ | - | Phosphotidylinositol |
| PKC | - | Protein Kinase C |
| PVA | - | Polyvinyl Alcohol |
| PVP | - | Polyvinylpyrrolidone |
| RA | - | Retinoic Acid |
| RGD | - | Arg-Gly-Asp (based on alphabetical code for amino acids) |

| | | |
|---|---|---|
| RNase | - | Ribonuclease |
| rpm | - | Revolution(s) Per Minute |
| $r_s$ | - | Fluorescence Anistropy |
| RT | | Room Temperature |
| RT-PCR | - | Reverse Transcriptase-Polymerase Chain Reaction |
| SCLC | - | Small Cell Lung Carcinoma |
| SDS | - | Sodium Doedecyl Sulphate |
| sec | - | Second(s) |
| SF | - | Serum-Free |
| SF Briclone | - | Serum-Free Briclone |
| SFM | - | Serum-Free Medium |
| SHE | - | Syrian Hamster Embryo |
| SMC | - | Smooth Muscle Cells |
| SRB | - | Sulforhodamine B |
| SS | - | Serum-Supplemented |
| SSM | - | Serum-Supplemented Medium |
| TBS | - | Tris Buffered Saline |
| TCA | - | Trichloroacetic acid |
| Td | - | Thymidine |
| TEM | - | Transmission Electron Microscopy |
| TEMED | - | N, N, N', N'-Tetramethyl-Ethylenediamine |
| TGF-α | - | Transforming Growth Factor α |
| TGF-β | - | Transforming Growth Factor β |
| TMA-DPH | - | 1-[4-(trimethylamino) phenyl]-6-phenylhexa-1,3,5-triene |
| TNF-α | - | Tumour Necrosis Factor - α |
| Tris | - | Tris(hydroxymethyl)aminomethane |
| uPA | - | Tissue Plasminogen Activator |
| V-SCLC | - | Variant Small Cell Lung Carcinoma |
| v/v | - | volume/volume |
| VCAM | - | Vascular Cellular Adhesion Molecule |
| VE-cadherin | - | Vascular Endothelial Cadherin |
| w/v | - | weight per volume |

# 1.0 INTRODUCTION

## 1.0 INTRODUCTION

The growth of cells in serum-free medium is of particular benefit to study cellular functions in a completely defined system. Serum, which is an obligatory component of most cell culture media, is very poorly defined and often there is variability in composition between different batches. Extracellular matrix (ECM) proteins appear to be important supplements to SFM to control growth and differentiation of many normal and transformed cell lines, especially tumour cell lines.

Cellular adhesion molecules, especially integrins, are gaining wider recognition as important factors for normal cellular functions including growth and differentiation. Alterations in the structure of the ECM and in the expression of various adhesion molecules have been implicated in pathogenesis of disease and tumour progression. Integrins appear to be consistently down-regulated in poorly-differentiated epithelial carcinomas.

## 1.1 SERUM-FREE MEDIUM

### 1.1.1 Background

Mammalian cells, in general, require serum for growth *in vitro*. When cells are removed from their original tissues and placed in culture, the medium must provide environmental conditions similar to those to which the cell has been exposed to in the body. Only then will the cells be able to survive and proliferate *in vitro*. This extracellular medium must provide the essential requirements for growth and survival; it must provide nutritional, hormonal and adhesion factors. Serum is an extremely complex mixture of many small and large biomolecules with different physiologically balanced growth-promoting and growth-inhibiting activities. It provides hormonal factors capable of stimulating cell growth and differentiation, attachment and spreading factors essential for cell survival, and transport proteins such as albumin and transferrin which carry hormones, vitamins, lipids, trace elements and other essential nutrients. It is usually added to most basal media at concentrations ranging from 5 to 20%.

In most of the serum-free media (SFM) formulations developed, insulin and transferrin appear to be the most important components. Many cell lines require spreading and attachment factors such as fibronectin and collagen. Hormones and growth factors appear to be specific for different cell lines. Bovine serum albumin (BSA) is often added to serum-free formulations but this component is not totally defined and its use in 'defined' media is quite controversial. Many cell lines have an obligatory lipid requirement, while other cell lines grow perfectly well in media without any lipids present. Cells in culture appear to have more of a requirement for the water soluble vitamins rather than the fat soluble vitamins. A number of trace elements are required in serum-free formulations especially selenium, iron, zinc and cadmium. Conditioned media are sometimes used in serum-free formulations to support the growth of specific cell lines that otherwise would not grow in media lacking serum. It is thought that one of the main reasons why cells do not achieve the same growth rates and same cell densities in serum-free media as cells grown in serum-supplemented media is due to trypsinisation damage during passaging of the cells. Cells in serum-supplemented media (SSM) tend to recover faster from such damage than cells in SFM.

#### 1.1.1.1 Advantages of growing cells in SFM

There are many advantages to growing cells in serum-free conditions. Selective culture of differentiated and functional cell types is possible from heterogenous populations of primary cultures without stimulation of variant sub-populations (Suzuki *et al.*, 1989;

Birkenfeld *et al.*, 1988). This takes advantage of different rates of cellular attachment and proliferation especially in primary cultures where fibroblast overgrowth can be a major problem in preventing the isolation of epithelial cells (Bottenstein and Sato, 1978). SFM has also been used to allow the selective growth of tumours from fresh clinical samples (Carney *et al.*, 1984; Oie *et al.*, 1996).

Serum introduces an ambiguous factor into cell cultivation because of its undefined composition and tends to vary between batches. Serum can be extremely expensive often accounting for up to 84% of the media costs (Griffiths, 1986) thus using SFM may provide cost savings. It is also a potential source of contamination from viruses, fungi and especially mycoplasma. The use of serum can often reduce the difficulty and cost of purification of required product on an industrial scale with less serum protein contamination of the final product. This leads to difficulties in reproducing experimental results on a small scale and in maintaining consistent yields of cells and their products on a large scale. The international regulations for good manufacturing procedures (GMP-procedures) for vaccine production restrict the amount of serum constituents in the final product when making injectible vaccines, since allergic reactions could be provoked (Bjare, 1992).

Defined media are of great advantage when investigating cellular processes affected by growth factors, cellular mediators and other specific agents as the background of possible effects caused by serum components can be diminished. Vonen *et al.* (1992) found that in the long-term cultivation of rat pancreatic islet cells, a higher daily insulin and somatostatin secretion rate was achieved in serum-free medium than in serum supplemented medium. They concluded that serum contains insulin degrading factors.

Serum often suffers from batch-to-batch variation in biological activity, protein or lipid concentration *etc.*, which can lead to significant problems in *in vitro* studies and on an industrial scale. HepG2 cells, a human heptocellular carcinoma cell line, are useful for model studies such as drug-metabolising enzyme induction. Cytochrome P450s of HepG2 cells are induced by many carcinogens or mutagens. As a method of human risk assessment, the effects of environmental chemicals can be observed in these cells. However, if serum-supplemented medium is used for such studies, the cell conditions are always unstable because of the ill-defined nature of serum and batch to batch variation. Serum-free culture allows the creation of constant culture conditions which lead to stable data vital for such drug investigations (Nakama and Yamada, 1993). The SHE (Syrian Hamster Embryo) system has been widely employed to identify, quantify and study the mechanism of action of chemical carcinogens. A major problem with one of the assays used in the system (MTA - morphological transformation assay) is the

absolute necessity to screen many batches of FBS for use in the assay. Kaighn *et al.* (1993) developed a SFM for the clonal growth of SHE cells which avoided the problem of serum variability and facilitated the investagation of autocrine factor expression at various stages of neoplastic progression in the SHE system.

Chemically defined media may be very useful in controlling the proliferation and differentiation of cells (Rahemtulla *et al.*, 1989; Reyne *et al.*, 1989). Hoshi *et al.* (1991) developed a serum-free medium to study the mechanisms of the growth and function of granulosa cells which are important for the formation of ovarian follicles and maturation of oocytes. The complex, ill-defined nature of serum is a complication when assessing the effect(s) of regulatory agents, such as hormones and neurotransmitters, on differentiated properties of nervous system cells in culture. Bottenstein and Sato (1979) developed a serum-free medium for the growth of the rat neuroblastoma cell line, B104.

Another main advantage of serum-free culture is that it allows for selection of cells which have been transformed with the SV40 virus. The growth of NIH-3T3 cells in serum-free medium is critically dependant on the presence of insulin, EGF and HDL. Growth-restrictive conditions (*i.e.* media lacking HDL, EGF or insulin) allows for the selection of cells transformed with SV40 virus (Chiang *et al.*, 1985).

#### 1.1.1.2 Disadvantages of growing cells in SFM

There are also many disadvantages to the use of serum-free media in the culture of mammalian cells. Serum-free media are often highly specific to one cell type and so medium optimisation may be required for each cell line. Often lag phases are longer and cell growth rate, maximum cell density and cell number are often lower (Ahearn *et al.*, 1992).

In the serum-free growth of many cell lines, supplementing the medium with hormones and growth factors can prove to be very expensive, even more expensive than supplementing with serum.

Protease inhibitors in serum may help protect the cells from enzymes such as trypsin. Cell recovery from trypsinisation damage is much slower in serum-free medium than in serum-supplemented medium.

### 1.1.2 MEDIA SUPPLEMENTS

The media supplements required in SFM will vary from one cell type to another due to the different origins of the cells from different sites in the body, or whether they are normal or transformed. In addition, cells grown in SFM are usually much more density-dependent than cells grown in serum-supplemented medium. Media supplements can be categorised into the following groups: growth promoters including hormones, growth factors and cytokines; attachment factors; transporter molecules; lipids; trace elements and vitamins.

#### 1.1.2.1 Growth promoters

##### 1.1.2.1.1 Insulin

Insulin is the most common hormone supplement of culture media. It is considered to be essential for the growth of nearly all cells in culture including hybridomas (Glassy *et al.*, 1988). It is a relatively small polypeptide with a molecular weight of about 5700Da and is known to have multiple effects on cell metabolism. It is known to be involved in cell membrane substrate transport, the stimulation of uridine and glucose uptake and the biosynthesis of nucleic acids particularly RNA, proteins and fatty acids. The classical insulin responses such as increased fatty acid and glycogen synthesis are seen in the serum-free medium for a number of cell lines (Barnes and Sato, 1980a,b). Insulin is included in most culture media at superphysiological levels ranging from 5-10µg/ml. High concentrations are needed due to its short half-life and its inactivation by cysteine via the reduction of disulphide bonds on the insulin molecule. In the mouse melanoma cell line, MR2, insulin caused maximal stimulation of growth at 5µg/ml in Ham's F12 (with cysteine) and at 0.5µg/ml in Ham's F12 in which the cysteine has been replaced by cystine. Mather and Sato (1979) reported that the incorporation of glucose into both glycogen and fatty acids is actually stimulated by increasing glucose concentrations. It is not known, however, if either of these phenomena is directly or indirectly responsible for the growth-stimulatory effect of insulin in MR2 cells as these cells are not derived from classical insulin target tissues such as fat, liver, *etc.* Hayashi *et al.* (1978) have shown that insulin also increases glucose incorporation into both glycogen and fatty acids in the rat pituitary cell line, $GH_3$. Another reason why insulin might be required in such high doses is that it may be mimicking insulin-like factors such as somatomedin C for some cell lines, and high insulin concentrations may be necessary in order to occupy receptors which have a high affinity for insulin-like factors and a lower affinity for insulin (Barnes and Sato, 1980a).

Suzuki *et al.* (1989) have shown that neonatal rat cardiac myocytes show a requirement for insulin when grown in serum-free medium. They found that insulin elicited an increase in DNA and protein synthesis in these cells. The stimulatory effect of insulin was found to be dose-dependant with a concentration of 10µg/ml being optimal for maximal growth of these cells. Insulin was required for serum-free growth of a human colon carcinoma cell line (Murakami and Masui, 1980), rat neuroblastoma cells (Bottenstein and Sato, 1979) and HeLa cells (Hutchings and Sato, 1978).

Hybridomas also require insulin for maximal growth under serum-free conditions (Glassy *et al.*, 1988). Murakami *et al.* (1982a) found that deletion of insulin from the media of the hybridoma cell line MPC11-BL completely inhibited growth. Leukaemic stem cells require insulin at a concentration of 8µg/ml for optimal growth (Dai *et al.*, 1992). The rat glioma cell line derived from a rat brain tumour can grow in serum-free media once insulin is present at a concentration of 25µg/ml (Wolfe *et al.*, 1980). BHK-21 cells require insulin at a concentration of 5µg/ml for optimal growth (Bradshaw *et al.*, 1983). SV-40 transformed Balb/c-3T3 cells also show a requirement for insulin (Rockwell *et al.*, 1980).

Mendiaz *et al.* (1986) have developed a serum-free medium for the growth of CHO-K1 cells. They have shown that insulin stimulated the growth of these cells in serum-free media over a wide range of concentrations from 1ng to 10µg/ml. On the basis of their results, they concluded that insulin itself was showing a true mitogenic effect and that insulin was acting through both the insulin receptor and the receptor for IGF-1.

Liu *et al.* (1993) have investigated the interactions of a number of cell lines including mouse fibroblasts with cell growth proteins, insulin and transferrin, which are immobilised on non-biodegradable polymer membranes. They found that immobilised insulin or coimmobilised insulin/ collagen possessed cell-growth acceleration activity almost equivalent to the addition of serum. One major benefit of this procedure is that the system is stable for repeated uses which would be beneficial in the large-scale production of biologicals from animal cells under serum-free conditions.

However, Zhou and Hu (1995) questioned the need for insulin supplementation to SFM for hybridoma cells. They found that insulin affected neither the cell growth nor the antibody production. However, elimination of insulin from the medium allowed antibody to accumulate to a concentration substantially higher than that in insulin-containing medium.

#### 1.1.2.1.2 Steroid hormones

The principal steroid hormones used in the serum-free culture of mammalian cells are the glucocorticoids, hydrocortisone and dexamethasone, oestradiol, testosterone and progesterone. The glucocorticoids have been found to inhibit or stimulate cells in culture depending on cell type and density. They may modulate cell proliferation by altering responsiveness to growth factors. Dexamethasone acted synergistically with IGF-1 to stimulate the growth of fibroblasts and smooth muscle cells (Concover *et al.*, 1989) but down-regulated the insulin receptor substrate-1 in 3T3-Li adipocytes (Turnbow *et al.*, 1994). Dexamethasone has been shown to be required for the serum-free growth of neonatal rat cardiac myocytes (Suzuki *et al.*, 1989); these authors found that dexamethasone in either hormone-free or hormone-supplemented, serum-free medium increased the ratio of beating to non-beating cells and markedly increased beating rate per cell. Dexamethasone at a concentration of 0.2µg/ml has been included in the serum-free media developed for the growth of human diploid fibroblasts (HDF) (Bettger *et al.*, 1981). In a study of the mode of growth stimulation of steroids, Baker *et al.* (1978) found that synthetic dexamethasone increased the binding capacity of cell membrane receptors for insulin and EGF. This increased binding enhanced the mitogenic action of these compounds. Hutchings and Sato (1978) have shown that hydrocortisone is an essential component of the serum-free medium developed for the growth of HeLa cells.

Prostaglandins $E_1$ and $E_2$ have been found to be stimulatory for the growth of the MCDK cell line under serum-free conditions. Evidence suggests that this stimulation is brought about by an increased cAMP level in the cell, since other agents which are known to increase intracellular cAMP, dibutryl cAMP, isobutryl methyl-xanthine and glucagon, are also stimulatory (Taub *et al.*, 1979).

The mouse melanoma cell line, MR2, has been found to grow in a serum-free medium containing insulin, NGF, FSH, testosterone and leutinising releasing hormone. Mather and Sato (1979) originally found that progesterone was stimulatory to the growth of these cells but later found that testosterone could replace progesterone and was, in fact, more stimulatory to growth. Progesterone has been found to be stimulatory to the growth of the rat neuroblastoma cell line, B104. The effects of progesterone in the central nervous system include modulation of single unit and electrocephalogram activity, as well as behaviour (Bottenstein and Sato, 1979). Oestrogen has been found to be stimulatory to the growth of MCF-7, breast carcinoma cells (Dickson *et al.*, 1986).

Triiodothyronine ($T_3$), a thyroid hormone, and hydrocortisone were found to significantly enhance the growth of a human colon carcinoma cell line, HC84S (Murakami and Masui, 1980). The intestinal hormone gastrin also gave some stimulation of growth. All of the prostaglandins inhibited growth apart from prostaglandin $E_1$. Progesterone, testosterone and oestradiol also inhibited cell growth (Murakami and Masui, 1980). This result and the demonstration that the hormone-supplemented, serum-free medium allows better growth of HC84S cells than does serum-supplemented medium raise the possibility that there are hormones present in serum that are inhibitory to the growth of human carcinoma cells and other cell lines.

#### 1.1.2.1.3 Growth Factors

There are a number of growth factors that are frequently added to serum-free formulations for the various cell lines. These include EGF, FGF, NGF, PDGF and IL-2 among many others.

EGF is a low molecular weight (6,100Da) acidic protein originally isolated from the submaxillary gland of adult male mice. Its physiological role is uncertain but it has been found to be mitogenic for many cell lines. The response range for the growth of cell lines *in vitro* is 1-100ng/ml (Barnes and Sato, 1980b). Steroids have been found to enhance the activity of EGF. Increased binding of EGF to the cellular membrane of human fibroblasts is found in the presence of the synthetic glucocorticoid steroid, dexamethasone (Baker *et al.*, 1978). The serum-free medium developed for the growth of NIH-3T3 cells contains EGF (Chiang *et al.*, 1985).

FGF is a class of polypeptides which was first isolated from bovine pituitary glands and subsequently from bovine brain (Gospodarowicz *et al.*, 1981a) and exists in both basic and acidic forms. This growth factor at a concentration of 200ng/ml was found to be required for the growth of BHK-21 cells in serum-free medium (Bradshaw *et al.*, 1983). FGF at a concentration of 50ng/ml is required for the growth of the rat glioma cell line, C6 (Wolfe *et al.*, 1980). FGFs are also potent mitogens for endothelial, mesoderm and neuroectodemal- derived cells.

NGF was one of the first growth factors to be described and is known to stimulate the growth and differentiation of the cells of the peripheral nervous system. NGF has been shown to have a minor but reproducible stimulatory effect on MR2, human melanoma cells (Mather and Sato, 1979).

IL-2 is a mitogenic protein which is released into the medium by T-lymphocytes and its target cells in terms of mitogenic activity include the T-lymphocytes from which it was extracted. A serum-free medium was developed by Brown *et al.* (1983) that supported the growth of a gibbon ape lymphoma T-cell line, MLA144. These cells produce IL-2 which they release into the serum-free medium. This serum-free, conditioned medium was then used to support the growth of IL-2-dependent cell lines.

PDGF is a serum protein derived from platelets during the clotting process (Stiles *et al.*, 1983). It is a potent mitogen for connective tissue including smooth muscle cells, fibroblasts and glial cells (Koher and Lipton, 1974). When PDGF was incorporated into the medium, a lower level of EGF was required for growth of mouse CBH/1OT1/2 cells (Carpenter and Cohen, 1979).

#### 1.1.2.2 Attachment factors

The attachment of cells to their extracellular matrix is important in maintaining the structural integrity of tissues and is also important in regulating gene expression controlling cellular proliferation and differentiation. Serum provides many attachment and spreading factors such as fibronectin and vitronectin; thus, the absence of serum from the media may have consequences for regulating growth and differentiation of the cells. Many cell lines are capable of synthesising attachments factors such as fibronectin thus SFM have been developed which do not contain exogenous attachment factors (Hahm *et al.*, 1990; Taub *et al.*, 1979). However, some cell lines especially primary lines appear to have a requirement for supplemented attachment factors (Gospodarowicz *et al.*, 1981b).

The extracellular matrix (ECM) is a complex network of collagenous, non-collagenous glycoproteins and proteoglycans. The role of collagens, fibronectin, laminin and vitronectin will be discussed in greater detail in Section 1.2.

#### 1.1.2.3 Transport protein

The main transport proteins used in most serum-free media are albumin and transferrin which transport lipids and iron respectively.

##### 1.1.2.3.1 Albumin

The role of albumin in the serum-free growth of mammalian cells in culture is quite controversial. No conclusive evidence has actually been published demonstrating a

need for a specific protein as a serum substitute. The physiological functions of albumin are diverse. It is involved in the maintenance of blood osmotic pressure, the transport of fatty acids and various hormones, tryptophan and cysteine. It is also a source of amino acids for peripheral tissues (Kan and Yamane, 1982).

Albumin has been used widely as an essential substitute for serum in defined media for a number of different cell types. Albumin has the ability to act as a carrier for the essential fatty acids and trace minerals. It can also transport hormones and peptide growth factors to the site of action. Albumin can also act as a detoxifying agent for $H_2O_2$ (Darfler and Insel, 1983) and excess trace elements (Kan and Yamane, 1982). It can also provide cells with protection against mechanical shear damage (Lambert, 1985). BSA also works in a serum-free system by buffering changes in pH during the culture time (Dai *et al.*, 1992).

The principal role of bovine serum albumin appears to be that of a carrier of fatty acids, protecting the cells against the toxic effects of fatty acids in free solution (Nilhausen, 1978). This group found that by removing the fatty acids from the albumin eliminated most of the growth stimulating effect of BSA on CHEF cells and recombination of albumin with the previously extracted and subsequently purified fatty acids returned the growth promoting activity to its original levels. These findings agree well with the view that albumin is a carrier and storer of fatty acids which are gradually made available to the cells in suitable, dilute non-toxic concentrations. Kan and Yamane (1982) also found that the growth promoting effect of BSA on human diploid fibroblasts (HDF) was virtually abolished when BSA was delipidised.

#### 1.1.2.3.2 Transferrin

Transferrin is a protein constituent of the β-globulin fraction of blood serum and has a molecular weight of 86,000Da. It is responsible for the transfer of iron in blood under normal physiological conditions. It is an obligatory ingredient of most serum-free media formulations as virtually every cell line, investigated under serum-free conditions, shows positive growth response to transferrin. Typical concentrations in serum-free media range from 1-30μg/ml. Transferrin interacts with specific cell surface receptors and has an intimate role in the facilitation of iron transport across the plasma membrane. There may also be additional *in vitro* functions such as the chelation of deleterious trace elements (Glassy *et al.*, 1988). It thus appears that the role of transferrin in growth stimulation is mainly due to its ability to increase the uptake or utilisation of the existing iron in the medium. Some of the stimulatory activity may be related to its ability to bind metal ions which may be present in the medium at

concentrations which are toxic (Barnes and Sato, 1980a; Iscove and Melchers, 1978). It is conceivable that the requirements of the MCF-7 and B104 cell lines for unusually high transferrin concentrations (Barnes and Sato, 1979; Bottenstein and Sato, 1979) may relate to a particular sensitivity of these cell lines to trace amounts of toxic metals introduced into the medium as contaminants of other medium components.

Mather and Sato (1979) investigated the growth of the mouse melanoma cell line, MR2, in a serum-free medium containing varying concentrations of transferrin from 0-50μg/ml. They found that the observed growth stimulatory effects were dependant on the presence or absence of $FeSO_4$ in the medium. In the absence of added iron, there was found to be a fifteen-fold increase in the cell growth at a transferrin concentration of 1μg/ml. However, at the same transferrin concentration in the presence of added $FeSO_4$, only a two-fold stimulation of growth was observed. They also carried out dose-response curves to both iron-free transferrin and iron-saturated transferrin (2 molecules of Fe/ molecule of transferrin) in Ham's F12 medium. They found no difference in the stimulation of growth between these two forms of transferrin most likely due to the iron-free transferrin binding sufficient contaminant iron from the medium during the course of the experiment over six days.

Transferrin is considered to be one of the essential growth promoting supplements in serum-free media for hybridomas (Glassy *et al.*, 1988). Transferrin at a concentration of 35μg/ml was used in the serum-free growth of the hybridoma cell line, MPC11-BL (Murakami *et al.*, 1982a). They found that transferrin at a concentration of 2μg/ml was required for the growth of the myeloma cell line, MPC-11.

Dai *et al.* (1992) showed that leukaemic stem cells require transferrin at a concentration of 1μg/ml. Murakami and Masui (1980) found that the human colon carcinoma cell line, HC84S, required transferrin at a concentration of 2μg/ml for optimal growth. Transferrin at a concentration of 5μg/ml was required for optimal growth of the SV40 transformed Balb/c-3T3 cell line. Iron, which is an essential component of the electron transport system and of several enzymes concerned with oxidation-reduction reactions within the cell, has been shown to be a critical nutrient for the successful proliferation of SV40 transformed cells (Rockwell *et al.*, 1980). BHK-21 cells require transferrin at a concentration of 1μg/ml (Bradshaw *et al.*, 1983). Deletion of transferrin from the SFM developed for C6, rat glioma cells, greatly reduced the growth response of this cell line (Wolfe *et al.*, 1980). HeLa cells require transferrin at a concentration of 5μg/ml (Hutchings and Sato, 1978). Mendiaz *et al.* (1986) developed a serum-free media for the growth of CHO-K1 cells. Transferrin was found not to be an essential requirement as $FeSO_4$ substituted equally well for it. Optimal transferrin concentration

for growth and long-term survival of neonatal rat cardiac muscle cells was found to be 1μg/ml (Suzuki *et al.*, 1989).

A different function of transferrin was noted when it was found to counteract the effect of LDL in its action on specific mitogen stimulation of lymphocytes. Another example of the diverse effects of transferrin is the interplay of LDL and transferrin in oxidative enzyme stimulated lymphocytes. The presence of LDL blocked the cell cycle but the block was relieved by the addition of transferrin (Scupham *et al.*, 1987; Bjare, 1992).

#### 1.1.2.4 Lipids

Serum is a rich source for the various lipids cultured cells generally need for their survival and particularly for their growth. Cell lines tend to differ in their requirements for essential fatty acids, phospholipids, lipoproteins, lecithins and cholesterol. Many cell lines have an obligatory lipid requirement, while other cell lines can grow perfectly well in media without any lipids present. The specific lipid demands have to be tested for each particular cell line (Bjare, 1992).

Lipids may be present in culture medium as components of added serum or they can be supplemented in the form of isolated lipoprotein fractions, free fatty acids complexed to serum albumin, or microemulsions composed of various fatty acids, phospholipids and cholesteryl esters. Incorporated lipids are utilised within the cell either as a source of cellular energy or as building blocks for cell growth especially in the formation of cellular membranes. Often the fatty acid composition of cellular storage lipids as well as of membrane phospholipids resembles that of lipids present in the medium. Modification of membrane phospholipid fatty acyl groups have been shown to lead to differences in cell function such as membrane enzymatic activity and membrane transport properties. This could lead to changes in the specific growth rate of cultured cells (Schmid *et al.*, 1991). Thus, care must be taken in supplementing basal media with various lipids.

Fatty acids and cholesterol are the basic building blocks for cells. The more complex lipids can be synthesised by the cell provided that these two basic precursors are available. Many cell lines are also able to synthesise *de novo* both cholesterol and many of the fatty acids that they require from Acetyl CoA (King and Spector, 1981). If lipids are available in the medium, cells take them up and *de novo* synthesis of fatty acids and cholesterol is supressed. When the medium lipids are in the form of serum, the lipid content of the membrane resembles that of the serum (Geyer *et al.*, 1962). Thus, most cell lines are capable of making fatty acid and cholesterol from non-lipid precursors but

supression of *de novo* lipid synthesis occurs when adequate supplies of these lipids are available in the culture medium.

#### 1.1.2.4.1 Fatty acids

The majority of cell lines show requirements for either essential or non-essential unsaturated fatty acids as opposed to saturated ones. The mechanism by which fatty acids stimulate growth is still relatively unknown. Exogenous lipids may be incorporated into the cell membranes, thus affecting their fluidity, which could influence the shear sensitivity of cells in culture. Polyunsaturated fatty acids such as linoleic or arachidonic acids have been shown to reverse the uncoupling of oxidative phosphorylation and the loss of respiratory control of HeLa cells incubated in lipid-free medium (Rockwell *et al.*, 1980; Gerschensen *et al.*, 1967). Therefore, the stimulation of growth by these unsaturated fatty acids may be simply due to their ability to maintain the integrity of the mitochondrial membrane. Rockwell *et al.* (1980) found that the cis-unsaturated fatty acids, linoleic, linolenic, oleic and arachidonic acids, were equally effective in stimulating the growth of SV40 transformed Balb/c-3T3 cells. Saturated fatty acids at similar concentrations were either inhibitory in the case of palmitic, or inactive in the case of stearic acid. Oleic acid was also found to be stimulatory to the growth of BHK cells (Bradshaw *et al.*, 1983) and a number of other cell lines (Yamada *et al.*, 1990). The rat glioma cell line, C6BU-1, was found to have a requirement for linoleic acid. It is thought that the growth promoting effects of linoleic acid are related to its conversion to either prostaglandin $F_{2\alpha}$ or a related compound. Prostaglandin $F_{2\alpha}$ is synthesised from arachidonic acid, a linoleate derivative, and has been found to initiate DNA synthesis in quiescent mouse 3T3 cells (King and Spector, 1981; Jimenez de Asua *et al.*, 1975).

#### 1.1.2.4.2 Cholesterol

Cholesterol is the most abundant of the lipid species in the plasma membranes of animal cells. It has a two-fold function, as a structural component of the membrane to maintain the integrity of the cells and as the regulator of fluidity of the plasma membrane playing an important role in the control of many cellular functions. Dividing cells require cholesterol for the formation of new plasma membrane either through endogenous biosynthesis or from an exogenous source such as serum.

Ohmori and Takahama (1989) demonstrated a cholesterol requirement for antibody production in murine lymphocytes in SFM. Cholesterol or cholesterol precursors such as lathosterol, 7-dehydrocholesterol and desmosterol have been found to support the

growth of NS-1 and X63 mouse myeloma cells in SFM (Sato *et al.*, 1988). Some cell lines are cholesterol auxotrophs thus requiring an exogenous supply of cholesterol (Chen and Kandutsch, 1983). Cholesterol can also have a negative effect on cell growth (Buntemeyer *et al.*, 1993). Darfler (1990b) found that cholesterol and desmosterol were growth inhibitory or had no positive effect on hybridoma cell growth. This suggests that cholesterol requirements are cell line specific.

#### 1.1.2.4.3 Lipoproteins

Lipoproteins, especially HDL and LDL, are often added to serum-free formulations. Nakama and Yamada (1993) found that the most effective factor on the growth of HepG2 cells was LDL and found that the growth requirement of HDL was ten times less than than for LDL for the same growth promoting activity.

HDL has been found to be a primary mitogen for the growth of ABAE (adult bovine aortic endothelial) and VSM (vascular smooth muscle cells) grown in serum-free media (Fugii and Gospodarowicz, 1983; Gospodarowicz *et al.*, 1981b; Tauber *et al.*, 1981; Giguere *et al.*, 1982). The primary mitogenic component of HDL was found to be phosphatidyl choline (Fugii *et al.*, 1983).

The optimal proliferation of both human EC (vascular endothelial cells) and SMC (smooth muscle cells) clearly requires LDL or HDL where the active concentrations range from 20-60μM for both cell types (Chen *et al.*, 1986). A simple conjugate of BSA-oleic acid could not substitute for the lipoproteins. Therefore a growth stimulatory feature for EC or SMC is encoded in the lipoprotein structure beyond the presence of a simple polyunsaturated fatty acid like oleic acid. These results suggest that both the protein and lipid component of lipoproteins contribute to the maximum support of the growth of human EC and SMC cells.

### 1.1.2.5 Trace elements

The role of various inorganic trace elements has only been partly elucidated. Defining the type and concentration of trace elements needed for optimal cell growth is a very difficult task because of the regular contamination of these elements in the other nutritional components of culture media. The list of essential or potentially beneficial trace elements includes; cobalt, copper, iron, manganese, molbydenum, zinc, selenium, nickel and vanadium.

Many of these trace elements act as enzyme cofactors. $SeO_3^{2-}$ is required to activate distinct enzymes essential for metabolic detoxification. Selenium is also thought to serve as an inactivator of free radicals. The primary effect of selenium on cultured cells has been suggested as protection against oxygen toxicity (Murakami *et al.*, 1982a). Murakami *et al.* reported that selenium was required for the optimal growth of the myeloma cell line, MPC-11, in serum-free medium. Ham has suggested that the requirements of cells for selenium is related to its presence as a cofactor for glutathione peroxidase, which appears to protect the cells from the toxic effects of lipid peroxides (Barnes and Sato, 1980a; Ham and McKeehan, 1979). Hamilton and Ham (1977) demonstrated that selenium appears to be an absolute requirement for the clonal growth of CHO cells. However, Mendiaz *et al.* (1986), in trying to develop a serum-free medium for the growth of their CHO-K1 cell line, were only able to demonstrate a minor effect of selenium. McKeehan *et al.* (1976) have found that selenium is essential for the clonal growth of diploid fibroblasts from human fetal lung (WI-38) in media containing small amounts of serum protein. Selenium has also been found to be required for the growth of certain human hepatoma cell lines (Nakabayashi *et al.*, 1982), rat neuroblastoma cells (Bottenstein and Sato, 1979) and hybridomas (Murakami *et al.*, 1982b; Glassey *et al.*, 1988).

#### 1.1.2.6 Vitamins

Information in the literature is quite scant on the role of vitamins in serum-free cell culture. It does appear, however, that the water soluble vitamins are of more importance than the fat soluble vitamins for optimal growth of cells in culture. The requirements do vary from one cell line to another but it appears that the ingredients in the common media formulations satisfy most cell lines. Eagle (1955) reported that mouse L cells and HeLa cells had a strict requirement for seven vitamins (choline, folic acid, nicotinimide, panthothenate, pyridoxal, riboflavin, and thiamine) and thus included them in his basal medium. If a single vitamin was omitted from the culture environment, the cells would undergo degenerative changes within 5 to 15 days, resulting in eventual cell death. Media for serum-free culture are often enriched with a few additional vitamins especially vitamin $B_{12}$ and biotin. Biotin becomes increasingly important when serum is reduced or eliminated because of its function in lipid biosynthesis as the prosthetic group of acetyl CoA carboxylase. Matsuya and Yamane (1986) noted a vitamin $B_{12}$ requirement at low cell densities for a number of mouse cell lines that disappeared at higher cell densities. Inositol is often referred to as a vitamin supplement in cell culture media. Lockart and Eagle (1959) showed that a requirement for inositol became apparent when cells were cultured at low cell densities. These authors were the first to recognise differences in the nutritional requirements of cells

undergoing clonal growth at low cell densities and those of cells in higher density cultures. The main functions of the B vitamins included in culture media are as cofactors or coenzymes associated with the many enzyme systems involved in carbohydrate, lipid, protein and nucleic acid metabolism.

The lipid constituent choline is classically included with the vitamins but it is usually required in amounts that greatly exceed those for other vitamins. It is a constituent of phospholipids and a precursor of the putative neurotransmitter acetylcholine. Choline was demonstrated to have a strong stimulatory effect upon the growth of human epidermal keratinocytes in the presence of myo-inositol in a defined culture medium (Gordon *et al.*, 1988). CHO cells have been found to respond to supplemented choline (Esko *et al.*, 1983).

There are many conflicting reports about the role of ascorbic acid in cell culture media. It may be beneficial, particularly to cells that synthesise collagen in culture, but its instability to oxidation makes it difficult to work with (Ham and McKeehan, 1979). Ascorbic acid was found to be stimulatory to the growth of a human colon carcinoma cell line in SFM (Murakami and Masui, 1980). It can function as a water-soluble chain-breaking anti-oxidant and acts synergistically with vitamin E to protect both endo and plasma membranes from oxidative damage. Ascorbic acid can regenerate vitamin E by directly reducing tocopherol radicals generated when vitamin E scavenges free radicals (Constantinescu *et al.*, 1993).

The fat soluble vitamins, A, E, D and K, are rarely found in culture media even though the requirements for these fat soluble vitamins has been well established for higher animal species. Vitamin E has been added to some serum-free formulations usually as an anti-oxidant to protect membrane lipids from peroxidation (Smith, 1981). Bettger *et al.* (1981) included vitamin E and vitamin E acetate in the serum-free cultivation of HDF. It has also been found to inhibit the stimulation of *c-fos* levels in MCF-7, mammary carcinoma cells, induced by IGF-1 (Li *et al.*, 1994).

Vitamin A appears to have a more pronounced effect on cells in culture compared to the other fat-soluble vitamins. It has been shown to increase DNA synthesis in guinea pig epidermal cells (Christopher, 1974). Sporn *et al.* (1973) have reported significant increases in protein, RNA and DNA synthesis in mouse epidermal cultures exposed to retinyl acetate. Vitamin A also appears to have a role in the maintenance of differentiated function and inhibition of neoplastic transformation *in vitro* (Smith, 1981; Miyazaki *et al.*, 1984). It has also been found to promote differentiation of neuroblastoma cells (Ueno *et al.*, 1993).

### 1.1.2.7 Miscellaneous components

Many other components are often added to SFM. Ethanolamine has been shown to be an essential growth promoting compound for hybridomas in serum-free culture (Glassy *et al.*, 1988; Murakami and Masui (1982b). The rat neuroblastoma cell line, B104, has been shown to have a requirement for putrescine at a concentration of 100μM (Bottenstein and Sato, 1979).

Polyethylene glycol (PEG), which is a well-known fusogen for mammalian cells and microbial or plant protoplasts, has been found to stimulate the growth of a number of mammalian cell lines including Namalwa and CHO-K1 in SFM (Shintani *et al.*, 1988). It was suggested that it stimulated growth by lowering the surface tension of the medium and thus improving the transport of metabolites into the cells. Bertheussen (1993) found that Pluronic F68 at a concentration of 20μg/ml was optimal for the growth of the cell lines tested. Pluronic F68 is a solid with a molecular weight of 8300 Da. About 80% of the molecule (by weight) consists of hydrophilic polyoxyethylene groups and the remainder of hydrophobic polyoxypropylene groups. In serum supplemented culture, it is often added to prevent denaturation of serum proteins during agitation. In serum-free media, it may act as a solubilising agent to increase the solubility of sparingly water soluble components (Collett and Tobin, 1977). It also has the ability to form complexes with phenols and other small molecules (Marcus *et al.*, 1956). This may reflect a weak albumin-like property of detoxifying species in the medium.

Ohmori and Takahama (1989) have reported that β-cyclodextrin is effective as a serum substitute for inducing the primary antibody response to sheep erythrocytes in murine lymphocyte cultures. α-cyclodextrin has been used as a partial substitute for BSA in the serum-free culture of a human lymphoblastoid cell line (Yamane, 1982). β-mercaptoethanol has been proven to be an effective growth stimulatory supplement in hybridoma and lymphoma cultures (Glassy *et al.*, 1988).

Conditioned media may be defined as media that have used by one cell culture and are reused by another. The 'conditioning' of the medium is non-specific and includes the release of growth stimulating substances and specific nutrients by the first culture. These types of media are thus not defined. However, they are often useful for very selective strains that do not grow well in SFM (Bjare, 1992). In the serum-free growth of a number of human hepatoma cell lines, it was found that a conditioned medium from the HuH-7 cell line of liver origin was able to support the growth of the hepatoma cells under serum-free conditions (Bagnarelli and Clementi, 1987). CCRF-CEM, a

human T-cell line, was found to grow well in serum-free media at concentrations greater than $2x10^5$ cells/ml, while at lower cell densities the cultures rapidly underwent apoptosis or programmed cell death (Sandstrom and Buttke, 1993). The viability of low density CEM cells could be preserved by supplementing the medium with 'conditioned' medium from high density CEM cultures. Analysis of the conditioned medium resulted in the isolation of a 60kDa protein capable of sustaining CEM growth in the absence of serum and was subsequently identified as human catalase. Bradshaw *et al.* (1983) showed that at low cell densities, the growth of BHK-21 cells in SFM was poor unless it was supplemented with 'conditioned' medium from BHK-21 cells themselves.

### 1.1.3 Industrial relevance for SFM

There is huge potential for the use of serum-free medium in the production of commercially valuable products for the veterinary and pharmaceutical industries. Mammalian cell products include vaccines, monoclonal antibodies, interferons, hormones and tissue plasminogen activator. There are two traditional methods of production, monoclonal antibodies from hybridoma technology and expression of recombinant proteins in genetically engineered cell lines.

One of the biggest successes for SFM has been in the production of monoclonal antibodies (mAbs). For most of the hybridomas used industrially, there is little variation in the medium composition with insulin, transferrin, and ethanolamine being the most important components of the SFM (reviewed Merten and Litwin, 1991; Glassey *et al.*, 1988). It has also been possible to grow hybridomas in protein-free media (Schneider, 1989; Darfler, 1990; Zhou and Hu, 1995) with improved mAb secretion. Immobilisation of hybridoma cells using calcium alginate entrapment has been shown to increase mAb production in SFM (Lee and Palsson, 1990).

Bacterial and yeast expression systems have been traditionally used to produce a wide variety of recombinant proteins. However, post-translational modification especially glycosylation, is much more complex in animal cells compared to bacterial and yeast cells. Animal cells thus provide an ideal environment in which recombinant proteins can be properly transcribed and glycosylated. A wide variety of cell lines have been found to be suitable for transfection with various genes encoding particular therapeutic products including CHO-K1, BHK, MCDK, Vero and Namalwa. Various SFM have been used in the production of such therapeutics and viruses from these cells (reviewed Berg *et al.*, 1993).

BHK-21 cells have been adapted to suspension aggregate culture for the serum-free production of rabies vaccine in a perfusion-reactor system (Perrin *et al.*, 1995). A *ras*-amplified BHK-21 cell line can hyper-produce a human recombinant mAb in protein-free medium (Inoue *et al.*, 1996). Production of tPA has also been reported from BHK cells adapted to SFM (Wang *et al.*, 1995). HeLa cell have been cultured in protein-free medium for the production of a number of viruses including poliovirus 2 and 3 and herpes simplex virus 1 and 2 (Cinatl Jr. *et al.*, 1994). Namalwa cell adapted to growth in SFM have been used to produce β-interferon (Miyaji *et al.*, 1990a) and human lymphotoxin (Miyaji *et al.*, 1990b). A Vero cell line derived from the parental cells by SV40 transformation was found to be a useful host for protein production, such as human growth hormone (GH), in SFM (Ohno *et al.*, 1991).

Chinese hamster ovary cells (CHO) lacking the enzyme $dhfr^-$ are among the most frequently used recipient cells for stable transfection of genes encoding recombinant proteins (Kaufman, 1989; Zaworski and Gill, 1993). Often genetically engineered CHO cells are used for pharmaceutical or medical production of human glycoproteins because they can grow in suspension and can be adapted to grow in SFM which lightens down-stream processing and eliminates the potential for contaminating proteins with undefined FBS components. Recombinant CHO cell lines have been found to be capable of producing a wide range of products in SFM including human thyroid stimulating hormone (Cole *et al.*, 1993), human soluble thrombomodulin (Ogata *et al.*, 1993a, 1993b), monoclonal antibodies (Keen and Rapson, 1995), plasminogen activator and IgG kappa light chain (Zang *et al.*, 1995). Ito *et al.* (1996) successfully cultivated CHO cells over-expressing human insulin receptors on insulin immobilised to an artificial substrate in protein-free conditions.

Recent insights into cell cycle and cancer biology have allowed a clearer understanding of the basic mechanisms that control cell cycle progression. These can be manipulated to bypass growth factor and cytokine requirements for cultured cells when grown in serum- or protein- free media. It has been shown that transfection of CHO cells with a vector encoding cloned cyclin E resulted in the elimination of anchorage dependence and growth factor requirements in these cells (Renner *et al.*, 1995). Manipulation of transcription factors may also allow growth in serum-free or protein-free media. De-regulated E2F-1 transcription factor expression may have a predominant effect on cellular proliferation by bypassing the requirements for growth factor signalling. Lee *et al.* (1996) transfected CHO-K1 cells with an expression vector for E2F-1 with the resultant over-expression of the gene and growth in a protein-free medium.

### 1.1.4 SFM and differentiated cell types

The expression of specialised functions in cell culture *in vitro* is controlled by the composition of the surrounding microenvironment. The presence of various nutrients, growth factors or hormones (with inducer or repressor functions) and the composition of the extracellular matrix (ECM) all play key roles in the proliferation and maintenance of the differentiated phenotype of the specific cell type. The design of the cell culture environment which will determine the proliferative capacity and the expression of function of tissue-specific cells is dependent on the type of tissues that are the object of the study.

Cells that are derived from constantly regenerating tissue such as skin or bone marrow cells can be maintained in culture for sustained periods of time before the occurrence of terminal differentiation. The use of defined culture conditions allows the growth and differentiation of a cell type to be controlled. For example, Rahemtulla *et al.* (1989) reported on two SFM for the growth of normal human keratinocytes, where good growth was achieved in a SFM composed of 10ng/ml EGF and 0.3mM calcium, whereas differentiation was enhanced by a combination of low EGF (0.1ng/ml) and a high calcium (2mM) concentration. Haematopoiesis is a tightly regulated process whereby mature cells in the circulating blood are constantly re-generated. This is vital for the sustainence of life because of the relatively short life-span of these blood cells. The process is regulated by a cascade of haematopoietic growth factors especially the interleukins and CSFs, with many of these cytokines having multiple and overlapping functions. Many SFM have been described in the literature for the cultivation of progenitor and mature haematopoietic cells (Lebkowski *et al.* 1995; Sandstrom *et al.* 1994).

Epithelial cell types are often responsible for the recognised fuctions of an organ such as gas exchange in the lung. Many epithelial cell lines have been derived from liver, lung, pancreas, kidney, prostrate, mammary gland, *etc.* These can be maintained for months with limited cell divisions (Greunert *et al.*, 1990; McCallum and Lowther, 1996). B-estradiol (Peterson *et al.,* 1990), prolactin and progesterone (Ethier, 1986) are often required in defined culture for the maintenance of the differentiated phenotype in mammary cell lines. Normal human bronchial cells (Lechner *et al.*, 1982) and conducting airway cells (Robinson and Wu, 1991) which are important for research into the physiology of respiratory epithelia and the study of airway diseases have all been cultured in SFM. Type II pneumocytes have been successfully maintained in SFM (Borok *et al.,* 1994; Kumar *et al.*, 1995; Fraslon *et al.*, 1991). By using serum-free defined conditions, a potential *in vitro* model exists to study regulation of alveolar

epithelial cell function and differentiation. Serum has been found to accelerate loss of type II cell differentiated phenotype to type I cells and inhibit DNA synthesis in adult type II pneumocytes (Kumar *et al.*, 1995).

### 1.1.5 SFM and cancer

Tumour transformation is associated with a partial break-down of the normal regulatory systems governing cell proliferation and differentiation. As a consequence, malignant cells are often less dependent on external growth factors and may be refractory to differentiation signals. Autocrine stimulation of cellular proliferation may be an important element in the oncogenesis of various tumours including bladder carcinomas (Sporn and Roberts, 1985; Ruck *et al.*, 1994) and hepatomas (Bagnarelli and Clementi, 1987). FGF has been found to play a role in the autocrine growth of human oral squamous cell carcinoma (Myoken *et al.*, 1994). TGF-α overexpression causes autonomous cell proliferation and contributes to neoplasia in hepatocytes (Wu *et al.*, 1994) and in a gastric scirrhous carcinoma cell line (Yanagihara *et al.*, 1993). Transferrin has been found to be an autocrine growth factor secreted by a rat hepatoma cell line (Shapiro and Wagner, 1989). Addition of anti-transferrin antibodies resulted in a decrease in growth. A number of polypeptide growth factors have been identified in the conditioned medium (CM) of various cancer cell lines including EGF, TGF-α, TGF-β, PDGF, CSF, IGF and IL-2 (Goustin *et al.*, 1989).

A number of serum-free media have been described in the literature in the culture of lung cancer cells (Brower *et al.*, 1986; Gazdar, 1994; Collodi *et al.*, 1991). Over the last fifteen years many SCLC and NSCLC cell lines have been established by using serum-free, hormone and growth factor supplemented defined media, HITES and ACL-4 (Oie *et al.*, 1996). Masusda *et al.* (1991) successfully established twenty human NSCLC cell lines in ACL-4 SFM.

## 1.2 EXTRACELLULAR MATRIX

The interaction of normal cells with the extracellular matrix (ECM) components plays a crucial role in regulating many cellular processes such as anchorage, polarity, cytoskeletal organistation, state of differentiation and proliferation (Yamada, 1991). Mammalian organisms are divided into a series of tissue compartments separated by the ECM, which consists of the basement membrane (BM) and its underlying interstitial stroma (Yurchenco and Schittney, 1990).

The major components of the ECM include the cell adhesive or anti-adhesive molecules such as fibronectin, vitronectin, laminin and tenascin; the structural components such as collagens and elastin; and the proteoglycan family which is a complex array of proteins with glycosamino glycans side-chains. The ECM is an integral part of the machinery that regulates cell function, see Fig. 1.1. These processes are mediated by transmembrane receptors known as integrins, which will be discussed in greater detail in Section 1.4. Upon integrin ligation, changes in receptor triggered by ligand binding, in turn causes rearrangement of the cytoskeletal network and an intracellular cascade of signal transduction leading to changes in gene expression, and therefore the growth and differentiation status of the cells. The ECM can interact with hormones and growth factors to regulate growth and differentiation (Lin and Bissell, 1993; Lupetti *et al.*, 1996; Yao *et al.*, 1995; Nugent and Newman, 1989).

Under normal conditions, the structural integrity of the BM is largely maintained. However, alterations in its structure and signalling pathways could lead to the ECM playing a major role in carcinogenesis. During the progression from *in situ* to invasive cancer, the tumour cells gain the ability to invade the basement membrane, through to the interstitial stroma (reviewed Liotta *et al.*, 1991).

The major proteins of the extracellular matrix, fibronectin, collagen and laminin, will now be discussed in more detail.

### 1.2.1 Fibronectin

Fibronectin has been found to mediate cell adhesion, embryonic cell migration and wound healing (reviewed Hynes, 1990). It is encoded by one gene but exists in a number of variant forms that differ in sequence at three general regions of alternative splicing of its precursor mRNA. Fibronectin is produced by a wide variety of cell types, which secrete and organise themselves into extensive extracellular matrices. It is also found in blood as a soluble plasma glycoprotein. Fibronectin appears as a

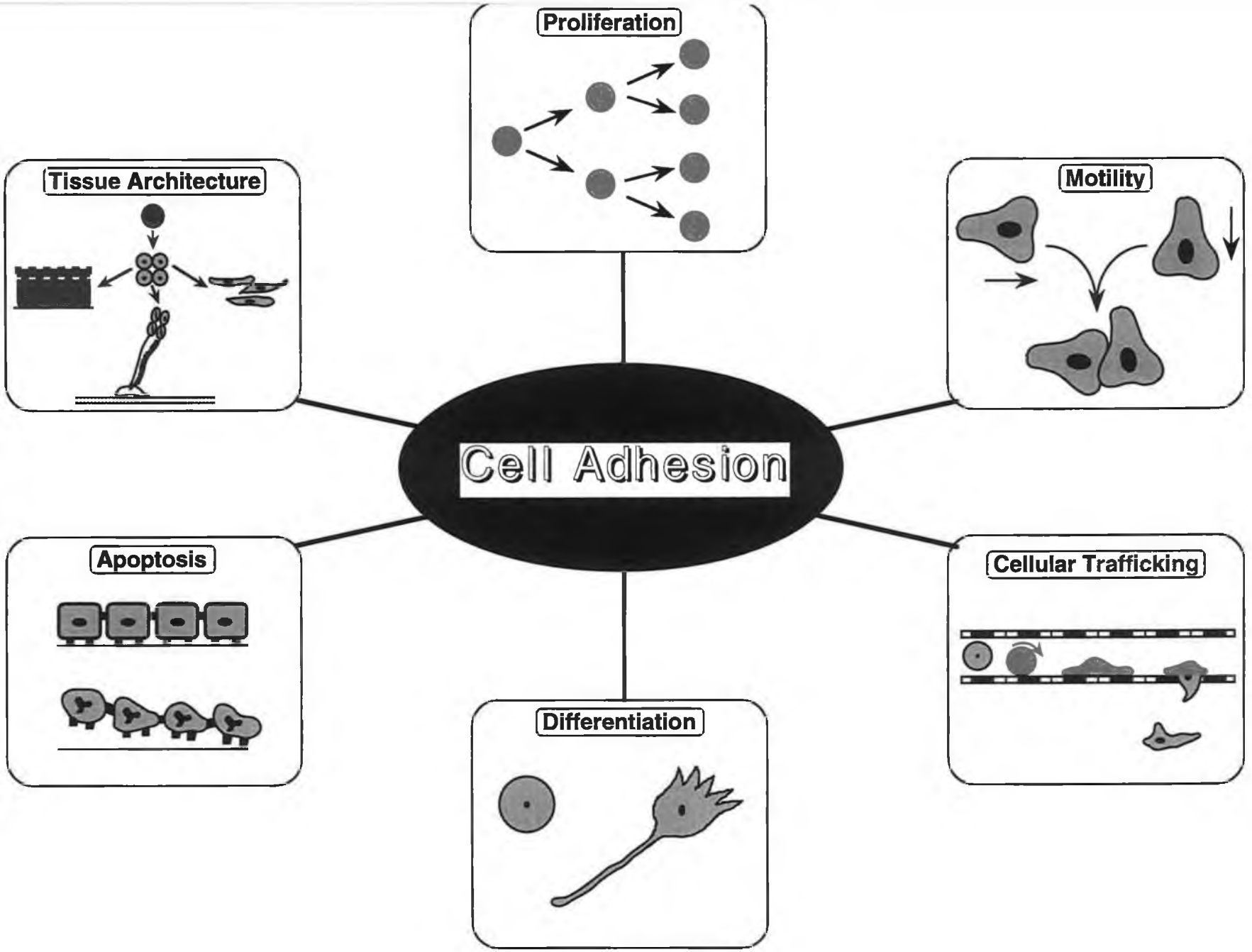


**FIG. 1.1** Role of cell adhesion in basic multicellular processes

multimeric glycoprotein consisting of a dimer of α and β chains of unequal length, linked together at the carboxyl end by disulphide bonds. Most of the cells adhere to fibronectin by binding to centrally located 'cell-binding' domain. The crucial sequence in this domain is the RGD (Arg-Gly-Asp) motif.

Fibronectin appears to have an important role in wound healing where it is found in large quantities associated with fibrin in the provisional matrix laid down during wound healing (Hynes, 1990). Keratinocytes then migrate over this fibronectin-rich matrix to initiate the healing process. It appears that fibronectin is capable of inhibiting terminal differentiation in human keratinocytes thus maintaining the cells in a continuous 'cycling' proliferative state enabling growth of new tissue (Adams and Watt, 1989).

Fibronectin and its integrin receptors appear to play a role in tumour progression (reviewed Akiyama *et al.*, 1995). Lupetti *et al.* (1996) found that interaction of metastatic melanoma cells with fibronectin can regulate cytokine gene expression with upregulation of IL-6 and IL-8. Interaction with fibronectin can upregulate GM-CSF expression in macrophages (Thorens *et al.*, 1987). Many cell lines can synthesise fibronectin so the need for fibronectin supplementation in serum-free medium varies from one cell type to another. Some cell lines apparently have lost their ability to produce fibronectin which may explain their frequent loss of anchorage dependence (Butler, 1986). Addition of fibronectin to culture medium can alter the phenotype of transformed cells towards a more normal morphology (Junker and Heino, 1987).

In serum-free medium, the requirement for fibronectin to promote proliferation or maintenance of the differentiated state of a cell has been clearly demonstrated in a number of cases. Orly and Sato (1979) have shown that to inoculate the rat ovarian follicular cell line, RF-1, directly into serum-free media, it was necessary to add fibronectin at a concentration of 8μg/ml to the culture medium. They found that in the absence of fibronectin, a high proportion of the cells became binucleated after the first cell cycle occurred. Fibronectin eliminates cell binucleation and considerably enhanced cell growth and thus facilitates cytokinesis of RF-1 cells so that their cell cycle is not disturbed and cell growth is enhanced. Grinnell (1976) has shown that fibronectin was found to promote cell attachment and spreading for a number of cell lines. Grinnell and Feld (1980) inhibited cellular synthesis of fibronectin in human fibroblasts by dithiothreitol. This adversely affected the initial spreading onto culture dish surfaces in serum-free medium. The addition of plasma fibronectin to these cells restored the ability of the cells to adhere to the substratum. Suzuki *et al.* (1989) found that their neonatal rat cardiac myocytes would not spread on plastic without serum being present. Fibronectin, at a concentration of 10μg/ml, significantly improved cell attachment,

spreading and maintenance of contractility of rat myocytes in serum-free medium. Rockwell *et al.* (1980) showed that fibronectin completely replaced serum preincubation of cells and serum precoating of tissue culture surfaces in allowing attachment, spreading and growth of SV40 transformed Balb/c-3T3 cells in serum-free medium. The proper attachment provided by fibronectin is an obligatory requirement for the growth of these cells.

### 1.2.2 Laminin

Laminin has been found to mediate cell adhesion, migration and differentiation (reviewed Timpl, 1989; Beck *et al.*, 1990). It is a large molecular weight glycoprotein which is found exclusively in the basement membrane. It consists of several different subunits linked together by disulphide bonds. The most extensively studied of these subunits are the 400kDa A chain, the 210kDa $B_1$ chain and the 200kDa $B_2$ chain which are found ubiquitously in BM. This and other forms of laminin appear to be asssembled from closely related genes encoding isoforms of certain chains rather than undergoing alternative splicing controlled by one gene in the case of fibronectin.

Laminin can interact with cells by a host of mechanisms including a variety of adhesive recognition sequences and receptors. Heparin-binding domains are present which are important for interaction with the large heparin sulphate proteoglycans present in the BM in large quantities (Ott *et al.*, 1982). Laminin can also interact with type IV collagen (Rao *et al.*, 1982). Complexes of laminin, heparin sulphate proteoglycans and other molecules especially type IV collagen provide structural organisation to the BM. Laminin contains many EGF-like repeats which may explain the role of laminin in promoting the growth of certain cells (Darmon, 1982).

Laminin appears to play an extremely important role in tumour progression and metastasis. Topley *et al.* (1993) found that by injecting a number of cell lines with Matrigel (reconstituted basement membrane) or purified laminin the growth of subcutaneous xenografts in nude mice can be improved. Down-regulation of the laminin binding integrins also appears to play a role in tumour progression and metastasis (reviewed Ziober *et al.*, 1996).

Laminin has been found to modify gene expression in a number of cancer cell lines as a result of cell-matrix interactions. The over-expression of oncogenic *ras* and *myc* in SCLC cells resulted in the transition towards a more NSCLC classification when cultured on laminin. *Myc*-overexpressing SCLC cells which normally grow as aggregates adhered to laminin and exhibited an epithelial morphology and began to

express markers of both SCLC and NSCLC (Barr *et al.*, 1996). Laminin has also been found to induce attachment of a number of SCLC cell lines with the formation of neurite-like processes in the cells (Giaccone *et al.*, 1992). Interaction with laminin may be important for the malignant behaviour of lung cancer cells (Fridman *et al.*, 1990), as SCLC cells generally have a higher metastatic potential than NSCLC in patients (Minna *et al.*, 1989).

### 1.2.3 Collagens

Collagens are the most abundant proteins found in the basement membrane. Collagens are glycoproteins present in five major forms (types I to V). The most widely distributed collagen chains are the $\alpha_1$ (IV) and $\alpha_2$ (IV) chains with a more restricted distribution of $\alpha_3$ (IV), $\alpha_4$ (IV), $\alpha_5$ (IV) and $\alpha_6$ (IV) (reviewed Yurchenco and O'Rear, 1994). Type IV collagen provides the scaffolding for laminin and the other components of the basement membrane. The basal lamina is composed of enmeshed collagen and laminin polymers in which nidogen (entactin) acts as a bridge between these molecules to provide anchorage for diverse matrix components and tissue integrity.

Type IV collagen has been found to be capable of binding epithelial carcinoma cells (Palm and Furcht, 1982). It has also been shown to provide extended proliferation for mammary epithelial cells growing in serum-free medium (Kidwell *et al.*, 1982). It can also be used in native or denatured form to form a gel which can act as a pre-coat for cells.

### 1.2.4 Other glycoproteins of the extracellular matrix

Vitronectin is found in human serum and in the ECM (reviewed Tomasini and Mosher, 1991). This protein was originally known as serum spreading factor. The main vitronectin receptors are the integrins, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_3$, and the attachment of cells to vitronectin is mediated through the 'RGD' binding motif. The protein contains heparin-binding sites and also binds plasminogen activator and stabilises its activity (Tomasini and Mosher, 1991). These functions may help regulate the localisation and activity of this important inhibitor of plasminogen activator which is implicated in a host of tissue remodelling events including implantation, cell migration and tumour cell invasion. Most of the cell adhesive activity of serum used for cell culture can be attributed to vitronectin.

Thrombospondin is a large glycoprotein released when platelets are activated and secreted continuously by a a variety of cell types (reviewed Mosher, 1990). It is

capable of mediating or inhibiting cell adhesion and regulating the growth of certain cells.

Tenascin (also known as cytotactin) is important in morphogenetic events including embryonic migration, wound healing and tumourigenesis. Increased levels are observed during wound healing (Mackie *et al.*, 1988) and in association with certain tumours (Hoffman *et al.*, 1990).

Nidogen (also known as entactin) is distributed ubiquitously in basement membrane where is binds tightly to laminin. This entactin-laminin complex can then bind tightly to collagen to maintain structural integrity of the basement membrane (Yamada, 1991).

## 1.3 CELLULAR ADHESION MOLECULES

Cell-cell and cell-matrix interactions are mediated to a large extent by families of adhesion molecules called cadherins and cellular adhesion molecules (CAMs) and also by cell surface receptors called integrins. Cadherins and CAMs (as part of the immunoglobulin superfamily of adhesion molecules) will be briefly discussed in this section. An extensive review of integrins will be presented in Section 1.4 because of their importance in normal cellular functioning and alterations in expression which may be associated with the pathogenesis of disease and the progression to a malignant phenotype.

### 1.3.1 Cadherins

Cadherins are calcium-dependent transmembrane cell-cell adhesion receptors. They bind cells by means of homophilic interactions and are mainly responsible for establishing and maintaining intercellular connections (reviewed Takeichi, 1991).

There at least twelve known members of the cadherin family which are subdivided into subclasses, sharing a common basic structure. The three main subclasses are well characterised at the molecular level, and are E-cadherin, found on many types of epithelial cells; P-cadherin, found in the placenta and epidermis; and N-cadherin, found on nerve, heart and lens cells (Takeichi, 1991; Buxton *et al.*, 1993). Cadherins connect cells to each other by selective binding to identical cadherin types (Nose *et al.*, 1990).

The presence of cadherins is important for the maintenance of epithelial and endothelial structure (Buck and Horwitz, 1987), and alterations in their expression may thus be expected to be associated with loss of normal cell-cell integrity. In tumour metastasis, the detachment of cells from the primary tumour is an initial step which is caused by a disruption of normal mutual cell connections (Van Roy and Mareel, 1992; Streit *et al.*, 1996). Malignant transformation appears to be associated with a supression in cadherin receptors (reviewed Birchmeier and Behrens, 1994).

Intercellular adhesion of epithelial tissues is mainly regulated by the E-cadherin molecule and its associated cytoplasmic protein α-catenin that forms a linkage to the cytoskeleton and regulates the function of E-cadherin. Down-regulation of the E-cadherin/ catenin complex has been implicated in oesophageal cancer (Kadowaki *et al.*, 1994), gastric cancer (Streit *et al.*, 1996) and colon cancer (Vermeulen *et al.*, 1995b). The tumour supressor function of this complex has been demonstrated in skin tumours (Cano *et al.*, 1996) and lung carcinomas (Bohm *et al.*, 1994). Retinoic acid has been

found to upregulate the function of the invasion suppressor complex in human MCF-7 breast cancer cells (Vermeulen *et al.*, 1995a). The E-cadherin/catenin complex also plays an important role in embryonic development (Ranscht, 1994).

Thus a lot of experimental evidence exists to support the hypothesis that loss of cadherin expression or function is associated with increased invasiveness in transformed epithelial cells.

### 1.3.2 Immunoglobulin Family

The immunoglobulin (Ig) superfamily comprises a wide variety of cell adhesion molecules (CAMs) that share common structural features of antibody molecules (Springer, 1990). They mediate function *via* calcium-independent mechanisms. The most important members of the family are neural cell adhesion molecule (NCAM) (Montefort *et al.*, 1993), intercellular adhesion molecule (ICAM) (Long, 1992) and vascular cell adhesion molecule (VCAM) (Rice *et al.*, 1988). Most members of the Ig family are invoved in cell-cell recognition. Other members of the Ig family include molecules that function in cellular immunity, CD4 and CD8, and molecules associated with tumours, *e.g.* carcinombryonic antigen (CEA).

ICAM-1 may have a potential role in lymphocyte-carcinoma cell interactions. It mediates cell-cell adhesion by interacting with its counter-receptors $\alpha_L\beta_2$ and $\alpha_M\beta_2$, the leucocyte integrins. Increased expression of ICAM-1 has been correlated with melanoma tumour progression and an increased risk of metastasis (Johnson *et al.*, 1989).

## 1.4 INTEGRINS

### 1.4.1 Introduction

Integrins are a large family of heterodimeric transmembrane glycoproteins that bind to components of the extracellular matrix and can bind to cell-cell adhesion molecules such as CAMs of the immunoglobulin family (reviewed Hynes, 1992; Newham and Humphries, 1996). Integrins consist of two protein chains; the α chain is known to have 15 variants while the β chain has 8 identified variants at present. They can combine into at least 22 different integrins (see Fig. 1.2). The α subunits are homologous to one another but not to the β subunits, which form their own homologous group. The extent of similarity at the amino acid sequence level within the α and β subunit groups is 40-50%. During the mid to late 1980s, it became obvious from sequence data that a major glycoprotein complex on platelet cell surfaces (at that time, known as IIb and IIa) and two groups of glycoprotein antigens on lymphoid and myeloid cells (one group contained LFA-1 and Mac-1 and a second set of antigens originally identified on T-lymphocytes termed VLA antigens) shared common structural features and considerable sequence homology (Hynes, 1987).

Integrins are the most ubiquitous and versatile adhesion receptors expressed by almost every cell type. In addition to mediating cell adhesion, integrins are now known to function as signalling receptors, participating in a diverse array of cellular events including spreading, migration, proliferation, differentiation, apoptosis, and gene expression. Epithelial cells, *in vivo*, perform a number of important functions that require unique interactions with the ECM. These include wound repair, modulation of inflammation and establishment of tissue polarity and morphogenesis. Integrins have been implicated in a number of diseases and in cancer (reviewed Pignatelli and Stamp, 1995; Varner and Cheresh, 1996; Horwitz, 1997; Hillis and MacLeod, 1996). Neoplastic change seems to be associated with alterations in cellular integrin expression. Integrins play a role in a number of cellular processes that impact on development of tumours, including the regulation of proliferation and apoptosis, cellular motility and invasion, cell surface localisation of metalloproteinases, and angiogenesis.

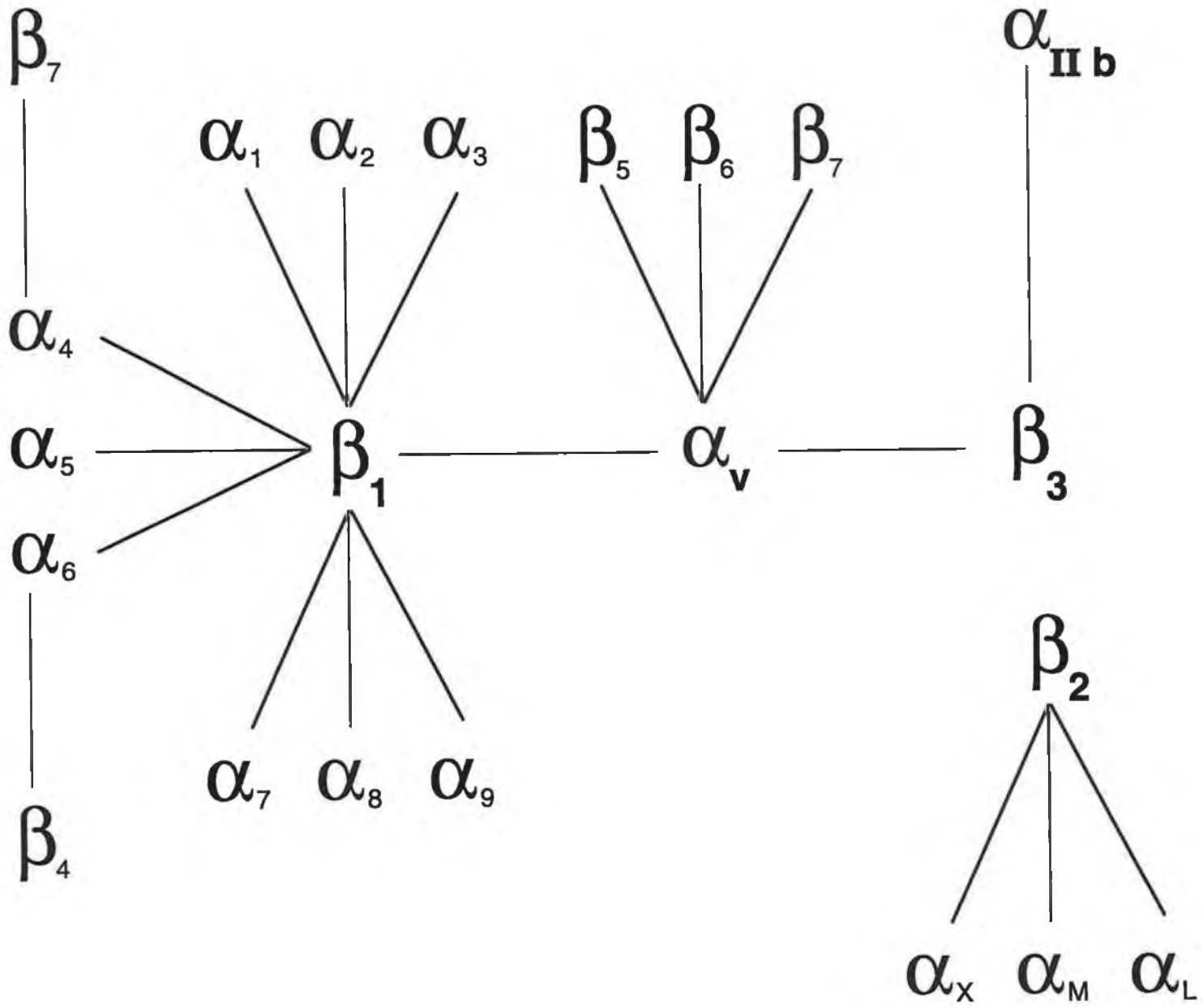


**FIG. 1.2** Integrin subunit association

### 1.4.2 Structure

The α subunits vary in size from 150 to 200kDa and are non-covalently associated with the β subunit which varies in size from 90 to 110kDa, with the exception of $\beta_4$ which is larger than 210kDa. The integrin receptor is a transmembrane glycoprotein with two large globular amino terminal extracellular domains that together make up an ellipsoidal head (N-domain). Each subunit provides a relatively thin leg which traverses the plasma membrane and ends in a relatively short cytoplasmic tail of less than 60 amino acids (C domain). The only known integrin which does not fit this category is the $\beta_4$ integrin which has a cytoplasmic domain of close to 1000 amino acids (Akiyama *et al.*, 1995).

The β subunits all show considerable similarity at the amino acid level (Hemler, 1990). They all contain 56 conserved cysteine residues except for $\beta_4$ which has 48. These cysteine residues are arranged in 4 repeating patterns which are thought to be linked internally by disulphide bonds. The α subunits exhibit a lower degree of similarity than the β chains. The N-terminal region of all α subunits contain a sevenfold tandem repeat of a homologous module composed of approximately 60 amino acids. The final 3 or 4 of these repeats show sequence homology to functional subunits known as EF-hands, found in $Ca^{++}$ binding proteins such as calmodulin. Seven α subunits including $\alpha_1$ and $\alpha_2$ chains of $\beta_1$ integrins plus the $\alpha_L$, $\alpha_M$ and $\alpha_X$ chains of $\beta_2$ integrins possess a 200-residue insert between the second and third repeats which shares sequence homology with the A domain of von Willebrand factor and a number of ECM components. This may contribute to the binding function of these integrins (Hynes, 1992; Hemler, 1990).

Several divalent cations regulate integrin-ligand binding including $Ca^{++}$, $Mg^{++}$ and $Mn^{++}$. The activity varies with the integrin. Integrins possess multiple cation binding sites and it is not clear whether occupancy of one or all of these sites is required for activation or ligand binding (Hillis and MacLeod, 1996). A characteristic feature of integrins is that they can exist in active or inactive states; they may be present at the cell surface but they are not necessarily active and capable of ligand binding. The transition from the inactive to active state appears to be accompanied by conformational changes and is regulated by divalent cation occupancy (Hillis and MacLeod, 1996). An understanding of the structural basis of integrin activation will be a major goal because it appears to be a critical step in initiating transmembrane signal transduction.

The α and β subunits could in theory associate to give more than 100 integrins. However, the actual diversity is much more restricted. The $\beta_1$ integrins primarily affect adhesion between cells and the ECM (Buck and Horwitz, 1987), the exception being $\alpha_4$

which mediates cell-cell adhesion via VCAM-1 (Elices *et al.*, 1990). The majority of $\beta_1$ integrins are receptors for ECM proteins such as collagens ($\alpha_1$, $\alpha_2$, $\alpha_3$), fibronectin ($\alpha_3$, $\alpha_4$, $\alpha_5$) or laminin ($\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_6$). These integrins are also known as very late antigens (VLA) because they were originally identified as a series of cell membrane antigens expressed by T-lymphocytes after long-term stimulation with phytohaemagglutin (Hemler *et al.*, 1987). The $\beta_2$ integrins are composed of three closely related molecules which are also known as leucocyte integrins because their expression is limited to leucocytes (Springer, 1990). They participate in cell-cell interactions (Hynes, 1987). The $\beta_3$ integrins consist of two members at the moment. The $\alpha_{IIb}\beta_3$ integrin is involved in platelet aggregation and has affinity for fibronectin, fibrinogen and von Willebrand factor (Ferrell and Martin, 1989). The $\alpha_v\beta_3$ receptor binds to many adhesion molecules including vitronectin and thrombospondin and appears to have an important role in angiogenesis (Brooks *et al.*, 1994). The binding specificity of a particular integrin may vary according to cell type which further adds to their complexity. $\alpha_2\beta_1$ is a receptor for collagen on platelets but is a collagen and laminin receptor on endothelial cells (Kirchofer *et al.*, 1990).

Integrins hold a cell in place by attaching at one end of the molecules of the ECM (or molecules on other cells) and at the other end to the cellular cytoskeleton. Binding is not very tight, but many relatively weak attachments form a stronger one. The most extensively studied site in the integrin ligands is the RGD sequence which refers to arginine, glycine and aspartate. This sequence is present in fibronectin, fibrinogen, thrombospondin, vitronectin, vWF, laminin and collagen type I (Rouslahti and Pierschbacher, 1987; Humphries, 1990)

Integrin ligands appear to use very short (3 or 4 amino acids) sequences as recognition motifs for receptor binding. An additional binding site in fibronectin and in some cell-surface Ig superfamily ligands have identified a second common motif 'LDVP' (Humphries, 1990). This binds to $\alpha_4\beta$ integrin. The 'DGEA' site in collagen type I was also recognised (Staatz *et al.,* 1991) among others (reviewed in Newham and Humphries, 1996; Danen *et al.*, 1996). All these sequences have been shown to possess a degree of cross-reactivity in their binding to a variety of integrins. However the exact mechanisms by which integrins bind different ligands are not yet fully understood.

### 1.4.3 Integrin Signalling

Integrins function as signalling receptors to direct cell adhesion and regulate other aspects of cell behaviour, including cell proliferation and differentiation, and to determine cell survival and cell cycle events (including apoptosis). The biochemical pathways initiated by integrin-ligand interactions are now being extensively elucidated. Integrins can mediate the bidirectional transfer of information from the outside to the inside of the cell, and also from the inside to the outside of the cell, which has been termed "outside-in" and "inside-out" signalling respectively. The pathways involved in integrin signalling have been extensively reviewed in the last few years (LaFlamme and Auer, 1996; Dedhar and Hannigan, 1996; Parsons, 1996; Yamada and Miyamoto, 1995; Dedhar, 1995; Garratt and Humphries, 1995).

Although the cytoplasmic domains of both integrin subunits are required for binding, the β chain appears particularly important. Phosphorylation of this subunit may be a means by which the function of this integrin is regulated (Hibbs *et al.*, 1991). $\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$, $\beta_5$ and $\beta_6$ chains all possess potential sites for phosphorylation due to the presence of tyrosine residues. Thus, the β subunit is primarily responsible for integrin clustering to focal adhesions (LaFlamme *et al.*, 1992).

When integrins become complexed with a ligand they redistribute into clusters. This serves to increase avidity or strength of interaction between cell and ligand, and also sequesters integrin cytoplasmic domains into focal adhesions. These are sites of interaction between the ECM and signalling molecules. Integrins co-localise with a complex organisation of cytoskeletal components that serves both to link the cytoskeleton to the focal adhesions and as a site for the congregation of secondary messenger mediators, linker/adapter proteins, kinases and phosphatases.

#### 1.4.3.1 "Outside-in" Signalling

Occupation of integrins at focal adhesions results in tyrosine phosphorylation of focal adhesion kinase (FAK) and regulates actin cytoskeleton reorganisation. $p21^{rho}$ is a member of the Ras superfamily of GTPases which regulates actin cytoskeleton rearrangement and assembly of focal adhesion plaques in response to growth factors and ligand binding to the ECM (Ridley and Hall, 1992). Assembly of focal adhesions requires the direct binding of the cytoplasmic domains of the β integrin subunit with the focal adhesion proteins, α-actinin, talin and FAK. FAK can bind to paxillin which is tyrosine phosphorylated in an adhesion-dependent fashion (Parsons, 1996). FAK has

binding sites for the SH2 domain of Src and for phosphatidylinositol-3-kinase (Clark and Brugge, 1995).

The phosphorylation of FAK is believed to initiate a cascade of phosphorylation events and new protein interactions required for adhesion and formation of adhesion-dependent signalling complexes. The adhesion-dependent activation of MAP kinases appear to be important in the pathway by which integrins regulate cell proliferation. MAP-K can then be activated by Ras which can in turn activate a number of transcription factors including *c-myc* that can regulate growth and differentiation (Davis, 1993).

Another early event triggered by integrins is the activation of lipid secondary messenger pathways. Activation of tyrosine kinase dependent phospholipase C-γ (PLC-γ) has been demonstrated which in turn hydrolyses phosphatidylinositol ($PIP_2$) to inositol triphosphate and diacylglycerol which are secondary messengers involved in the control of intracellular $Ca^{2+}$ and the activation of protein kinase C (PKC) (Kanner *et al.*, 1993; Daniel *et al.*, 1992). $PIP_2$ can also regulate other cell processes, such as modulating the function of several actin-binding proteins of the cytoskeleton.

Thus, regulation of adhesion is clearly a complex process under the control of several regulatory pathways. The $\beta_2$ integrin cytoplasmic domains have been found to contain all the information required for its localisation to focal adhesion plaques and for the initiation of many of the integrin-mediated signalling events. By defining the pathways that are altered by integrin occupancy, it is conceivable that specific targets will be identified for therapeutic intervention.

### 1.4.3.2 "Inside-out" signalling

This refers to changes in the activation state of integrins *via* intracellular signalling. The ability of cells to regulate their adhesiveness is critical in many situations. Modulation of the ligand-binding activity of integrins plays a role in these responses and is critically dependent on the cytosolic environment with which the tails of the integrins are associated. There appear to be regions of the cytoplasmic tails which may be crucial for the mediation of this "inside-out" signalling. A highly conserved "GFFKR" motif exists in many α subunits (O'Toole *et al.*, 1994; Kassner *et al.*, 1994).

The integrin $\alpha_6\beta_4$ is part of an epithelial adhesive structure that is distinct from focal adhesions - the hemi-desmosome complex. The extremely long cytoplasmic tail part of the $\beta_4$ chain can reach to the actin cytoskeleton directly.

### 1.4.4 Role of Integrins

Ligation of integrins by their ECM protein ligands induces a cascade of intracellular signals, including an increase in intracellular pH and intracellular $Ca^{++}$ levels (Schwartz, 1992), tyrosine phosphorylation of pp125 FAK (Kornberg *et al.*, 1993), activation of p34/cdc2 (Symington, 1993) and cyclin A (Guadagno *et al.*, 1993), synthesis of protein kinase C (Vuori and Rouslahti, 1993), activation of MAPK (Morino *et al.*, 1995; Schlaepfer *et al.*, 1994; Chen *et al.*, 1994a), p21 ras (Kapron-Bras *et al.*, 1993), and transcription factors such as NF-KB (Yebra *et al.*, 1995).

As well as signal transduction, integrins have many roles in mediating the expression of genes involved in proliferation, apoptosis, differentiation, invasion and metastasis.

#### 1.4.4.1 Role of integrins in proliferation

Most adherent cells are incapable of proliferating without signals from the ECM, most of them being transmitted *via* integrins. Integrin signalling has been found to regulate cell-cycle progression. In fibroblasts, the requirements for adhesion maps to the $G_0/G_1$ transition and to mid-to-late $G_1$ before the initiation of DNA synthesis (Guadagno *et al.*, 1993). Integrin-mediated signalling activates expression of MAP kinases, which have been implicated in cellular proliferation (Davis, 1993), as discussed previously. Cyclin A expression, which is required for entry into S phase of the cell cycle, is regulated by adhesion (Guadagno *et al.*, 1993).

There may be a possible tumour supressor role associated with the $\alpha_5$ integrin. Fibronectin matrix assembled by this integrin is primarily responsible for the supression of malignant properties of cells expressing elevated levels of this integrin (Rouslahti, 1996; Varner *et al.*, 1995; Symington, 1990; Giancotti and Rouslahti, 1990).

Since the intracellular domain of the β integrin sub-unit signals FAK phosphorylation, it is thus likely that integrins play an important role in regulating cell proliferation. The $\beta_1$ cytoplasmic domain has been shown to cause a stimulation in cell proliferation (Pasqualini and Hemler, 1994). A spliced $\beta_1$ subunit (β1C) has been shown recently to be growth inhibitory, preventing cyclin A expression and DNA synthesis (Meredith *et al.*, 1995).

### 1.4.4.2 Role of integrins in apoptosis

Programmed cell death or apoptosis is a normal part of development, homeostasis, and the pathogenesis of some diseases (Steller, 1995). Integrin ligation has been shown to regulate the expression of bcl-2, a key regulatory component in the supression of apoptosis (Zhang *et al.*, 1995). Expression of the $\beta_4$ cytoplasmic domain in cells has been shown to activate p21 and induce growth arrest (Clarke *et al.*, 1995).

There is evidence that integrin occupancy can trigger signals that inhibit apoptosis. This inhibition of apoptosis by the ECM is dependent upon the expression and function of particular integrin heterodimers, and these requirements appear to be cell type specific. Inhibition of $\alpha_v\beta_3$ function during angiogenesis with an anti-$\alpha_v\beta_3$ antibody both inhibited proliferation and induced apoptosis whereas treatment with anti-$\beta_1$ antibodies did not (Brooks *et al.*, 1994). Howlett *et al.* (1995) reported that interruption of normal mammary morphogenesis by anti-integrin antibodies was associated with an inhibition of cell growth and induction of apoptosis. Engagement of specific $\beta_1$ integrins prevented apoptosis in mammary epithelial cells by inhibiting the expression of interleukin 1β-converting enzyme (ICE), a known inducer of apoptosis in mammalian cells (Boudreau *et al.*, 1995).

### 1.4.4.3 Role of integrins in tissue morphogenesis and differentiation

Epithelial branching morphogenesis is a process by which a continuous epithelium, embedded in mesenchyme, forms tubules and branches into surrounding mesenchyme. This is responsible for the architecture of many organs, including the lung. Integrin receptors may mediate the interaction between the ECM molecules and the epithelium to allow normal branching to occur (reviewed by Gumbiner, 1996). There appear to be major changes in the expression of ECM molecules, such as laminins and integrins (during morphogenesis of the lung), which may be important for normal development (Virtanen *et al.*, 1996).

The $\alpha_1$ integrin has been found to be expressed on developing smooth muscle and endothelium. The $\alpha_3$ and $\alpha_6$ integrins are expressed pericellularly in young epithelia, while as maturation progresses, these subunits become basally restricted. These integrins thus have roles in the maturation and polarisation of the epithelium (Wu and Santoro, 1996). The $\alpha_2$ integrin is expressed at lung branch tips and may have a role in the formation of the basement membrane at growing tips or the extension of epithelia into surrounding mesenchyme (Wu and Santoro, 1996; Virtanen *et al.*, 1996). *In vitro*, $\alpha_2\beta_1$ integrin has been found to play an important role in epithelial branching

morphogenesis in mammary epithelial cells (Keely *et al.*, 1995; Zutter *et al.*, 1995; Howlett *et al.*, 1995) and kidney epithelial cells (Saelman *et al.*, 1995).

Integrins must be functional for development to proceed normally. As the cells of an embryo proliferate and differentiate to form tissues of the body, they add and subtract integrins from their surface. Cells of the embryo also require integrins to help them travel to their final destination. "Knocked-out" integrins often result in deformation or death during development (Horwitz, 1997).

Adhesion molecules generally vary with the epidermal stratification and differentiation states. Integrins appear to be extremely important in the regulation of events leading to terminal differentiation of keratinocytes (reviewed by Jensen and Wheeloch, 1996). Basal keratinocytes of the skin contain three populations based on proliferation capacity; stem cells, transit-amplifying cells and committed cells. Stem cells can be identified and sorted *in vitro* based on higher expression of $\beta_1$ integrins (Jones and Watt, 1993; Jones *et al.*, 1995). In committed cells, integrin function appears to be inhibited and they are lost from the cell surface. This allows keratinocytes to be free to move upwards through the epidermis from the basement membrane. There appears to be *in vitro* evidence that inhibition of integrin synthesis occurs only after the basal keratinocyte has begun to differentiate further and detach fom the ECM. Expression of integrin receptors, especially $\alpha_2$, $\alpha_3$ and $\alpha_5$, is down-regulated by forced suspension culture of keratinocytes (Adams and Watt, 1989) and may represent detachment from the ECM *in vitro*.

Symington and Carter (1995) have provided evidence that $\alpha_3\beta_1$ mediated adhesion to epiligrin (epidermal BM component) may be capable of regulating keratinocyte differentiation. They found that using anti-$\alpha_3\beta_1$ antibodies can inhibit keratinocyte adhesion to epiligrin and induce involucrin synthesis, a marker of terminal differentiation in keratinocytes.

The role of integrins in expression of markers of differentiation has been most extensively studied in mammary epithelial cells. When mammary cells are grown on plastic dishes, they cannot be induced to produce milk proteins. However in 3D culture, $\beta_1$ interaction with laminin results in activation of β-casein gene expression with resultant milk production (Streuli *et al.*, 1991). An ECM response element has recently been identified in the β-caesin promoter (Schmidhauser *et al.*, 1992).

### 1.4.4.4 Integrins in Wound Healing, Infection and Inflammation

The effects of wounding on the local expression of integrins and their ligands has been most extensively studied in squamous epithelia of the skin. Cutaneous wounds contain a provisional matrix, rich in fibronectin, osteopontin and tenascin. Keratinocytes at the wound edge have been found to increase expression of several integrins, such as $\alpha_5\beta_1$, $\alpha_v\beta_6$ and $\alpha_v\beta_5$ (Sheppard, 1996).

Integrins have been found to be very important in processes that involve cell adhesion and motility, such as extravasation of circulating leucocytes into infected or damaged tissues, which results in inflammation (reviewed by Horwitz, 1997). At the site of infection, white blood cells of the immune system are plucked from the blood by endothelial cells. Migration of these leucocytes initially involves selectins, which causes the white blood cells to slow down and roll along the endothelial lining of the vessel wall. Inside-out signalling causes $\alpha_4\beta_1$ and $\beta_2$ subunit integrins of the leucocytes to gain affinity for molecules of the immunoglobulin family - ICAM's - on the endothelial cells. These attachments help the leucocytes to stop, squeeze between endothelial cells thus allowing them to cross the blood vessel wall into the damaged or infected tissue.

### 1.4.5 Regulation of Integrin Expression

The up- and down- regulation of certain genes in various cancers appears to be manifested at the transcriptional level. However, mechanisms controlling integrin gene expression are quite poorly understood. A number of growth factors and cytokines have been found to regulate the expression of integrins (see Table 1.4.1 and Table 1.4.2).

TGF-$\alpha$ has been shown to upregulate expression of $\alpha_2$ and $\alpha_3$ integrins, while downregulating $\alpha_v$ and $\alpha_6$ integrins (Lin *et al.*, 1994). If tumour cells lose their ability to bind ECM proteins, growth factors such as TGF-$\alpha$ and FGF may exert growth stimulatory activities, due to loss of the normal regulatory control, mediated by ECM proteins, therefore inappropriate expression of growth factors seen in malignant tissue whose cell-matrix adhesion receptors are altered, may have deleterious effects on the maintenance of normal tissue architecture and growth (Pignatelli and Stamp, 1996).

Many tumours are heterogenous populations with ECM heterogeneity within these tumours, which results in differences in integrin expression. Growth factors and cytokines also vary with tumour type and may directly or indirectly affect integrin expression. TGF-$\beta$ has also been found to upregulate certain integrins and

downregulate others. These changes promote synthesis of ECM, causing changes in integrin expression and hence influence gene expression (Heino and Massague, 1989). Integrin receptors on melanoma cells are subject to cytokine mediated modulation and type of response to each cytokine depends on the integrin considered.

Regulation of cytokine effects by specific integrins has also been recently demonstrated for the angiogenic effects of bFGF, TGF-α and VEGF. Friedlander *et al.* (1995) demonstrated that *in vivo* angiogenesis induced by bFGF was dependent on $\alpha_v\beta_3$ integrin, whereas that induced by VEGF or TGF-α depended on $\alpha_v\beta_5$ integrin.

Adhesion of cancer cells to the vascular epithelium is an important step in the hematogenous metastasis of cancer. Heparin-binding EGF has been found to increase the expression of $\alpha_2$ and $\alpha_3$ integrins in human breast cancer cells (Narita *et al.*, 1996) and oesophageal cancer cells (Sato *et al.*, 1996) already expressing EGF-R. The interpretation from the findings of these groups is that cancer cells receive signals from the cytokines such as HB-EGF, produced by endothelial cells following initial adhesion of cancer cells *via* selectins. This causes a secondary increase in the expression of cell adhesion molecules and leads to augmentation in the adhesive activities of cancer cells at the vessel walls.

EGF-R has also been implicated in the upregulation of expression of the $\alpha_2\beta_1$ integrin and enhance cell adhesion and migration on type I collagen (Fujii, 1996). Signals down-stream of EGF-R can modulate integrin-mediated adhesion to ECM proteins in both an inhibitory and stimulatory manner in mammary carcinoma cells (Genersch *et al.*, 1996). EGF-R encodes the c-erbB proto-oncogene which has also been implicated in cancer metastasis as well as cellular proliferation.

ECM and growth factors are important in morphogenesis and wound repair. In an *in vitro* model designed to mimic wound healing, Xu and Clark (1996) cultured normal human dermal skin fibroblasts in relaxed collagen gels and fibronectin-rich cultures or stressed fibrin gels, and stressed collagen gels. Integrin subunit mRNA levels were measured before and after stimulation with PDGF-BB, a potent mitogen and chemoattractant for fibroblasts. The fibronectin-rich cultures and fibrin gel induced $\alpha_3$ and $\alpha_5$ mRNA expression. The stressed and relaxed collagen gels attenuated these responses while promoting maximal integrin $\alpha_2$ expression. ECM alterations during wound healing or any new tissue formation causes cells to respond differently to repeated growth factor stimuli. During cutaneous wound repair *in vivo*, the provisional matrix of fibronectin, fibrinogen, fibrin and vitronectin forms in the wound area (Gailit and Clark, 1994). In the fibrin network, platelets release PDGF that stimulates proliferation and chemoattraction of fibroblasts (Deuel *et al.*, 1991). Activated

fibroblasts must move from surrounding collagenous connective tissue into a fibrin/ fibronectin- filled wound and subsequently synthesise new collagenous matrix. Thus fibroblast response to these ECM molecules and growth factors is essential for the healing process (Xu and Clark, 1996).

TGF-β has been found to increase $\alpha_6$ and $\beta_1$ mRNA expression in alveolar epithelial cells in a dose-dependant manner. It has also been found to increase the levels of the laminin $\beta_1$ chain mRNA (Kumar *et al.*, 1995). The cytokine thus appears to be important in regulating embryonic formation and tissue morphogenesis and remodelling (Sporn and Roberts, 1990; Border and Rouslahti, 1992).

**Table 1.4.1 Upregulation of integrin expression by cytokines**

| *Cytokine* | *Integrin* | *Cell type* | *Reference* |
|---|---|---|---|
| FGF | $\alpha_1$ | rat PC12 cells | Zhang *et al.* (1993) |
| NGF | $\alpha_1$ | rat PC12 cells | Zhang *et al.* (1993) |
| EGF-R | $\alpha_2$ | cutaneous squamous carcoma | Fujii (1996) |
| IFN-$\gamma$ | $\alpha_1\beta_1$ | monocytes | Rubio *et al.* (1995) |
| TGF-$\beta$ | $\beta_1$ | osteocarcoma | Cervella *et al.* (1993) |
| TNF-$\alpha$ | $\alpha_1,\alpha_2,\alpha_5$ | melanoma | Mortarini *et al.* (1991) |
| | $\alpha_2$ | bladder cancer | Nista *et al.* (1996) |
| IL-1$\beta$ | $\alpha_1,\alpha_2,\alpha_5$ | melanoma | Mortarini *et al.* (1991) |
| TGF-$\beta_1$ | $\beta_1\alpha_3$ | melanoma | Mortarini *et al.* (1991) |
| | $\alpha_1,\alpha_2,\alpha_3,\alpha_5,\beta_1$ | WI-38 fibroblasts | Heino *et al.* (1989) |
| | LFA-1 | monocyte leukemia | Ignotz *et al.* (1985) |

**Table 1.4.2 Downregulation of integrin expression by cytokines**

| *Cytokine* | *Integrin* | *Cell type* | *Reference* |
|---|---|---|---|
| IFN-$\gamma$ | $\alpha_2,\alpha_6,\beta_1$ | lung cancer | Van Valen *et al.* (1996) |
| TNF-$\alpha$ | $\alpha_2,\alpha_6,\beta_1$ | lung cancer | Van Valen *et al.* (1996) |
| | $\alpha_6$ | endothelial cells | Delfilippi *et al.* (1994) |
| IL-1$\beta$ | $\alpha_4$ | melanoma | Mortarini *et al.* (1991) |
| IFN-$\alpha$ | $\alpha_4$ | melanoma | Mortarini *et al.* (1991) |

### 1.4.6 Molecular Biology of integrins

Many of the α and β integrin subunits have been sequenced and many cDNA clones are available. Ignatius *et al.* (1990) reported on the rat $\alpha_1$ clone which is homologous to the human form of the $\alpha_1$ subunit. The human $\alpha_1$ structure has since been elucidated (Briesewitz *et al.*, 1993). Takada and Hemler (1989) cloned cDNA for the $\alpha_2$ integrin gene. The rat $\alpha_1$ and human VLA-2 $\alpha_2$ subunit have been found to share greater than 50% sequence homology that may underline some of their similar ligand properties. The promoter regions of a number of integrin α subunits have been described and characterised (Birkenmeier *et al.*, 1991; Rosen *et al.*, 1991; Shelly and Arnaout, 1991; Uzan *et al.*, 1991). A common feature demonstrated by these promoters is the lack of TATA and CCAAT boxes and high G+C content, with the exception of the $\alpha_4$ integrin promoter (Rosen *et al.*, 1991). The promoter region of the $\alpha_5$ integrin also lacks these TATA and CCAAT boxes (Birkenmeier *et al.*, 1991).

The promoter region of $\alpha_2$ integrin seems to be the most characterised and was found to lack TATA and CCAAT boxes, but contains an abbreviated initiator sequence and 6 Sp1 binding sites. Consensus binding sequences for AP-1 and AP-2 complexes, a Pu-1 box, a GATA, and 2 palindromic motifs with potential to bind the oestrogen receptor are also present (Zutter *et al.*, 1994). Characterisation of the $\alpha_2$ promoter suggests a complex pattern of positive and negative regulatory elements which determine cell type and differentiation-specific expression (Zutter *et al.*, 1994).

The regulatory region of the human $\beta_1$ gene consists of two promoters tandemly located which appear to be independently regulated. The two promoters drive the expression of the unique $\beta_1$-gene resulting in the synthesis of at least two mRNAs, that diverge only in the complete 5'-untranslated region and share the same coding sequence, as is indicated by the isolation of two cDNAs with this structure. The longer mRNA transcript is ubiquitously present in human tissues and is 20-fold more abundant than the shorter transcript, which is expressed only in a subset of tissues (Cervella *et al.*, 1993). All the characterised integrin gene promoters drive the expression of reporter genes in a tissue-specific manner, which corresponds to the tissue-specific expression pattern of the respective genes (Ziober *et al.*, 1996).

### 1.4.7 Integrins and Disease

There have been many good recent reviews on the role of integrins in various diseases (Horwitz, 1997; Hills and MacLeod, 1996). Integrins have been associated with inflammation as already discussed. The expression of $\alpha_4\beta_1$ on immune cells is associated with chronic inflammation such as autoimmune disease, multiple sclerosis, rheumatoid arthritis and asthma. The $\beta_2$ integrins, $\alpha_M\beta_2$ (Mac-1) and $\alpha_L\beta_L$ (LFA-1), are the main integrins on neutrophils. These cells are usually the first at the site of injury and infection and are responsible for many of the symptoms of acute inflammation. Compounds have been targetted against $\alpha_4\beta_1$ and $\beta_2$ integrins to interfere with integrin-mediated adhesion of white blood cells to endothelial cells (Featherstone, 1996). Monoclonal antibodies against LFA-1 and Mac-1 ($\beta_2$ integrins) have been successfully used to treat pulmonary fibrosis (Piguet *et al.*, 1993). Antibodies against $\alpha_4\beta_1$ integrin have been used to treat airway inflammation such as asthma (Albelda, 1993a).

A disease known as leucocyte adhesion deficiency (LAD) results in individuals lacking $\beta_2$ integrin or producing a defective version of this integrin. These people suffer repeated life-threatening infections, as leucocytes cannot migrate to the sites of injury or trauma. People who lack $\alpha_{IIb}\beta_3$ integrin which is involved in platelet aggregation suffer from a disease known as Glenzmann's thrombasthemia. Platelets are incapable of aggregating properly causing excessive bleeding. On the other hand, excessive thrombus formation as a result of too much $\alpha_{IIb}\beta_3$ integrin production can lead to heart attacks or strokes if it obstructs a blood vessel. The first product of integrin research (ReoPro - Centocor, Leiden, The Netherlands) is an antibody against this integrin, to be used as a potent anti-thrombotic agent in patients with cardiovascular complications (Newham and Humphries, 1996).

Integrins have been implicated in other diseases such as osteoporosis (Horton and Roden, 1995), where overactivity of cells such as osteoclasts, which bind to and degrade bone, is a result of overexpression of $\alpha_v\beta_3$ integrin. In skin diseases such as psoriasis, normal skin integrins such as $\beta_1$ and $\beta_4$ are normally confined to the basal layer whereas in psoriatic lesions, both these integrins are found in suprabasal layers of lesional skin (Gailit and Clark, 1993; Andrew *et al.*, 1992). Some microbes apparently enter cells at least in part by latching onto integrins, including influenza virus, meningitis and diarrheoa.

### 1.4.8 Integrins and cancer

Integrins are required for tissue morphogenesis and disruption of expression or function may lead to loss of differentiation of malignant cells and their ability to invade normal tissue (Albelda, 1993b; Pignatelli and Vessey, 1994). It appears that there is no single integrin expression profile that correlates with malignancy. Rather, for different types of cancer, different stages in integrin expression seem to be associated with transformation and tumour progression (Pignatelli and Stamp, 1995). However, there appears to be a trend for the down-regulation of $\beta_1$ integrins in epithelial carcinomas (reviewed Ziober *et al.*, 1996).

The $\alpha_2\beta_1$ integrin is consistently lost or decreased in moderately and poorly differentiated colorectal tumours as described in Table 1.4.3. The potential tumour supressor function of this integrin has recently been shown in breast cancer where antisense mRNA reduction of $\alpha_2\beta_1$ integrin induced a transformed phenotype (Keely *et al.*, 1995). Zutter *et al.* (1995) transfected this integrin into a poorly-differentiated mammary carcinoma that lacked $\alpha_2$ integrin expression, with a dramatic change in cellular phenotype. The cells changed from a fibroblastoid, spindle-shaped, not-contact inhibited, motile and invasive cell to an epitheloid, polygonal-shaped, contact-inhibited, less motile and invasive cell. Re-expression of the $\alpha_2\beta_1$ integrin was found to restore the ability of the cells to differentiate into gland-like structures in 3D matrices (reconstituted Matrigel) and markedly reduced the *in vivo* tumourigenicity of the cells.

**Table 1.4.3 Loss of $\alpha_2$ integrin in poorly differentiated carcinomas**

| *Tumour type* | *Reference* |
|---|---|
| Colorectal | Koukoulis *et al.* (1993)<br>Nigam *et al.* (1993)<br>Pignatelli *et al.* (1990)<br>Koretz *et al.* (1991) |
| Oral carcinoma | Kosmehl *et al.* (1995) |
| NSCLC | Damjanovich *et al.* (1992) |
| SCLC | Pellegrini *et al.* (1994) |
| Breast cancer | Zutter *et al.* (1995)<br>Koukoulis *et al.* (1991)<br>Pignatelli *et al.* (1991) |

In some cancers, high levels of the $\alpha_2\beta_1$ integrin has been associated with metastasis. Highly metastatic melanoma cells have been found to over-express this integrin (Klein *et al.*, 1996; Albeda *et al.*, 1990) which appears to have an important role in melanocytic tumour progression. Increased expression was shown in advanced stage carcinoma (Duncan *et al.*, 1996). Transfection of this integrin into a rhabdomyosarcoma cell line resulted in a more metastatic cell type that showed increased adhesion to collagen and laminin (Chan *et al.*, 1991). This integrin may play a dual role in poorly differentiated epithelial tumours by inhibiting early stages but facilitating late events in the metastatic process (Danen *et al.*, 1995a). Chen *et al.* (1994b) looked at the integrin expression in various clones from a human NSCLC primary tumour. Parental cells expressed low amounts of $\alpha_1$, $\alpha_2$ and $\beta_1$ integrins. However, one of the clones was found to express high amounts of the $\alpha_1$ and $\alpha_2$ integrins. Different clonotypes from the same tumour may have different invasive and metastatic potentials. In primary anal carcinomas, no $\alpha_2$ integrin expression was detected but cells from metastatic tissuse showed $\alpha_2$ positivity (Anastassiou *et al.*, 1995).

Loss of $\alpha_1$ integrin has been observed in lung adenocarcinomas, compared to high expression in normal lung pneumocytes (Roussel *et al.*, 1994). Down-regulation of $\alpha_3$ integrin has been observed in NSCLC (Smythe *et al.*, 1995; Chen *et al.*, 1994b), breast carcinoma (Pignatelli *et al.*, 1991) and colorectal carcinoma (Pignatelli *et al.*, 1990). In CHO cells, a clone expressing low levels of $\alpha_3\beta_1$ grew most rapidly in nude mice while cells with elevated levels of the integrin grew much more slowly (Schreiner *et al.*, 1991). Transfection of $\alpha_3\beta_1$ integrin into rhabdomyosarcoma also repressed the transformed phenotype (Weitzman *et al.*, 1996). Elevated levels of $\alpha_5\beta_1$ integrin in CHO resulted in a supression of the transformed phenotype (Giancotti and Rouslahti, 1990).

Up-regulation, down-regulation and loss of $\alpha_6$ integrin has been implicated in tumour progression and metastasis in many cell types. This integrin plays a key role in anchoring cells to the basement membrane. The upregulation of this integrin appears in tumours where normal tissue express low or undetectable levels (see Table 1.4.4). A ribozyme that selectively degrades $\alpha_6$ integrin mRNA was transfected into a human fibrosarcoma cell line and resulted in a change to a less adherent and less invasive cell type (Yamamoto *et al.*, 1996). The down-regulation of $\alpha_6$ has been implicated in epithelial tumours that normally express high levels of this integrin (see Table 1.4.4). In lung cancer, the results are somewhat variable but high $\alpha_6\beta_4$ expression has been shown in highly metastatic Lewis lung carcinoma (Perrotin *et al.*, 1990) and SCLC (Feldman *et al.*, 1991) while low levels of expression have been reported in NSCLC

(Smythe *et al.*, 1995). The difference in expression may be due to the fact that mesenchymal cells (*e.g.* fibroblasts) have no need to interact with BM and thus have no need to express $\alpha_6$ integrin but when the cells become transformed they must interact with BM to invade tissue, hence increased expression. In epithelial cells which are associated with BM, for carcinomas to detach from the BM, a reduction in $\alpha_6$ expression may be necessary but on development of the secondary metastasis, expression of $\alpha_6$ may be required for attachment to the BM and to initiate invasion.

**Table 1.4.4 Changes in $\alpha_6$ integrin expression in cancer**

| *Tumour type* | *Reference* |
|---|---|
| ***Upregulation of $\alpha_6$*** | |
| Fibrosarcoma | Lin *et al.* (1993)<br>Yamamoto *et al.* (1996) |
| Melanoma | Danen *et al* . (1995b) |
| Liver cancer | Volpes *et al.* (1993) |
| Skin cancer | Tennenbaum *et al.* (1993) |
| ***Downregulation of $\alpha_6$*** | |
| Breast | D'Ardenne *et al.* (1991) |
| Colon | Stallmach *et al.* (1992) |
| Prostate | Knox *et al.* (1994) |
| Kidney | Korkonen *et al.* (1992) |
| Pancreas | Weinel *et al.* (1995) |

Integrins have been found to have a role in angiogenesis of tumours. The formation of blood vessels serve to nourish the cells and provide access routes into bloodstream for metastatic cells (Horwitz, 1997). To form new blood vessels, proliferating endothelial cells must form adhesive attachments to one another and to the matrix around them. There appears to be increasing evidence that the $\alpha_v\beta_3$ integrin appears in quantity on these endothelial cells that are forming new blood vessels and this display keeps proliferating cells from dying. As a result there is often an upregulation of this integrin associated with invasive cancers. Increased expression has been observed in metastatic melanomas (Albeda *et al.*, 1990; Danen *et al.*, 1995a; Natali *et al.*, 1997), colorectal carcinoma (Nigam *et al.*, 1993), and breast carcinoma (Pignatelli *et al.*, 1992a).

There appears to be an association of integrin expression with expression of metalloproteinases (MMPs) (reviewed Heino, 1996). MMPs appear to play an important role in cancer cell invasion and metastasis (Liotta, 1986; Ura *et al.*, 1989). Collagenase MMP-2 has been shown to bind directly to $\alpha_v\beta_3$ and is thus localised in

proteolytically active form on the surface of invasive tumour or endothelial cells (Brooks *et al.*, 1994; Seftor *et al.*, 1993). Clarke *et al.* (1997) have shown that production of MMPs is associated with increased $\alpha_v$ expression. There appears to be an association between $\alpha_2\beta_1$ integrin expression and positive regulation of MMP-1 (Riikonen *et al.*, 1995). Using antibodies to $\alpha_3$ integrin resulted in increased levels of MMP-2 and greater invasive capabilities in Matrigel assays of glioblastoma cells (Chintala *et al.*, 1996).

## 1.5 DIFFERENTIATION

Cellular differentiation can be defined as the process leading to the expression of the phenotype characteristic of the functionally mature cell *in vivo*. In other words, 'a cell acquires or displays a new phenotype without changing its phenotype' (Ham and Veomett, 1980). As differentiation progresses, cell division is reduced and eventually lost (reviewed Davila *et al.*, 1990). The principle cells which differentiate are stem cells capable of rapid growth and division. These cells are multipotent and thus have the potential to differentiate into two or more different cell phenotypes. In general they possess unlimited proliferative potential but they can remain quiescent under certain microenvironment conditions (Davila *et al.*, 1990). A progenitor cell represents the progeny of stem cells which possess more limited proliferation and differentiation potential. This cell is usually only involved in a single cell lineage.

The destiny of a cell during embryogenesis and development is regulated by genetic programs and restricts the number of lineages that stem cells have the potential to form. Ham and Veomott (1980) proposed that 'determination' is a process whereby a cell becomes committed to differentiate into a specific lineage. A determined or committed cell initially may not appear phenotypically different; this only occurs after its program has been expressed (MacLean and Hall, 1987).

A cell can differentiate in a manner that results in either the irreversible loss of its proliferative capacities (terminally differentiated) or some of its proliferative capacity has been retained while being fully differentiated (non-terminally differentiated). Dedifferentiation is the process whereby a cell loses its differentiated phenotype and transdifferentiation occurs when a cell dedifferentiates and then redifferentiates into a new distinct phenotype (Davila *et al.*, 1990).

*In vivo* and *in vitro* studies support the role for differentiation defects early in the process of carcinogenesis. This may be due to uncoupling of the integrated control of differentiation and proliferation in cancer cells, where such developments can result in a relative insensitivity to differentiation signals and increased sensitivity to proliferation signals. Two scenarios thus exist for the possible origins of cancer; from dedifferentiation of mature cells which retain the capacity to divide or from maturation arrest of immature cells. With increasing tumour progression, the histology of the tumour often indicates poorer differentiation and from a prognostic standpoint, patients with poorly differentiated tumours will generally have a lower survival rate than those with differentiated tumours.

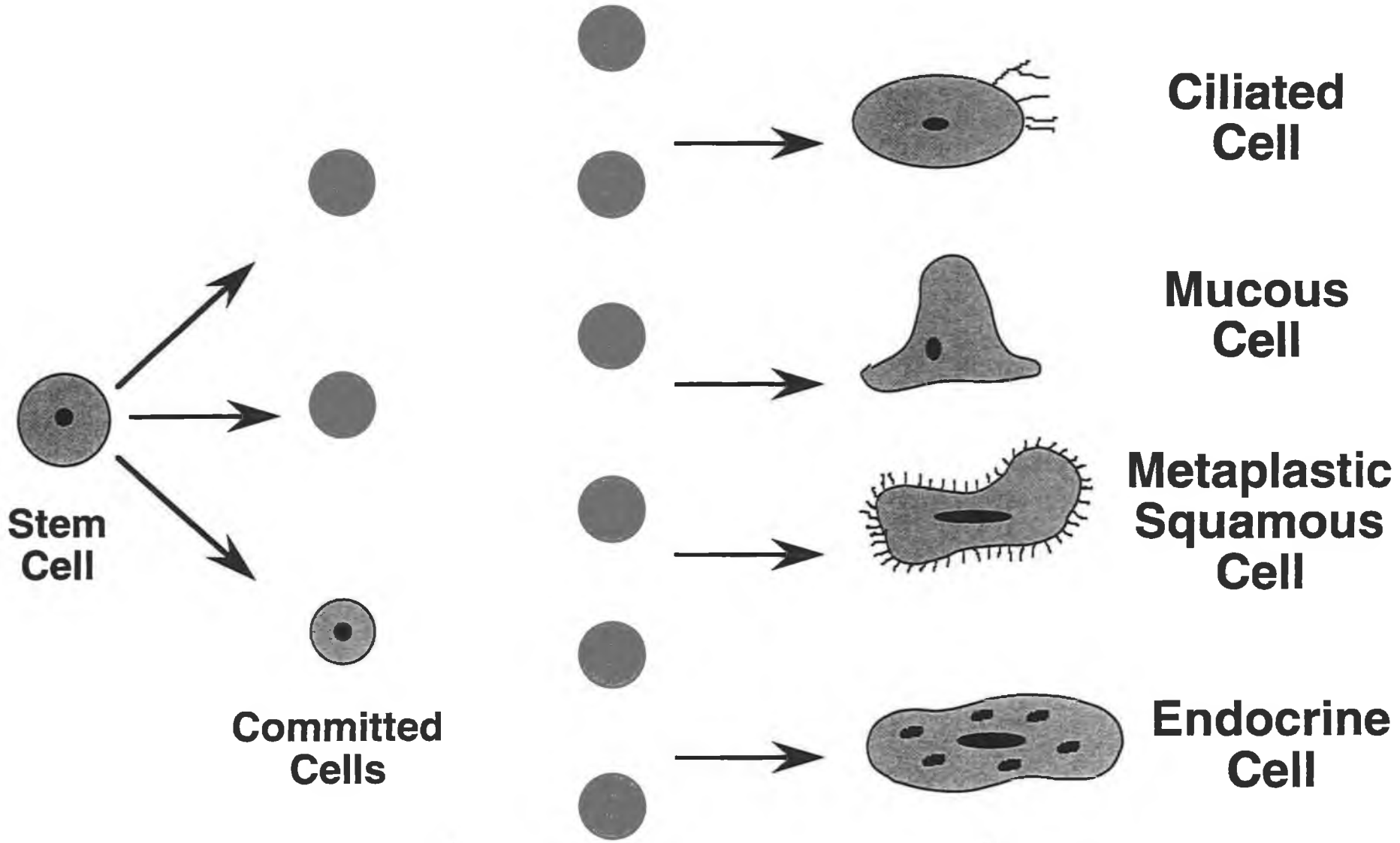


**FIG. 1.3** Stem cell theory of the origin of lung epithelial cells

The pathways involved in the haematopoietic system and their disruption leading to leukaemia have been extensively studied (reviewed in Sawyers *et al.*, 1991). Leukaemia may be defined as the uncontrolled proliferation of haematopoietic cells that do not retain the capacity to divide into normal mature blood cells. In the adult, the pluripotent stem cells of the haematopoietic system are located in the bone marrow in a quiescent state. The proliferation, differentiation and survival of haematopoietic stem cells depend on a number of different growth factors and cytokines produced by the bone marrow and stroma of other cell types (reviewed Metcalf, 1989). HL60 promyelocytic cell line can be chemically induced to differentiate into a variety of cell types that functionally and morphologically resemble their normal counterparts (reviewed Collins, 1987). HL60 can differentiate into four types of myeloid cells depending on the inducing agent. DMSO and retinoic acid induce granulocytic differentation. Vitamin $D_3$ can induce differentiation into monocytes, phorbol esters induce macrophage-like colonies to develop, while GM-CSF can promote eosinophil differentiation in these cells.

In the respiratory system, a single stem cell type may be a precursor of different cell types such as Clara cells, alveolar type II cells and pulmonary neuroendocrine cells depending on specialised culture conditions (Emura, 1996). Fetal syrian hamster cells appear to have the potential to differentiate into Clara cells by cultivating the cells in a collagen-gel supplemented with retinoic acid, into type II pneumocytes by using hormone-supplemented medium containing an agar overlay supplemented with insulin and hydrocortisone, and into neuroendocrine cells by reducing the oxygen pressure in the air to 5% concentration. McDowell (1987) and Emura (1997) have discussed the possible existence of a common stem cell for the lung epithelium, as outlined in Fig. 1.3.

The presence of common overlapping features between lung cancer cell types supports the theory of a common stem cell origin for lung epithelial cancers. Gazdar *et al.* (1981) outlined how all the cells of the bronchial epithelium and the tumours which arise from them could have a common stem cell origin. The transforming events which lead to carcinogenesis affect undifferentiated or partially-differentiated cells. There are many reports in the literature of evidence for the presence of stem cell populations in tumour cell lines (Khan *et al.*, 1991). Gazdar *et al.* (1988) suggested that the overlapping expression of markers between different tumour types is indicative of a common stem cell origin of lung epithelial cancers.

Induction of differentiation has been considered as a possible component of therapy for cancer (Waxman *et al.*, 1988). Natural retinoids have been found to play an important role in the maintenance of the normal differentiated state of the adult lung (Chytil, 1992; Zachman, 1995; Chytil, 1996). Retinoids thus play an important role in cancer differentiation and chemoprevention (Lotan, 1996).

### 1.5.1 Differentiation specific markers - Keratins

The proteins of the keratin family, which is composed of at least 20 different proteins (not including hair keratins) are expressed in a very cell-specific manner in epithelial tissue (Moll *et al.*, 1982; Coulombe, 1993). A particular epithelium may be characterised by its specific pattern of keratin expression. The keratin family can be generally subdivided into acidic type I keratins (keratins 9-20) and neutral to basic type II keratins (keratins 1-8) (Schaafsma and Raemakers, 1994; Moll *et al.*, 1982). Type I and type II keratins are always co-ordinately and pair-wise expressed, in approximately equal amounts, which is due to the fact that the keratin filament is a heterotetramer of two type I and two type II polypeptides (Steinart and Roop, 1988). Keratins 5 and 14 are generally co-expressed in the undifferentiated basal cell layer of stratified squamous epithelia while keratins 8 and 18 are constituitively expressed in essentially all simple epithelia.

The 20 cytokeratins can be grossly subdivided into stratified epithelium-type (keratins 1-6, 9-17) and simple epithelium-type (keratins 7, 8, 18, 19, 20) according to predominant occurrence (Moll, 1992). Many epithelial tumours retain a profile of keratin expression characteristic of their differentiation state (Broers and Raemaekers, 1994). However, alterations in keratin expression during malignancy has been observed (Trask *et al.*, 1990; Moll, 1992; van der Gaast *et al.*, 1994). SCLC-V cells in particular, despite being of epithelial origin, often express no detectable amounts of keratin filaments (Broers *et al.*, 1985) and can instead express neurofilaments or vimentin intermediate filaments.

Epithelial cells possess specific keratin profiles characteristic of their cell type, stage of development, disease status and degree of differentiation. Keratins appear to be the first intermediate filament proteins detectable during foetal development, with appearance of keratins 8 and 18 first (Franke *et al.*, 1982). It appears that the more specialised keratins which are expressed at later stages of development have probably evolved from K8 and K18 (Blumenberg, 1988). Co-expression of cytokeratins with other intermediate filaments (vimentin, neurofilaments, glial filaments) are characteristic of certain epithelial differentiation lineages (Moll, 1992). Blobel *et al.* (1984) showed

that the keratin pattern clearly distinguishes different domains of the normal respiratory tract as well as different types of lung carcinomas.

In general, *in vivo*, poorly-differentiated tumours exhibit increased invasiveness and have a worse prognosis compared to well differentiated tumours (Behrens, 1994). The loss or alteration of epithelial features such as keratins and cell adhesion molecules such as beta-1 integrins and cadherins involved in cell-cell and cell-matrix interactions appears to facilitate cell migration and metastasis.

#### 1.5.1.1 The simple epithelial keratins

Keratins 8 and 18 are the first to appear during embryogenesis (Franke *et al.*, 1982) and they have also been shown to occur in early mesenchymal tissues and developing organs such as the heart (van Muijen *et al.*, 1987). In foetal skin maturation, keratins 8, 18 and 19 appear first (van Muijen *et al.*, 1987; Moll *et al.*, 1982). All adenocarcinomas express K8 and K18 and frequently express K19. Neuroendocrine carcinoma has been shown to express K8 and K18 consistently with K19 expressed in a more restricted fashion (Moll *et al.*, 1982; Broers *et al.*, 1988; Wetzels *et al.*, 1991). Trask *et al.* (1990) showed that breast cancer cells produce K8, K18 and K19 whereas normal breast cells produce K5, K6, K14 and K17. Keratin 8 expression may be a prognostic marker in invasive breast carcinomas (Takei *et al.*, 1995).

The particular localisation of keratin 19 in various epithelia has led to the hypothesis that it could be a marker for epithelial progenitors (Bartek *et al.*, 1986), including stem cells of the skin (Michel *et al.*, 1996). In human adult skin, K19 is restricted to the inner root sheath of the hair follicle and has not been detected in the epidermis (Stasiak *et al.* 1989), while in human foetal skin, K19 is observed in cells of the basal layer (Dale *et al.*, 1985; Oliver *et al.*, 1990). Increased expression of $\alpha_3\beta_1$ integrin on the surface of these K19 positive cells was shown by double-labelling immunofluorescence. This is consistent with previous reports where stem cells of the skin express increased amounts of $\beta_1$ integrin than differentiated keratinocytes (Jones and Watt, 1993; Jones *et al.*, 1995). Keratin 19 is an informative sub-type specific marker because the basal cells of non-keratininising epithelia express keratin 19 *in vivo* and in culture. However, epidermal keratinocytes normally do not express keratin 19 (Schön and Rheinwald, 1996).

Keratin 19 also appears to be associated with the fully differentiated type II cell phenotype (Paine *et al.*, 1995). In freshly isolated type II cells there is abundant synthesis of K19 and low-level synthesis of K18. As the cells differentiate to the type I

phenotype, K18 synthesis is increased and that of K19 is virtually lost (Paine *et al.*, 1988). Keratin 19 expression is frequently associated with the less differentiated proliferating cell in epithelial tissue (Stasiak *et al.*, 1989). Cyfra-21 is a K19 fragment that is soluble in serum and may be useful as a circulating tumour marker, *e.g.* in gynecologic malignancies (Inaba *et al.*, 1995). A high level of Cyfra-21 may correlate with advanced disease stage in NSCLC (Wieskopf *et al.*, 1995). Diffuse staining of K19 may be a potential marker of poorly-differentiated oesophageal squamous carcinomas (Lam *et al.*, 1995). The detection of K19 gene expression by RT-PCR has been reported as a sensitive tool for detecting occult metastasis to the lymph nodes and bone marrow of patients with primary epithelial tumours of the bowel and breast (Burchill *et al.*, 1995; Gunn *et al.*, 1996; Krüger *et al.*, 1996). The presence of micrometastases in bone marrow worsens the outcome of breast cancer treatment significantly .

Keratin mRNA appears to be expressed more widely than its protein (Tyner and Fuchs, 1986; Stoler *et al.*, 1988). In normal stratified oral epithelia, post-transcriptional control of K7, K8 and K18 has been reported (Su *et al.*, 1994). Protein expression of these keratins was observed in dysplastic and malignant epithelia suggesting a release from a post-transcriptional block on K8 and K18 translation (Su *et al.*, 1994). Alternatively rapid degradation of K8 and K18 protein may be occuring in normal epithelia but may be supressed in dysplasia and malignancy. A retinoic acid-dependent post-transcriptional mechanism modulates K19 intermediate filament expression in stratified squamous epithelia (Crowe, 1993).

### 1.5.2 Differentiating inducing agent - Bromodeoxyuridine

Bromodeoxyuridine (BrdU) has been used for 20 years as a tool for measuring DNA synthesis in isolated chromosomes and in cells and tissues (extensively reviewed Dolbeare, 1995a; Dolbeare 1995b; Dolbeare 1996). BrdU is a thymidine (Td) analogue which can compete with Td for incorporation into DNA. It appears to be capable of modulating differentiation in a number of different cell lineages. Its activity is reversible if removed from the culture medium or if thymidine is added to the growth medium in equimolar concentrations (Ashman and Davidson, 1980). This indicates that BrdU is not acting as a mutagen, but is reversibly blocking specific differentiation pathways. The ability of BrdU to modulate differentiation may be related to its incorporation into DNA, which may implicate the involvement of inhibition of expression of differentiation-specific genes (Harding *et al.*, 1978).

Induction or inhibition of differentiation depends on the particular cell type and the target genes. Decreased *c-myc* expression as a result of BrdU treatment has been demonstrated in terminally differentiated leukaemic cells (Yen and Forbes, 1990) and in differentiating melanoma cells (Valyi-Nagi *et al.*, 1993).

BrdU has been found to supress differentiation and promote invasiveness of melanoma cells (Thomas *et al.*, 1993). The integrin repertoire expressed by melanoma cells is relevant to their invasive phenotype. Expression of $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins and metalloproteinases were found to be increased, while induction of $\alpha_3$ and $\alpha_v\beta_3$ integrins was shown in these cells. The production of melanin was repressed by BrdU. BrdU-treated cells were found to cause extensive invasion of Matrigel (Thomas *et al.*, 1993). However BrdU has been found to induce differentiation of melanoma cells, with a resultant change in morphology. Flattening of the cells, with a 20-fold increase in cell surface area and a 17-fold increase in nuclear area was observed (Valyi-Nagy *et al.*, 1993). Tumourigenicity of these cells in nude mice was supressed when the cells lines were treated with BrdU. Colony formation in soft agar of these BrdU-treated cells was also decreased by more than 10% at a 10μM concentration compared to untreated cells (Valyi-Nagy *et al.*, 1993). BrdU has been shown to upregulate expression of the actin gene and increase cell adhesiveness in B16 melanoma cells (Gomez *et al.*, 1995). Actin may be an important gene in melanoma growth where actin expression appears to be inversely correlated with melanoma metastatic potential (Shimokawa-Kuroki *et al.*, 1994).

BrdU has been found to block myogenesis and differentiation through a myogenic regulatory gene, MyoD1, in a mouse myoblast cell line (Tapscott *et al.*, 1989). It appears that although BrdU was incorporated into the muscle structural genes, these genes were apparently transcribed normally.

BrdU has been shown to induce terminal differentiation in HL60 leukemic cells (Yen and Forbes, 1990). BrdU induces the early events, leading to precommitment to the onset of terminal differentiation along the myeloid or monocytic pathways. BrdU has also been found to induce differentiation in human myeloid leukemia cells (Koeffler *et al.*, 1983).

Neuroblastoma have the potential to undergo three major differentiation pathways (neuronal, Schwannian or melanocytic) (Tsokos *et al.*, 1987). BrdU has been found to induce Schwannian differentiation in neuroblastoma (Sugimoto *et al.*, 1988; Emsui *et al.*, 1989; Reynolds and Maples, 1985). BrdU treatment of neuroblastoma cells has

been shown to upregulate expression of human melanoma-associated antigen (Feyles *et al.*, 1991a).

Feyles *et al.* (1991b) examined the effects of BrdU on a SCLC cell line with features of neuroendocrine differentiation. Treatment resulted in induction of substrate-adherant growth, along with an epithloid morphology and increased expression of neuroendocrine markers.

## 1.6 AIMS OF THESIS

In this thesis, SFM was used to develop models to grow various cultured cell lines in totally defined conditions. As outlined in Section 1.1.1.1, there are many disadvantages to the use of serum in supporting growth, differentiation status and metabolic activities of cells in culture.

The aims of the thesis were as follows:

**1.** CHO-K1 is a commercially important cell line that has been successfully used to produce a number of recombinant human therapeutic proteins. Investigations were carried out to develop a system to grow CHO-K1 in SF suspension culture. Experiments were carried out to improve suspension culture growth using shear protective agents such as Pluronic F68 and also to use microcarriers to try to alleviate cellular aggregation of the cells by using low calcium SFM.

**2.** The SFM developed by Mendiaz *et al.* (1986) was found not to support the long-term growth of CHO-K1. In this SFM, the cells can only be maintained for up to one month at most. Experiments were carried out to try to extend the subcultivation period of this cell line in SFM using inexpensive compounds to try to avoid using expensive growth factors or hormones. The low calcium SFM used in suspension culture experiments was also tried in long-term subcultivation experiments.

**3.** Transformation of normal to malignant cells is associated with a partial break-down of the normal regulatory systems governing cell proliferation and differentiation. SFM was developed for the growth of the poorly-differentiated lung carcinoma cell line, DLKP. A model would then be available to investigate response of these epithelial cancer cells to cytotoxic, chemotherapeutic drugs and to growth stimuli, in SFM without interference from ill-defined serum. The availability of a SFM then allowed experiments to be carried out using attachment factors, which are important in mediating many processes within the cell including proliferation and differentiation. DLKP appears to have three clonal populations that interconvert with each other and may be a model for the *in vivo* phenomenon of tumour heterogeneity. It is possible that these DLKP morphologies represent distinct phenotypes of the lung and the interconverting clones could be used as a model to examine cellular heterogeneity in lung cancer. SFM was also studied for the growth of the individual clones and DLKPA, the multiple drug resistant variant of DLKP. This would be of particular use to study mechanisms involved in MDR and drug transport in a completely defined system. The effects of different chemotherapeutic drugs on the cells could be tested without risk of masking effects that can be associated with the use of serum.

At this point in the project, a new system, also based on DLKP, became available in our laboratory through the research of Dr. Shirley McBride. Treatment of DLKP with BrdU was shown to induce the expression of epithelial-specific proteins including keratins 8 and 18.

**4.** It was decided to investigate the possibility that similar treatment could induce the expression of other epithelial-specific proteins including adhesion molecules such as integrins. Growth of DLKP in SFM influenced the morphology of the cells especially when attachment factors such as laminin or fibronectin were added to the SFM. The differentiation-inducing agent, bromodeoxyuridine, also influenced morphology with the cells appearing more flattened and spread compared to untreated DLKP cells. Experiments were carried out to investigate changes in the expression of integrins and the adhesive properties of the cells themselves, by investigating the interactions of the cells with various extracellular matrix proteins. This may have important implications for the study of tumour progression and metastasis.

**5.** Induction of keratins 8 and 18 was previously observed in DLKP cells by treating them with bromodeoxyuridine. Further studies were carried out on these cells to determine if similar induction could be demonstrated in DLKPA cells. Studies were also performed to determine if keratin induction was a result of cytotoxic stress rather than a differentiating effect of BrdU. There appears to be strong evidence that K8 and K18 expression is regulated at a post-transcriptional level. Studies were carried out using protein synthesis inhibitors to try to determine if a protein may be causing de-stabilisation or rapid degradation of translational products.

# 2.0 MATERIALS AND METHODS

## 2.1 WATER

Ultrapure water was used in the preparation of all media and solutions. This water was purified by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP) to a standard of 12 - 18 MΩ/cm resistance.

## 2.2 GLASSWARE

Solutions pertaining to cell culture and maintenance were prepared and stored in sterile glass bottles. Bottles (and lids) and all other glassware used for any cell-related work were prepared as follows:- all glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (AGB Scientific) for at least 1 hour. This is a deproteinising agent which removes proteinaceous material from the bottles. Following scrubbing and several rinses in tap water, the bottles were then washed by machine using Neodisher detergent, an organic, phosphate-based acid detergent. The bottles were then rinsed twice with distilled water, once with ultrapure water and sterilised by autoclaving.

## 2.3 STERILISATION

Water, glassware and all thermostable solutions were sterilised by autoclaving at 121°C for 20 minutes (min) under pressure of 1bar. Thermolabile solutions were filtered through a 0.22μm sterile filter (Millipore, millex-gv, SLGV-025BS). Low protein-binding filters were used for all protein-containing solutions.

## 2.4 MEDIA PREPARATION

Medium was routinely prepared and sterility checked by Joe Carey. The basal media used during routine cell culture were prepared according to the formulations shown in Table 2.4.1. 10x media were added to sterile ultrapure water, buffered with HEPES and $NaHCO_3$ and adjusted to a pH of 7.45 - 7.55 using sterile 1.5M NaOH and 1.5M HCl. The media were then filtered through sterile 0.22μm bell filters (Gelman, 121-58) and stored in 500ml sterile bottles at 4°C. Sterility checks were carried out on each 500ml bottle of medium as described in Section 2.5.5.

The basal media were stored at 4°C up to their expiry dates as specified on each individual 10x medium container. Prior to use, 100ml aliquots of basal media were

supplemented with 2mM L-glutamine (Gibco, 25030-024) and 5% foetal calf serum (Sigma, F-7524 Batch # 122H3363; Biowhittaker, 14-601F Batch # 53B0007) and this was used as routine culture medium. This was stored for up to 2 weeks at 4°C, after which time, fresh culture medium was prepared.

**Table 2.4.1** Preparation of basal media

| | **DMEM** (Gibco, 12501-029) | **Hams F12** (Gibco, 21700-109) | **MEM** (Gibco, 21430-020) |
|---|---|---|---|
| **10X Medium** | 500ml | Powder | 500ml |
| **Ultrapure $H_20$** | 4300ml | 4700ml | 4300ml |
| **1M HEPES*** Sigma , H-9136 | 100ml | 100ml | 100ml |
| **7.5% $NaHCO_3$** BDH, 30151 | 45ml | 45ml | 45ml |

* HEPES = N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

Ham's F12 medium was supplemented with 5% FCS. MEM was supplemented with 5% FCS, 2mM L-glutamine, 1x NEAA (Gibco, 11140-035) and 1x sodium pyruvate (Gibco, 11360-039). For most cell lines, ATCC (Ham's F12/ DMEM (1:1)) supplemented with 5% FCS and 2mM L-glutamine was routinely used.

## 2.5 CELL LINES

All cell culture work was carried out in a class II down-flow re-circulating laminar flow cabinet (Nuaire Biological Cabinet) and any work which involved toxic compounds was carried out in a cytoguard (Gelman). Strict aseptic techniques were adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items used in the cabinet. Each cell line was assigned specific media and waste bottles and only one cell line was worked with at a time in the cabinet which was allowed to clear for 15min between different cell lines. The cabinet itself was cleaned each week with industrial detergents (Virkon, Antec. International; TEGO, TH.Goldschmidt Ltd.), as were the incubators.

The cell lines used during the course of this study, their sources and their basal media requirements are listed in Table 2.5.1. Lines were maintained in 25cm$^2$ flasks (Costar; 3050) or 75cm$^2$ flasks (Costar; 3075) at 37°C and fed every two to three days.

### 2.5.1 Subculture of Adherent Lines

During routine subculturing or harvesting of adherent lines, cells were removed from their flasks by enzymatic detachment.

Waste medium was removed from the flasks and rinsed with a pre-warmed (37°C) trypsin/EDTA (TV) solution (0.25% trypsin (Gibco, 25090-028), 0.01% EDTA (Sigma, EDS) solution in PBS A (Oxoid, BR14a)). The purpose of this was to inhibit any naturally occurring trypsin inhibitor which would be present in residual serum. Fresh TV was then placed on the cells (2ml/25cm$^2$ flask or 4ml/75cm$^2$ flask) and the flasks incubated at 37°C until the cells were seen to have detached (5-10 min). The trypsin was deactivated by addition of a equal volume of growth medium (*i.e.* containing 5% serum). The entire solution was transferred to a 30ml sterile universal tube (Sterilin; 128a) and centrifuged at 1,000 rpm for 5 min. The resulting cell pellet was resuspended in pre-warmed (37°C) fresh growth medium, counted (Section 2.5.3) and used to re-seed a flask at the required cell density or to set up an assay.

### 2.5.2 Subculture of suspension cells

Cell lines growing in suspension did not require enzymatic detachment. Sometimes it was necessary to detach some of the cells which had become loosely adhered to the flask surface by gently tapping the flask. This was often the case for H82 cells. The cell suspension was then removed to a sterile universal, centrifuged at 1000rpm for 5 min and used to re-seed fresh flasks or set up assays.

**Table 2.5.1** Cell lines used during the course of this study

| Cell line | Basal medium | Cell type | Source |
|---|---|---|---|
| DLKP (and all its derivatives) | ATCC | Human poorly-differentiated lung carcinoma | Dr. Geraldine Grant, NCTCC |
| SKLU-1 | MEM | Human lung adenocarcinoma | ATCC[2] |
| HL60* | RPMI-1640[1] | Human leukaemic line | ATCC |
| H82* | RPMI-1640 | Human SCLC-V | ATCC |
| MCF-7 | ATCC | Human breast carcinoma | ATCC |
| A549 | ATCC | Human lung adenocarcinoma | ATCC |
| CHO-K1 | Ham's F12 | Chinese Hamster Ovary - fibroblastic-like | ATCC |

* These cells grow in suspension

[1] RPMI-1640 (Gibco, 52400-025) supplemented with 10% FCS and 2mM L-glutamine

[2] ATCC = American Type Culture Collection

### 2.5.3 Cell Counting

Cell counting and viability determinations were carried out using a trypan blue (Gibco, 15250-012) dye exclusion technique.

An aliquot of trypan blue was added to a sample from a single cell suspension in a ratio of 1:5. After 3 min incubation at room temperature, a sample of this mixture was applied to the chamber of a haemocytometer over which a glass coverslip had been placed. Cells in the 16 squares of the four outer corner grids of the chamber were counted microscopically, an average per corner grid was calculated with the dilution factor being taken into account and final cell numbers were multiplied by $10^4$ to determine the number of cells per ml (volume occupied by sample in chamber is 0.1cm x 0.1cm x 0.01cm *i.e.* 0.0001cm$^3$ therefore cell number x $10^4$ is equivalent to cells per ml). Non-viable cells were those which stained blue while viable cells excluded the trypan blue dye and remained unstained.

### 2.5.4 Cell Freezing

To allow long term storage of cell stocks, cells were frozen and cryo-preserved in liquid nitrogen at temperatures below -180°C. Once frozen properly, such stocks should last indefinitely.

Cells to be frozen were harvested in the log phase of growth (*i.e.* actively growing and approximately 50 - 70% confluent) and counted as described in Sections 2.5.3. Pelleted cells were re-suspended in serum and an equal volume of a DMSO/serum (1:9, v/v). This solution was slowly added dropwise to the cell suspension to give a final concentration of at least $5x10^6$ cells/ml. This step was very important as DMSO is toxic to cells. When added slowly the cells had a period of time to adapt to the presence of the DMSO, otherwise cells may have lysed. The suspension was then aliquoted into cryovials (Greiner, 122 278) which were then quickly placed in the vapour phase of liquid nitrogen containers (approximately -80°C). After 2.5 to 3.5 hours, the cryovials were lowered down into the liquid nitrogen where they were stored until required.

### 2.5.5 Cell Thawing

Immediately prior to the removal of a cryovial from the liquid nitrogen stores for thawing, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells to reduce their exposure time to the DMSO freezing solution which is toxic at room temperature. The suspension was centrifuged at 1,000 rpm. for 5 min, the DMSO-containing supernatant removed and the pellet re-suspended in fresh growth medium. A viability count was carried out (Section 2.5.3) to determine the efficacy of the freezing/ thawing procedures. Thawed cells were placed into tissue culture flasks with the appropriate volume of medium (10ml/25$cm^2$ flask and 15ml/75$cm^2$ flask) and allowed to attach overnight. After 24 hours, the cells were re-fed with fresh medium to remove any residual traces of DMSO.

### 2.5.6 Sterility Checks

Sterility checks were routinely carried out on all media, supplements and trypsin used for cell culture. Samples of basal media were inoculated into Columbia (Oxoid, CM331) blood agar plates, Sabauraud (Oxoid, CM217) dextrose and Thioglycollate (Oxoid, CM173) broths which should between them detect most contaminants including bacteria, fungus and yeast. Growth media (*i.e.* supplemented with serum and L-glutamine) were sterility checked at least 2 days prior to use by incubating samples at

37°C which were subsequently examined for turbidity and other indications of contamination.

## 2.6 *MYCOPLASMA* ANALYSIS

*Mycoplasma* examinations were carried out routinely (at least every 3 months) on all cell lines used in this study. These analyses were performed by Mary Heenan and William Nugent.

### 2.6.1 Indirect Staining Procedure

In this procedure, *Mycoplasma*-negative NRK cells (a normal rat kidney fibroblast line) were used as indicator cells. As such, these cells were incubated with supernatant from test cell lines and then examined for *Mycoplasma* contamination. NRK cells were used for this procedure because cell integrity is well maintained during fixation. A fluorescent Hoechst stain was utilised which binds specifically to DNA and so will stain the nucleus of the cell in addition to any *Mycoplasma* DNA present. A *Mycoplasma* infection would thus be seen as small fluorescent bodies in the cytoplasm of the NRK cells and sometimes outside the cells.

NRK cells were seeded onto sterile coverslips in sterile Petri dishes at a cell density of $2x10^3$ cells per ml and allowed to attach over night at 37°C in a 5% $CO_2$, humidified incubator. 1ml of cell-free (cleared by centrifugation at 1,000 rpm for 5 min) supernatant from each test cell line was then inoculated onto a NRK Petri dish and incubated as before until the cells reached 20 - 50% confluency (4 - 5 days). After this time, the waste medium was removed from the Petri dishes, the coverslips washed twice with sterile PBS A, once with a cold PBS/Carnoys (50/50) solution and fixed with 2ml of Carnoys solution (acetic acid:methanol-1:3)for 10 min. The fixative was then removed and after air drying, the coverslips were washed twice in deionised water and stained with 2ml of Hoechst 33258 stain (BDH)(50ng/ml) for 10 min.

From this point on, work proceeded in the dark to limit quenching of the fluorescent stain.

The coverslips were rinsed three times in PBS. They were then mounted in 50% (v/v) glycerol in 0.05M citric acid and 0.1M disodium phosphate and examined using a fluorescent microscope with a UV filter.

### 2.6.2 Direct Staining

The direct stain for *Mycoplasma* involved a culture method where test samples were inoculated onto an enriched *Mycoplasma* culture broth (Oxoid, CM403) - supplemented with 16% serum. 0.002% DNA (BDH; 42026), 2mg/ml fungizone (Gibco,15290-026), $2x10^3$ units penicillin (Sigma, Pen-3) and 10ml of a 25% (w/v) yeast extract solution - to optimise growth of any contaminants and incubated at 37°C for 48 hours. Sample of this broth were then streaked onto plates of *Mycoplasma* agar base (Oxoid, CM401) which had also been supplemented as above and the plates were incubated for 3 weeks at 37°C in a $CO_2$ environment. The plates were viewed microscopically at least every 7 days and the appearance of small, "fried egg" -shaped colonies would be indicative of a mycoplasma infection.

## 2.7 SERUM-FREE MEDIA ASSAYS

### 2.7.1 Preparation of SFM

Serum-free medium was prepared for routine work using Ham's F12 basal medium supplemented with the components listed in Table 2.7.1. All serum-free media were made up in sterile plastic 150ml containers (Sterilin, 165C) and stored for up to 2 weeks at $4^0$C in the dark.

**Table 2.7.1** Supplements to serum-free medium

| Supplement | Final concentration (μM) |
|---|---|
| $FeSO_4$ | 5.0 |
| $H_2SeO_3$ | $3.0x10^{-2}$ |
| $CuSO_4.5H_2O$ | $1.0x10^{-3}$ |
| $MnSO_4.5H_2O$ | $1.0x10^{-3}$ |
| $Na_2SiO_3$ | $5.0x10^{-2}$ |
| $NH_4VO_3$ | $5.0x10^{-3}$ |
| $(NH_4)_6Mo_7O_{24}.4H_20$ | $1.0x10^{-3}$ |
| $NiCl_2.6H_20$ | $5.0x10^{-4}$ |
| $ZnSO_4.7H_2O$ | $5.0x10^{-2}$ |
| $SnCl_2.H2O$ | $5.0x10^{-4}$ |
| $CaCl_2$ | $6.0x10^{-2}$ |
| Linoleic acid | $3.0x10^{-2}$ |
| NEAA | $1.0x10^{-2}$ |
| Transferrin | 5.0μg/ml |
| Insulin | 10.0μg/ml |
| L-glutamine | 2mM |

The reconstitution of the reagents required for SFM and growth assays are described in Table 2.7.2

**Table 2.7.2** Reconstitution of reagents required for SFM

| PRODUCT | SIZE | CAT. NO. | RECONSTITUTION |
|---|---|---|---|
| Bovine insulin | 100mg | Sigma, I-882 | Add 9.9ml of sterile $H_2O$ plus 100µl glacial acetic acid. Stock = 10mg/ml |
| Sodium selenite | 1mg | Sigma, S-9133 | Add 20ml sterile medium or PBS A. Stock = 20µg/ml |
| holo-transferrin (iron saturated) | 100mg | Sigma, T-2158 | Add 10ml sterile medium or PBS A. Stock = 10mg/ml |
| Trypsin Inhibitor | 500mg | Sigma, T-6522 | Add 36ml of PBS A or sterile medium. Stock = 10x. |
| Type IV Collagen | 5mg | Sigma, C-5533 | Add 10ml of PBS A. Stock = 500µg/ml |
| Fibronectin | 5mg | Sigma, F-2006 | Add 10ml of PBS A. Stock = 500µg/ml |
| Laminin | 1mg | Sigma, L-2020 | Dilute to 500µg/ml in 2ml PBS A. |

## 2.7.2 Subculture of cells in SFM

Routinely growing cells were harvested by trypsinisation. An equal volume of prewarmed soybean trypsin inhibitor (Sigma T-6522) was used to inhibit the activity of trypsin. A volume of trypsin inhibitor at 1.388mg/ml was sufficient to inactivate an equal volume of the 0.25% trypsin solution used. The cell solution was then removed to a universal and centrifuged at 1500rpm for 7 minutes. The pellet was then resuspended in 8ml of basal medium. The centrifugation step was repeated a second time to remove as much residual serum proteins as possible. The supernatant was gently decanted off and the pellet was resuspended in fresh, pre-warmed SFM and inoculated in 25cm$^2$ vented flasks (Costar 3056). Cells were incubated in an atmosphere of 5% $CO_2$ at 37$^0$C.

### 2.7.3 Cell freezing in SFM

Cryopreservation studies were carried out using Methylcellose 4000cp (Fluka AG, 18-5844-37) and Polyvinylpyrollidone (Sigma, PVP-360) to replace serum. Stock solutions of 1% methylcellulose (10x) and 30% polyvinylpyrrolidone (PVP) (10x) were made up in PBS-A and autoclaved. The cryopreservative solutions were then made up at 2x concentrations from these stocks. These 2x stock solutions were 0.2% MC or 6% PVP supplemented to 20% DMSO in SFM previously filter-sterilised. 10mls volumes were made up at a time and stored at -20$^0$C until use.

Cells to be frozen were usually in the log phase of growth and fed with fresh SFM 24 hours prior to freezing. The procedure is as described in Section 2.5.4 except that no serum was used in the procedure. Before addition of the cryopreservative solutions, the cells were resuspended in SFM rather than serum.

### 2.7.4 Cell thawing in SFM

Cells were thawed after removal from the liquid nitrogen tanks, as described in Section 2.5.5, and immediately transferred to a universal containing 9ml of SFM. The cell suspension was then centrifuged at 800rpm, the supernatant carefully removed and the cells resuspended in 8ml of SFM. Viability was assessed using haemocytometer counting as outlined in Section 2.5.3. The cells were then inoculated into 25cm$^2$ vented flasks and allowed to attach. The cells were then re-fed with fresh SFM 24 hours after thawing.

## 2.8 SUSPENSION CULTURE

500ml glass spinner flasks from Techne (Cambridge, UK) were used for the majority of the serum-free suspension culture experiments. 500ml spinner flasks from Bellco (New Jersey, USA) were used for the fluorescence anistropy experiments. Before glass spinner flasks could be used for any cell work they needed to be treated to prevent cells adhering to the glass.

### 2.8.1 Siliconisation of spinner flasks

A small amount (approximately 10-15ml) of 2% dimethyldichlorosilane in 1,1,1 trichloroethane (BDH, 33164) was added to the flasks which were then rotated by hand to ensure even coating of all surfaces (including the agitator). This procedure was carried out with care in a fume hood. The flasks were allowed to air-dry for approximately one hour, after which time they were rinsed three times in ultrapure water. A small amount of UHP water was left in the flask after the final rinse and the two side arm caps left a little open to allow steam ventilation during antoclaving. Flasks were then loosely covered with tin-foil and sterilised at 121$^0$C at 1bar absolute pressure for 20 minutes.

### 2.8.2 Culturing in spinner flasks

After sterilisation, flasks were rinsed with pre-warmed medium and incubated at 37$^0$C ready for use. Cells in the exponential phase of growth were trypsinised in the normal manner as in 2.5.1 for serum-supplemented cells and in 2.7.2 for serum-free cells. Counts were carried out and 100ml of medium containing cells diluted to the required density were inoculated into the spinner. Flasks were incubated at 37$^0$C on a special magnetic spinner unit (Techne) at an average speed of 40rpm for most experiments.

### 2.8.3 Sampling from spinner flasks

Sampling was carried out in the laminar flow cabinet making sure to keep the agitator rotating to ensure a representative sample from the spinner flask was being obtained. Usually a 1ml sample was taken and centrifuged at 1000rpm for 5 minutes. The supernatant was then carefully removed and 1ml of pre-warmed trypsin was added to the cells and incubated at 37$^0$C for 5-10 minutes. The cells were then pipetted gently to obtain a single cell suspension and counted as described in Section 2.5.3. Trypsin was added to try to break up cell aggregates which is a common phenomenon of suspension culture CHO-K1 cells.

### 2.8.4 Subculture of suspension culture cells

Every 8-9 days, the cells were decanted into 50ml centrifuge tubes and an aliquot was removed for counting using trypsin to break up the cell aggregates. Cells were then re-seeded at the original inoculation density using fresh SFM.

### 2.8.5 Preparation of microcarriers

0.3g/l of microcarrier beads (Cytodex 3; Pharmacia, 17-0485-01) were added to a siliconised glass bottle containing 30ml of PBS A. The bottle was left at 37$^0$C for 2-3 hours with occasional agitation. The supernatant was then removed after allowing the beads to settle and replaced with fresh PBS A. The beads were then allowed to settle again, the PBS A removed and replaced with another 10ml of fresh PBS A. The beads were then autoclaved. After sterilisation, the bottle was then placed in a laminar flow cabinet and the PBS A was carefully removed and replaced with pre-warmed SFM. The microcarriers were agitated and allowed to settle before the supernatant was removed and replaced with 10ml of fresh pre-warmed SFM. The beads were then transferred to a sterile siliconised spinner flask.

### 2.8.6 Culturing in microcarrier culture

Cells were diluted to the required volume and added to 90ml of pre-warmed SFM. The cells were then transferred to the sterile spinner flask containing 10ml of microcarrier beads. The spinner flasks were placed on the spinner unit and agitated at 40rpm.

### 2.8.7 Sampling from microcarrier culture

Sampling was carried out while ensuring that the beads and cells were well mixed in order to obtain a representative sample from the flask. This was very important as the microcarrier beads tend to settle very quickly. 1ml samples were removed from the well mixed spinner and centrifuged at 1500rpm for 5 minutes. The supernatant was carefully removed using a pasteur pipette and the pellet was resuspended in 200µl of 0.1%(w/v) crystal violet in 0.1M citric acid (Sigma, C-3029). The samples were incubated at 37$^0$C for 1-2 hours to allow the hypertonic solution to burst the cells and release the nuclei which the crystal violet stained. The beads were then pipetted gently up and down to dislodge the nuclei from the beads. Samples were loaded onto a haemocytometer and the nuclei were counted as for whole cells. The microcarrier beads are too large to enter the counting chamber and do not interfere with cell counts.

## 2.9 PLASMA MEMBRANE FLUIDITY MEASUREMENTS

Steady state fluorescence polarisation was used to determine the plasma membrane fluidity (PMF) of the cells. This technique involves labelling the membrane with a fluorescent probe (TMA-DPH) and then exciting the membrane with polarised monochromatic light. The movement of the probe depends on the fluidity of the membrane.

A stock solution of $5x10^{-4}$M TMA-DPH (Sigma, T-0775) was prepared by adding 1.15mg of TMA-DPH to 5ml of dimethylformamide (DMF) (Sigma, 27,054-7) and stored in the dark at $4^0$C for up to one month.

The method involved washing cell monolayers or suspension culture samples (in pelleted form) three times in PBS A. The monolayer cells were trypsinised in 2.5% trypsin at $10^0$C for approximately 1 minute and then transferred to a sterile universal and centrifuged at 1000rpm for 5 minutes. The resultant pellet was resuspended in PBS A and the viability was checked. Cells were diluted to $1x10^6$ cells per ml. To 1ml of cell suspension, 5µl of TMA-DPH solution was added to give a final concentration of probe of $1x10^{-6}$M. The fluorescence anistropy value of the cell suspension/ probe mixture was immediately read on a Perkin-Elmer fluorimeter.

## 2.10 GROWTH ASSAYS IN SFM

### 2.10.1 Miniaturised growth assays

Miniaturised assays were of two types, depending on plate size (24-well (Greiner, 662160) and 96-well (Costar; 3599) plates). For 24-well plate assays, results were determined by haemocytometer counts. For 96-well plates, results were determined using a combination of the Suforhodamine B and Crystal Violet Dye Elution assays.

Cells in the exponential growth phase were fed 24 hours prior to harvesting for all assays. Trypsin inhibitor was used to inhibit the trypsin. The cells were then centrifuged at 1000rpm for 5 minutes and the pellet resuspended in basal medium. If the cells were being used directly from serum-supplemented flasks, a second centrifugation step was required to remove as much residual serum as possible before inoculating into SFM. The pellet was then resuspended in the required volume of SFM.

For growth curve assays, DLKP and DLKPA were seeded at $1x10^4$ per well while CHO-K1 cells were seeded at $0.5x10^4$ per well in triplicate in 24 well plates. One plate was taken down for analysis each day over a 7-10 day growth period and cells were counted using a haemocytometer.

For 96-well growth stimulatory assays, DLKP and DLKPA cells were both seeded at $3x10^3$ cells per well in SFM. Cells were added in 100µl aliquots per well using 8-well multichannel pipettes. The plates were divided so that each variable was set up with 8 repeats and 12 variables per plate. Two controls were set up per plate, a positive control with medium supplemented with 5% FCS and a negative control with basal or SFM. The variables to be tested were made up at a 2x final concentration required. 100µl aliquots were then added to the respective wells on the 96-well plate already containing cells plus medium. The concentration of the variables to be tested was thus diluted to the required 1x concentration. When all variables were plated out, the plates were placed in a $CO_2$ incubator and incubated for the required amount of time for the assay. At the end of the assay, plates were taken down as described in section 2.10.2.

### 2.10.2 End-point for SFM 96-well assays

The end-point assay used was a combination of the Suforhodamine B (SRB) and Crystal Violet (CV) Dye Elution assays extensively studied by Dr. Angela Martin (PhD, 1992).

At the end of the cell growth period, cells were first fixed by layering 50µl of 50% TCA (Sigma, T-9159) directly on top of the incubation medium, and the plates were incubated at $4^0$C for 2 hours. The wells were then rinsed 5 times with tap water to remove solutes, flicked to remove liquid and then allowed to air-dry thoroughly. Cells were then stained with 200µl/ well of sulforhodamine B (0.4% in 1% acetic acid) (Sigma, S-9012) for 30 minutes and then rinsed four times in 1% glacial acetic acid and allowed to dry. When dry, 200µl/ well of 10mM Tris buffer, pH 10.5 was added. Alternatively, it was found to be possible to stain the fixed cells with crystal violet (BDH, 42555) (0.25% solution in UHP water). In this case, 200µl of crystal violet solution was added to each well and left to stain for 5-10 minutes. The plates were then left to dry before being eluted from the plates by adding 100µl of 33% glacial acetic acid to each well. The absorbance of both dyes (SRB and CV) was read on an ELISA plate reader at dual wavelengths of 570nm and 620nm. (570nm is the wavelength for maximum absorbance of the dye and 620nm is specific for the absorbance from the plastic in the plates themselves).

For most assays, crystal violet was used because of the shorter incubation times required for staining the cells with this dye compared to using SRB.

### 2.10.3 Subculture assays in SFM

To study the long-term growth of cells in SFM, subculture experiments were required. For all cell lines, cells were inoculated into 25cm$^2$ vented flasks at a density of 2.5x10$^5$ per flask using 8ml of pre-warmed test medium. CHO-K1 cells were incubated for 4 days while DLKP and DLKPA were incubated for 7 days. The medium was replaced every 2-3 days when required. Cells were then trypsinised as described in Section 2.7.2. When the cell number was determined, fresh flasks were inoculated with 2.5x10$^5$ cells per flask. This was continued for the length of time required in the assay.

For all subculture assays, cells were inoculated at a density of 2.5x10$^5$ per flask. Cell counts were carried out once a week for DLKP and all its derivatives (DLKP-I, DLKP-M, DLKP-SQ, DLKPA) with feeding every 3-4 days when necessary. CHO-K1 were passaged every 4 days. The extent of proliferation of these cells under SFM conditions was determined by haemocytometer counting (Section 2.5.3).

### 2.10.4 Subculture 'crisis' assays

Cells were grown for three passages in serum-free medium as described in Section 2.10.3. Cells were reseeded in the fourth subculture at 2.5x10$^5$ cells per flask with medium supplemented with one of the factors described in Table 2.10.1. Cells were trypsinised every 4 days and counted to determine the extent of proliferation achieved with supplemented factors.

**Table 2.10.1** Compounds tested in 'crisis assays'

| Component tested | Concentration used | Source |
|---|---|---|
| SF Briclone | 5% v/v | NCTCC |
| CM CHO-K1 | 5% v/v | Section 2.11.1 |
| LDL | 100µg/ml | Sigma, L-2139 |
| BME vitamins (100x) | 1x | Gibco, 21040-035 |
| Pluronic F68 | 20µg/ml | Sigma, P-1300 |
| DNase I | 100µg/ml | Boehringer Mannheim, 1284932 |
| Fibronectin | 10µg/ml | Sigma, F-2006 |

### 2.10.5 Subculture Calcium assays

The low calcium medium used in these subculture assays was composed of Ham's F12: MEM.S (Gibco, 31380-025) (1:1) supplemented with the components listed in Table 2.7.1. A 'home-made' calcium-free was made using commercially available supplements listed in Table 2.10.2. This medium was then supplemented with $CaCl_2$ to required concentrations for subculture assays.

**Table 2.10.2** 'Home-made' basal medium formulation

| Component of medium | Volume required for 100ml of medium | Source |
|---|---|---|
| Earle's balanced salts solution* (10x) | 10ml | Gibco, 14165-021 |
| MEM vitamins (100x) | 1ml | Gibco, 11120-037 |
| MEM amino acids (50x) | 2ml | Gibco, 11130-036 |
| Phenol red (0.5%) | 0.2ml | Sigma, P-0290 |
| $NaHCO_3$ (2.2g/100ml) | 10ml | |
| $MgCl_2$ (2g/100ml) | 1ml | |
| ultrapure water | 75ml | |

* Earle's salts without Ca, Mg and phenol red

Subculture assays were carried out using these media supplemented with SF supplements as outlined in Table 2.7.1.

## 2.11 TOXICITY ASSAY

Due to the nature of the compounds tested in the assays, precautions were taken to limit the risks involved in their handling and disposal. All work involving toxic compounds was carried out in a Gelman "Cytoguard" laminar air flow cabinet (CG Series). All chemotherapeutic drugs were stored and disposed of as described in table 2.11.1.

**Table 2.11.1** Chemotherapeutic drugs used in study

| Cytotoxic drug | Supplier | Inactivation | Storage |
|---|---|---|---|
| Adriamycin | Farmitalia | Hyperchlorite inactivation followed by autoclaving | Store at $4^0$C |
| Vincristine | David Bull Laboratories Ltd. | Autoclave | Store at $4^0$C |
| VP-16 | Bristol Myers Pharmaceuticals | Incineration | Store at RT |

Cells were harvested from a flask in the exponential phase of growth. A cell suspension of $3x10^4$ cells/ml was made up in growth medium and 100µl added into 96-well plates and allowed to attach overnight at 37℃, 5% $CO_2$. Drug dilutions (including a control with no drug) were prepared at 2x their final concentration in growth medium and 100µl added to each well in replicas of eight, giving final a concentration of 1x drug. The plates were incubated for 7 days at 37℃ and 5% $CO_2$ or until the control wells reached 80% confluency.

Cell survival was determined using the end-point assay developed for SF culture of DLKP and DLKPA as described in Section 2.10.2. The concentration of drug which causes 50% cell kill (the $IC_{50}$ value) was calculated.

## 2.12 CONDITIONED MEDIUM COLLECTION AND FRACTIONATION

Medium in which cells have been growing can be termed 'conditioned medium' (CM). This medium contains waste products of metabolism as well as many growth factors and other secretory products. CM may be growth stimulatory to cell lines. The CM collected were SF Briclone and from CHO-K1 cells.

### 2.12.1 Collection of Conditioned Medium

Cells were harvested from $75cm^2$ flasks and seeded into tissue culture roller bottles (Falcon, 3027) at seeded at a concentration of $2x10^6$ per 100ml of serum-supplemented growth medium. The roller bottles were incubated at $37^0C$ on a roller bottle apparatus which rotated the bottles at approximately 0.25rpm for the first 24 hours to allow cell attachment and at 0.75rpm after this time. The medium was replenished every 2-3 days until the cells were approximately 50% confluent. The spent medium was then removed, the cells rinsed twice with 100ml of sterile PBS A and then incubated for 1 hour with PBS A to remove as much of residual serum components as possible. The PBS A was then removed and replaced with basal medium supplemented with 2mM L-glutamine. The roller bottles were incubated overnight.

This medium was then removed and replaced with fresh basal medium supplemented with L-glutamine and the cells were allowed to condition this medium for 48 hours. This medium was collected, centrifuged at 1000rpm for 10 minutes to remove any cell debris and stored at $4^0C$. A fresh 100ml volume of basal medium was added to the cells and incubated for a further 48 hours. After this time, the medium was harvested as described before. The two fractions were combined and stored at $-20^0C$ until assayed.

### 2.12.2 Ultrafiltration of SF Briclone

The Diafflo membrane used in the study was a YM3 36mm (AN 06177A) which had a molecular weight cut-off of 3kDa. The membrane was pre-treated by soaking for approximately 1 hour in about 250ml of UHP water, with 3 changes of water in that time, to remove residual sodium azide which was used as a preservative. The membrane was then placed in an ultrafiltration cell (Amicon) and rinsed again by running 100ml of PBS A or UHP water through the system three times. 150ml of SF Briclone was run through the system. Pressure of 40psi was provided by $N_2$ gas and a flowrate of 17ml per 15 minutes was used. When 135ml of filtrate was collected, the

gas was turned off and the retentate was poured into a universal (15ml of 10x retentate). The fractions were stored at $4^0$C until required.

At the end of the procedure, the membrane was rinsed with UHP water or PBS A, removed form the cell and soaked in water for a further 30 minutes. The membranes were then stored in a solution of 1.5M NaCl at $4^0$C until required again.

## 2.13 DIFFERENTIATION STUDIES

Differentiation studies were carried out using 5-bromodeoxyuridine (BrdU) (Sigma, B5002). BrdU powder was reconstituted in UHP water to a stock concentration of 10mM and the resultant solution was filter sterilised through a sterile 0.22µm filter, aliquoted into sterile Eppendorfs and stored at $-20^0$C for up to 1 year.

### 2.13.1 Differentiation assays - Adherent cells

In all experiments, cells were plated onto 6-well plates (Costar, 3516) at densities of $5x10^3$ per well for DLKP, DLKPA, A549 and $1x10^4$ per well for SKLU-1. 1.5ml of medium was sufficient for each well. The cells were allowed to attach and form colonies by incubating at $37^0$C, 5% $CO_2$ for 48 hours. The plates were covered with parafilm to prevent contamination. The medium was then removed and replaced with BrdU-containing medium at the required concentrations, usually from 0-10µM BrdU. Plates were wrapped in aluminium foil because of the light-sensitive nature of BrdU and incubated for up to 7 days. Medium was replaced every 2-3 days over the course of the assay. All waste medium was retained for disposal by incineration.

At the end of the assay, medium was removed from the cells, the plates were rinsed 3 times with PBS A and the cells were fixed with methanol as described in Section 2.14.1. Immunocytochemistry was the carried out using a range of antibodies as described in Section 2.14.4.

### 2.13.2 Differentiation assays - Suspension cells

Suspension culture cells, HL60 and H82, were plated into 6-well plates at densities of $5x10^3$ per well. In order to change the medium in these plates, it was necessary to transfer the cells plus medium to sterile universals and centrifuge at 1000rpm for 5 minutes to pellet the cells. The cells were then resuspended in 1.5ml of BrdU-containing medium and returned to the wells.

At the end of the assay, the cells in suspension were used to prepare cytospins as described in Section 2.14.2. Any cells which had adhered during the course of the 7 day treatment (H82 cells) were fixed as described in Section 2.14.1.

## 2.14 IMMUNOCYTOCHEMISTRY

### 2.14.1 Fixation of cells

This procedure was used for all cells whether they were grown on 6-well plates, multiwell slides, or cytospins had been prepared from them. For fixation, cells were rinsed 3 times with PBS A and then incubated at -20$^0$C for 7 minutes using ice-cold methanol. The methanol was the removed from the cells and the cells were allowed to air-dry for a number of hours or overnight and then stored at -20$^0$C until required. This method appeared to be successful for all antibodies investigated during the course of the study.

### 2.14.2 Preparation of cytospins

Suspension culture cells were harvested, centrifuged at 1000rpm for 5 minutes and resuspended in PBS A. The cells were rinsed a further 2 times in PBS A and then were resuspended in cytospin buffer (components described in Table 2.13.1) to a final density of 1x10$^6$ cells/ml. The cell were spun onto clean glass slides at 400rpm for 4 minutes using a cytocentrifuge. The cells were then allowed to air-dry before being fixed as described in Section 2.14.1.

**Table 2.14.1** Components of cytospin buffer

| Component | g/l |
|---|---|
| NaCl | 8.00 |
| KCl | 0.40 |
| $CaCl_2$ | 0.14 |
| $MgCl_2$ | 0.10 |
| $MgSO_4$ | 0.10 |
| $Na_2HPO_4$ | 0.06 |
| $KH_2PO_4$ | 0.06 |
| $NaHCO_3$ | 0.35 |
| Glucose | 1.00 |
| Ultrapure water | 1000ml |

### 2.14.3 Preparation of multiwell slides

Multiwell glass slides (Lennox, L10C7E) were used when the cells to be examined were adherent and they were used in non-differentiation assays. These slides were used because they enabled cells to be cultured on the slides *in situ*, allowing cell morphology and antigen localisation to be examined after the immunocytochemical procedure. This can sometimes be lost during the harsh preparation of cytospins, where some cell surface antigens may be lost during trypsinisation. These slides contained 10 wells, each of which could be analysed with a separate antibody.

The slides were prepared by soaking in RBS detergent for 1 hour. They were then scrubbed and rinsed thoroughly with UHP water and wrapped in aluminium foil. The slides were then sterilised by baking for at least 2 hours at $180^0$C.

Cells were harvested and seeded onto the sterilised slides at a density of $1x10^4$ per 50µl per well. Each slide was then placed in a sterile Petri dish which was sealed with parafilm and incubated in the $CO_2$ incubator for 24-48 hours until the confluency required was achieved. The cells were then fixed in methanol as described in Section 2.14.1.

### 2.14.4 Immunocytochemical procedure

The avidin-biotin complex (ABC) immunoperoxidase technique combined with the diaminobenzidine (DAB) visualisation procedure was used in all immunocytochemistry experiments. The ABC method involves application of a biotin-labelled secondary antibody to cells probed with a primary antibody, followed by the addition of avidin-biotin-peroxidase complex which results in a high staining intensity due to the formation of an avidin-biotin lattice which contains peroxidase molecules. The peroxidase enzyme then reacts with a DAB solution to give an insoluble, brown-coloured precipitate. The formation of this brown precipitate-coloured precipitate is indicative of primary antibody reactivity.

The procedure used is as follows:
Cell preparations (cytospins, multiwell slides, 6-well tissue culture plates) which had been previously fixed in methanol and frozen at -20$^0$C were allowed to thaw and equilibrate at room temperature. A grease pen (DAKO, S2002) was used to encircle cells on cytospins, multiwell slides and in tissue culture plates to contain the various solutions involved. The cells were incubated for 5 minutes with a 3% $H_2O_2$ solution to quench any endogenous peroxidase activity that may be present in the cells and could lead to false positive results. The cells were then rinsed with UHP water and placed in Tris-buffered saline (TBS) (0.05M Tris/HCl, 0.15M NaCl, pH 7.6) for 5 minutes. The slides were then incubated for 20 minutes at room temperature (RT) with normal rabbit serum (DAKO, X092) diluted 1:5 in TBS to block non-specific binding. This was then removed and 25-30µl of optimally-diluted primary antibody was placed on the cells. The slides and tissue-culture plates were placed on a tray containing moistened tissue paper and incubated at 37$^0$C for 2 hours. The primary antibodies used in the study are listed in Table 2.14.2. The slides were then rinsed in TBS/ 0.1% Tween (Sigma, P-1379), x3 in 15 min, and then incubated for 30 min with biotinylated rabbit anti-mouse immunoglobulins (DAKO, E354) diluted 1:300 in TBS. The slides were rinsed as before and incubated with strepABComplex/ Horse Radish Peroxidase (HRP) (DAKO, K377) for 30 min at RT, after they were rinsed x3 in TBS/ 0.1% Tween in 15 min. The cells were then incubated with a DAB solution (DAKO, S3000) for 10-15 min. Excess DAB solution was then rinsed off with UHP water and the slides were counterstained with a 3% methyl green (Sigma, M-5015). Slides were then mounted using a commercial mounting solution (DAKO, S3023).

**Table 2.14.2** Primary antibodies used in immunocytochemical studies

| Antibody | Dilution/ Concentration | Supplier | Catalogue no. |
|---|---|---|---|
| pan-keratin | 1/100 | Sigma | C-2562 |
| Keratin 8 | 1/200 | Sigma | C-5301 |
| Keratin 18 | 1/800 | Sigma | C8541 |
| Keratin 19 | 1/50 | Sigma | C-6930 |
| ESA | 1/300 | Sigma | E-6011 |
| Desmosomal protein | 1/400 | Sigma | D-1286 |
| α-transglutaminase | 1/20 | Biogenesis | 5560-6006 |
| Neurofilaments | 1/20 | Immunotech | 0168 |
| NCAM | 1/10 | DAKO | M799 |
| 5-HT serotonin | 1/5 | DAKO | M758 |
| EGF-R | 1/5 | DAKO | M886 |
| $\alpha_1$ integrin | 1/3 | Serotec | MCA1133 |
| $\alpha_2$ integrin | 1/50 | Serotec | MCA1186 |
| $\alpha_3$ integrin | 1/100 | Serotec | MCA794 |
| $\alpha_4$ integrin | 1/50 | Serotec | MCA697 |
| $\alpha_5$ integrin | 1/100 | Serotec | MCA1187 |
| $\alpha_6$ integrin | 1/100 | Serotec | MCA956 |

## 2.15 PROTEIN INHIBITOR EXPERIMENTS

Cycloheximide (Sigma, C-7698) and Actinomycin D (Boehringer Mannheim, 102 008) protein synthesis inhibitors were used in these assays. A 10mg/ml stock solution of cycloheximide was prepared in PBS A, filter-sterilised, aliquoted and stored at -20$^0$C until required. A 5mg/ml stock solution of actinomycin D was prepared by dissolving the powder in cold, ultrapure water and leaving it to dissolve on ice for 30 minutes. The solution was then filter-sterilised and stored in the dark at -20$^0$C because of its light-sensitive nature.

Cells were seeded into 6-well plates as described in Section 2.12.1. Cycloheximide and actinomycin D was added to the cells at a number of different concentrations for various time periods as outlined in more detail in Section 3.4.4. After treatment, cells were fixed with methanol before immunocytochemical analysis was performed.

## 2.16 WESTERN BLOT ANALYSIS

Proteins for western blot analysis were separated by SDS-polyacylamide gel electrophoresis (SDS-PAGE).

### 2.16.1 Sample preparation

Cells were inoculated into 75cm$^2$ flasks at a density of 1x10$^5$ cells per flask and allowed to attach and form colonies. BrdU-containing medium, at concentrations from 0-10μM, was then added to the cells after 48 hours. Cells were treated with BrdU for up to 7 days, with the medium replaced every 2-3 days. The cells were then harvested by trypsinisation, washed three times in cold, sterile PBS A, pelleted and stored at -80$^0$C until required.

Cells were then lysed in buffer containing 62.5mM Tris-HCl pH 6.8, 12.5% glycerol, 2% Nonidet P40 (Sigma, N6507), 2.5mM phenylmethylsulphonyl fluoride (PMSF) (Sigma, P7626), 1.25mM EDTA, 12.5μg/ml leupeptin (Sigma, L2884), 116μg/ml aprotinin (Sigma, A1153) for 30 min on ice. The extracts were used immediately for western blot analysis.

### 2.16.2 Gel electrophoresis

Resolving and stacking gels were prepared as outlined in Table 2.16.1 and poured into clean 10cm x 8cm gel cassettes which consisted of 1 glass and 1 aluminium plate, separated by 0.75cm plastic spacers. The resolving gel was poured first and allowed to set. The stacking gel was then poured and a comb was placed into the stacking gel in order to create wells for sample loading. Once set, the gels could be used immediately or wrapped in aluminium foil and stored at $4^0$C for 24 hours.

Before samples were loaded onto the stacking gels, equal cell numbers (usually $2x10^4$ cells per lane) were further lysed in 2x loading buffer (2.5ml 1.25M-Tris/HCl, 1.0g SDS, 5.8ml glycerol and 0.1% bromophenol blue (Sigma, B8026) made up to 25ml with distilled water). The samples were then loaded including 6µl of molecular weight colour protein markers (Sigma, C-3437). The gels were run at 250V, 45mA for approximately 1.5 hours. When the bromophenol blue dye front was seen to have reached the end of the gels, electrophoresis was stopped.

**Table 2.16.1** Preparation of electrophoresis gels

| Components | Resolving gel (7.5%) | Resolving gel (12%) | Stacking gel |
|---|---|---|---|
| Acrylamide stock* | 3.8ml | 5.25ml | 0.8ml |
| Ultrapure water | 8.0ml | 6.45ml | 3.6ml |
| 1.875M-Tris/HCl, pH 8.8 | 3.0ml | 3.0ml | - |
| 1.25M-Tris/HCl, pH 6.8 | - | - | 0.5ml |
| 10% SDS (Sigma, L-4509) | 150µl | 150µl | 50µl |
| 10% Ammonium persulphate (Sigma, A-1433) | 60µl | 60µl | 17µl |
| TEMED (Sigma, T-8133) | 10µl | 10µl | 6µl |

* Acrylamide stock = 29.1g acrylamide (Sigma, A-8887) and 0.9g NN'-methylene bis-acrylamide (Sigma, N-7256) made up to 100ml with distilled water

### 2.16.3 Western blotting

Following electrophoresis, the acrylamide gels were equilibrated in transfer buffer (25mM Tris, 192mM glycine (Sigma, G-7126) pH 8.3-8.5 without adjusting) for 20 min. Protein in gels were transferred onto Hybond ECL nitrocellulose membranes (Amersham, RPN 2020D) by semi-dry electroblotting. Five sheets of Whatman 3mm filter paper (Whatman, 1001824) were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus. Excess air was removed from between the filters by moving a glass pipette over the filter paper. Nitrocellulose, cut to the same size of the gel, was soaked in transfer buffer and placed over the filter paper, making sure there were no air bubbles. The acrylamide gel was placed over the nitrocellulose and five more sheets of presoaked filter paper were placed on top of the gel. Excess air was again removed by rolling the pipette over the filter paper. The proteins were transferred from the gel to the nitrocellulose at a current of 34mA at 15V for 23 min.

All incubation steps from now on, including the blocking step, were carried out on a revolving apparatus to ensure even exposure of the nitrocellulose blot to all reagents.

The nitrocellulose membranes were blocked for 2 hours at room temperature with fresh filtered 5% non-fat dried milk (Cadburys; Marvel skimmed milk) in TBS/ 0.1% Tween. pH 7.5.

After blocking, the membranes were rinsed with PBS A and incubated with primary antibody (keratin 19 diluted 1:150 in TBS or ESA diluted 1:300 in TBS) overnight at $4^0$C. The primary antibody was removed and the membranes rinsed 3 times with TBS/ 0.1% Tween. The membranes were then washed for 15 min, and then twice for 5 mins in TBS/ Tween. Bound antibody was detected using enhanced chemiluminescence (ECL).

### 2.16.4 Enhanced chemiluminescence detection

Protein bands were developed using the Enhanced Chemiluminescence Kit (ECL) (Amersham, RPN2109) according to the manufacturer's instructions.

Secondary antibody (1/1,000 dilution of anti-mouse IgG peroxidase conjugate (Sigma, A-6782) in TBS) was added for 1 hour. The secondary antibody was removed and the membranes were washed as before. A sheet of parafilm was flattened over a smooth surface, *e.g.* a glass plate, making sure all air bubbles were removed. The membrane

was then placed on the parafilm, and excess fluid removed. 1.5ml of ECL detection reagent 1 and 1.5ml of reagent 2 were mixed and covered over the membrane. Charges on the parafilm ensured the fluid stayed on the membrane. The reagent was removed after one minute and the membrane wrapped in cling film. The membrane was exposed to autoradiographic film (Kodak; X-OMAT S, 500 9907) in an autoradiographic cassette for various times, depending on the signal. The autoradiographic film was then developed.

The exposed film was developed for 5min in developer (Kodak, LX24), diluted 1:6.5 in water. The film was briefly immersed in water and transferred to a Fixer solution (Kodak, FX-40) diluted 1:5 in water, for 5min. The film was transferred to water for 5 min and then air-dried.

## 2.17 IMMUNOPRECIPITATION

Immunoprecipitation was carried out using a cellular labelling and immunoprecipitation kit (Boehringer Mannheim, 1647652) according to manufacturer's instruction.

### 2.17.1 Sample preparation

Cells were treated with BrdU as described in Section 2.15.1 and harvested in a similar manner, pelleted and stored at -80$^0$C until required.

Pre-cooled lysis buffer was added to the cells and the cells were sonicated (10 pulses) on ice and incubated at 4$^0$C for 30min. The lysate was then centrifuged at 13000rpm for 10 min at 4$^0$C. The supernatant was transferred to a fresh Eppendorf and labelled with 25µl of biotin-7-NHS stock solution for 15 min at RT, as described in the manual accompanying the kit. The reaction was stopped by adding 50µl of stop solution (50mM $NH_4Cl$) and incubated for 15 min at 4$^0$C.

### 2.17.2 Sample immunoprecipitation

The lysates were pre-cleared using 50µl of Protein A or Protein B beads per ml of sample for 3 hours to completely remove proteins that may bind to the agarose beads and result in non-specific binding. The samples were then centrifuged at 13000rpm for 20 seconds to pellet the beads. The supernatants were collected and divided into two aliquots. A control irrelevant monoclonal antibody (Ab to EGF-R) was added to one of the samples while the antibody to be tested was added to the second aliquot (see Table

2.17.1 for volume of antibody added). Samples were then rocked at room temperature for 1 hour. 50µl of protein A (Boehringer Mannheim, 1719408) or protein G (Boehringer Mannheim, 1719416) agarose beads, depending on the binding specificity of the Ab to be tested (see Table 2.17.2), were then added to the samples. The precipitated protein was then allowed to bind to the beads by incubating the mixture on a belly-dancer overnight at $4^0$C.

The complexes were then collected by brief centrifugation at 13000rpm for 20 seconds. The supernatant was carefully removed. The protein-bead complexes were washed with a number of different stringency buffers. 1ml of wash buffer 1 (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% NP-40, 100µg/ml PMSF, 1µg/ml each of leupeptin and aprotonin) was added to the samples and rocked for 20 min. The complex was then pelleted by centrifugation as before and washed with 1ml of wash buffer 2 (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% NP-40) in a similar manner to the first wash. A third wash was carried out using a buffer containing 50mM Tris-HCl, 500mM NaCl and 0.1% NP40.

**Table 2.17.1** Volume of antibody required to precipitate proteins

| Antibody tested | Volume of antibody used |
|---|---|
| Keratin 19 | 10µl per ml of sample |
| $\alpha_1$ integrin | 50µl per ml of sample |
| $\alpha_2$ integrin* | 20µl per ml of sample |

***Note:** Antibodies used were the same as those used for immunocytochemistry except for the $\alpha_2$ integrin where a separate antibody had to be used (Serotec, MCA2026) because of the unsuitability of the first antibody for immunoblotting and immunoprecipitation techniques

**Table 2.17.2** Agarose beads chosen to precipitate the required proteins

| Antibody used | Isotype of Ab | Avidity to Protein-A | Avidity to Protein-G | Beads chosen |
|---|---|---|---|---|
| Keratin 19 | mouse IgG2a | ++++ | ++++ | Protein-A |
| $\alpha_1$ integrin | mouse IgG1 | + | ++++ | Protein-G |
| $\alpha_1$ integrin | mouse IgG1 | + | ++++ | Protein-G |

After the final wash, 2x loading buffer was added to the pellets to give a final cell concentration of $2x10^5$ cells per 10µl. The proteins were then denatured by heating to $100^0$C for 3 min. The agarose beads were removed by centrifugation at 13000rpm for 20 seconds at room temperature in a microfuge and the supernatant was transferred to a fresh Eppendorf. Equal cell numbers were loaded on the gels, *i.e* $2x10^5$ cells per lane. The keratin 19 precipitaed proteins were run on 12.5% polyacrylamide gels while the $\alpha_1$ and $\alpha_2$ precipitated proteins were run on 7.5% gels as described in Section 2.16.2.

The proteins were then transferred to nitrocellulose membrane as described previously in Section 2.16.3 and were blocked for 3-4 hours at room temperature in 5% Marvel in TBS/ 0.1% Tween. The membranes were then rinsed briefly with PBS A and incubated for 1 hour in anti-biotin secondary antibody (monoclonal anti-biotin mouse IgG (Sigma, A0185) diluted 1:4000 in TBS). The precipitated proteins were then detected using ECL as described in Section 2.16.4.

## 2.18 EXTRACELLULAR MATRIX ADHERENCE ASSAYS

### 2.18.1 Reconstitution of ECM proteins

Collagen Type IV (Sigma, C-5533), fibronectin (Sigma, F-2006) and laminin (Sigma, L-2020) were reconstituted in PBS A to a stock concentration of 500µg/ml. Stocks were aliquoted into sterile Eppendorfs. Fibronectin and collagen stocks were stored at -$20^0$C while laminin stocks were stored at -$80^0$C. ECM gel (Sigma, E1270) was aliquoted and stored at -$20^0$C until use. This gel undergoes thermally activated polymerisation when brought to 20-$40^0$C to form a reconstituted basement membrane.

### 2.18.2 Coating of plates

Each of the ECM proteins, collagen, fibronectin and laminin, was diluted to 25µg/ml with PBS A and 250µl aliquots were placed into wells of a 24-well plate. The plates were tapped gently to ensure that the base of each well was completely covered with solution. The plates were then incubated at $4^0$C overnight. The ECM solutions were then removed from the wells, rinsed twice with sterile PBS A and 0.5ml of a sterile 0.1% BSA/ PBS A solution was dispensed into each well to reduce non-specific binding. The plates were incubated at $37^0$C for 20 min and then rinsed twice again with PBS A.

EHS is a liquid between 2-8$^0$C so aliquots of the gel were allowed to thaw in the fridge overnight before use. The EHS was then diluted 1:2 with cold DMEM and 250µl aliquots were quickly added to the wells of a 24-well plate. The gel was allowed to set at RT for 5-10 minutes. When the gel was completely set, the wells were rinsed twice with PBS A, blocked for 20 min with BSA as described above for the other ECM proteins.

### 2.18.3 Adherence assay

Cells were set up in 75cm$^2$ flasks and treated with BrdU at 0 and 10µM for 7 days as described in Section 2.16.1. The cells were harvested and resuspended in basal Ham's F12 medium. The cells were then plated at a concentration of 2.5x10$^4$ per well in triplicate and incubated at 37$^0$C for 15, 30 and 60 minutes. Control wells were those which had been coated but contained no cells. At each time point for the wells coated with fibronectin, collagen and laminin, the medium was removed from the wells and rinsed gently with PBS A. The cells were then stained with 0.5ml of 0.25% crystal violet dye for 10 min. The plates were then rinsed and allowed to dry. The dye was eluted with 200µl of 33% glacial acetic acid and 100µl aliquots were transferred to a 96-well plate and the absorbance was read on an ELISA reader at 570 and 620nm.

The extent of attachment of cells to EHS was determined using the MTT assay because of problems encountered with non-specific staining of the crystal violet dye to proteins of the EHS gel.

### 2.18.4 MTT assay

A Boehringer Mannheim cell proliferation kit (Cat no. I 465 007) was used in these experiments according to manufacturer's instructions.

Unattached cells were carefully removed and discarded from the wells. 0.25ml of basal Ham's F12 medium was then added to each well. 25µl of MTT labelling reagent was added to each well in subdued light and incubated at 37$^0$C for 4 hours. 250µl of solubilisation solution was then added to each well and left in incubator overnight at 37$^0$C. The samples were then read on the spectrophotometer at 575nm. Medium without cells and treated in this manner was used as the blank and negative control.

## 2.19 ADHESION BLOCKADE ASSAY

Wells of a 24-well plate were precoated with 5µg/ml of Type IV collagen for 1 hour at $4^0$C. The wells were then washed twice with sterile PBS A and blocked with 0.1% BSA/ PBS A for 45 min at $37^0$C. Cells were treated with BrdU and harvested as described in Section 2.16.1. They were then resuspended in Ham's F12 basal medium to a concentration of $4x10^4$ per ml. The cells were incubated with 10µg/ml of an anti-$\alpha_1$ integrin antibody (Upstate Biotech, 05246) for 30 min at $4^0$C. Control samples were cells incubated at $4^0$C without addition of antibody. The cells were then plated onto the wells at $2x10^4$ cells per well and incubated for 15 and 30 min at $37^0$C. The MTT assay was used to assess the attachment of the cells to collagen with and without Ab treatment, as outlined in Section 2.18.4.

## 2.20 RNA EXTRACTION

For all procedures using RNA, most glassware, solutions and plastics were treated with 0.1% diethyl pyrocarbonate (DEPC) before use which is a strong inhibitor of RNases.

RNA was extracted from the cells as follows:
Cells were seeded into 175cm$^2$ flasks (Nunc, I-56502A) at a density of approximately $2x10^6$ per flask and allowed to attach and form colonies for 48-72 hours at $37^0$C. The flasks were then treated with BrdU for time periods of 0, 15min, 1hr, 24hrs and 5 days. The cells were trypsinised and the pellet was washed once with PBS A. The cells were pelletted and lysed using 1ml of TRI REAGENT™ (Sigma, T-9424). The samples were allowed to stand for 5 min at RT to allow complete dissociation of nucleoprotein complexes. 0.2ml of chlorofom was then added per ml of TRI REAGENT™ used and the sample was shaken vigorously for 15 sec and allowed to stand for 15 min at RT. The sample was then centrifuged at 13000rpm for 15 min at $4^0$C. This step separated the mixture into 3 phases; the RNA was contained in the colourless upper aqueous layer. This layer was then transferred to a new Eppendorf and 0.5ml of isopropanol was added. The sample was mixed and allowed to stand at RT for 10 min before being centrifuged at 13000rpm for 10 min at $4^0$C. The RNA formed a precipitate at the bottom of the tube. The supernatant was removed and the pellet was washed with 1ml of 75% ethanol and centrifuged at $4^0$C for 5 min at 13000rpm. The supernatant was removed and the pellet was briefly allowed to air-dry. 20-30µl of DEP-C water was then added to the RNA to resuspend the pellet.

RNA concentration was calculated by determining its OD at 260nm and 280nm and using the following formula:-

$$OD_{260nm} \times \text{Dilution factor} \times 40 = \mu g/ml \text{ RNA}$$

The purity of the RNA extraction was calculated by determining its OD at 260nm and 280nm. An $A_{260nm} : A_{280nm}$ ratio of 2 is indicative of pure RNA. Only those samples with ratios between 1.7 and 2.1 were used.

## 2.21 REVERSE TRANSCRIPTASE REACTION

Reverse transcriptase (RT) reactions were carried out in laminar flow cabinets using micropipettes which were specifically allocated to this work.

cDNA was formed using the following procedure:-
1µl oligo $(dT)^{12-18}$ primers (1µg/µl) (Promega; C1101)
1µl total RNA (1µg/µl) (see 2.20)
3µl water
were mixed in a 0.5ml Eppendorf (Eppendorf, 0030 121.023), heated to 70°C for 10 min and then chilled on ice. To this, the following were added:-
4µl of a 5x buffer (250mM-Tris/HCl pH 8.3, 375mM-KCl and 15mM-$MgCl_2$)
2µl DTT (100mM) (Gibco; 510-8025 SA)
1µl RNasin (40U/µl) (Promega; N2511)
1µl dNTPs (10mM of each dNTP)
6µl water
1µl Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (40,000U/µl) (Gibco; 510-8025 SA).
The solutions were mixed and the RT reaction was carried out by incubating the Eppendorfs at 37°C for 1 hour. The MMLV-RT enzyme was then inactivated by heating to 95°C for 2 min. The cDNA was stored at -20°C until required for use in PCR reactions as outlined in Section 2.20.

## 2.22 POLYMERASE CHAIN REACTION

A standardised polymerase chain reaction (PCR) procedure was followed in this study. The Eppendorf tubes used (Eppendorf, 0030 121 023) and the sterile water were DEPC-treated. All reagents had been aliquoted and were stored at -20°C and all reactions were carried out in a laminar flow cabinet.

Each PCR tube contained the following:-
24.5µl water
5µl 10x buffer* (100mM-Tris/HCl, pH 9.0, 50mM-KCl, 1% Triton X-100)
3µl 25mM-$MgCl_2$*
8µl dNTPs (1.25mM each of dATP, dCTP, dGTP and dTTP) (Promega; U1240)
1µl each of first and second strand target primers (250ng/ml) (keratin 19 - Table 2.22.1)
1µl each of first and second strand endogenous control primer (250ng/ml) (β-actin)
0.5µl of 5U/µl *Taq* DNA polymerase enzyme*
5µl cDNA
*(Promega; N1862)

A drop of autoclaved mineral oil was placed in each reaction tube to prevent evaporation and the DNA was amplified by PCR as follows:

95°C for 1.5 min - to denature double-stranded DNA
30 cycles: 95°C for 1.5 min. - denature
55°C for 1 min - anneal
72°C for 3 min. - extend
72°C for 7 min. - extend
The reaction tubes were then stored at 4°C until analysed by gel electrophoresis as described in Section 2.23.

**Table 2.22.1** Primer sequences for PCR amplification of keratin 19 mRNA

| Keratin 19 | Primer sequence |
|---|---|
| Sense primer | GCGGGACAAGATTCTTGGTG |
| Anti-sense primer | CTTCAGGCCTTCGATCTGCAT |

as published Burchill *et al.* (1995)

## 2.23 ELECTROPHORESIS OF PCR PRODUCTS

A 3% agarose gel (NuSieve, GTG) was prepared in TBE buffer (5.4g Tris, 2.75g boric acid, 2ml 0.5M-EDTA pH 8.0 in 500ml water) and melted in a microwave oven. After allowing to cool, 0.003% of a 10mg/ml ethidium bromide solution was added to the gel which was then poured into an electrophoresis apparatus (BioRad). Combs were placed in the gel to form wells and the gel was allowed to set.

10µl loading buffer (50% glycerol, 1mg/ml xylene cyanol, 1mg/ml bromophenol blue, 1mM EDTA) was added to each 50µl PCR sample and 20µl was run on the gel at 80-90mV for approximately 2 hours. When the dye front was seen to have migrated the required distance, the gel was removed from the apparatus and examined on a transilluminator and photographed.

## 2.24 STATISTICAL ANALYSIS

All data was expressed as the mean of the number of replicates being analysed ± standard deviation (sd).

For all CHO-K1 suspension culture growth curves, see Section 3.1.1, all samples were taken in duplicate and the readings shown on the graphs are the average of two readings. The standard deviations, error bars, were not shown on the graphs. However, in all experiments the standard deviation was always less than 10%.

# 3.0 RESULTS

## 3.1 GROWTH OF CHO-K1 CELLS IN SERUM-FREE MEDIUM

Many serum-free media have been cited in the literature that are capable of supporting the growth of CHO-K1 cells. Ham's F12 medium was originally developed for the clonal growth of CHO cells in defined medium (Ham, 1965). In these studies, a modified Ham's F12 serum-free medium developed by Mendiaz *et al.* (1986) was used. This SFM was extensively investigated (Keenan, 1994) for the growth of CHO-K1 cells under various monolayer conditions.

In this thesis, the SFM was assessed for the growth of CHO-K1 cells in suspension culture. Investigations were also carried out to try and extend the subcultivation times of CHO-K1 cells. Keenan (1994) found that the modified SFM developed by Mendiaz *et al.* was not capable of supporting long-term growth of the cells, beyond on average 4 to 6 passages.

### 3.1.1 Growth in serum-free suspension culture

The serum-free medium used in these studies consisted of basal Ham's F12 supplemented with 5µg/ml transferrin, 10µg/ml insulin, 0.3µM linoleic acid, $6x10^{-2}$µM $CaCl_2$, $1x10^{-2}$µM NEAA, $3x10^{-2}$µM $Na_2SeO_3$, 5µM $FeSO_4$ plus the trace elements outlined in Table 2.7.1. The medium was buffered with 1.176g/l $NaHCO_3$ and 7.55mM HEPES. For all these experiments in spinner flasks, cell counts were taken every day and in duplicate where possible. The graphs for these suspension culture experiments are presented showing average cell counts without error bars (standard deviation was always less than 15%).

#### 3.1.1.1 Growth response of CHO-K1 cells in SFM

A growth curve was set up to compare growth of the cell line in basal medium, serum-free and serum-supplemented medium over a 7 day period in monolayer culture. Assays were set up in 24-well plates with 4 wells per variable in two separate experiments. Cell counts were carried out using a haemocytometer every day for 7 days where possible. The results are shown in Fig. 3.1.1. The basal medium, Ham's F12 was not capable of supporting proliferation of the cell line. The SFM was capable of growth of the cell line with an increase from 0.5 to $8.16x10^4$ cells/well on day 6. Growth in the SSM resulted in an increase from 0.5 to $38.9x10^4$ cells/well on day 5. These results show that the Ham's F12 based SFM is capable of supporting growth of CHO-K1 cells over a 7 day period in monolayer culture.

An experiment was then set up to assess the growth of CHO-K1 cells in this SFM in suspension culture. Cells in the exponential phase of growth were inoculated at a concentration of $2.5x10^5$ cells/ml into 100ml of medium in spinner flasks, either SFM or SSM, and aliquots of suspension culture were assessed for cell number and viability each day, for 7 days where possible. The following growth curve was obtained, see Fig. 3.1.2

It is clearly evident from Fig. 3.1.2 that the SFM does not support growth in suspension culture even though the medium supports good growth in monolayer culture (see Fig. 3.1.1). As expected, good growth was seen in serum-supplemented culture.

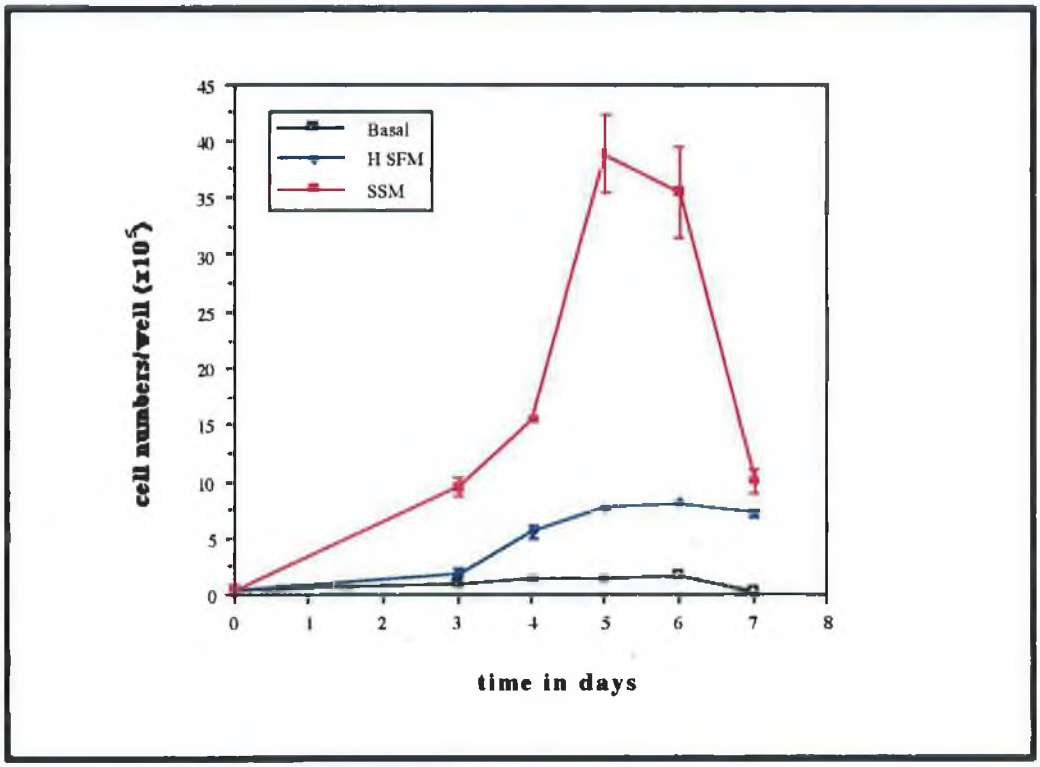


**Fig. 3.1.1** Growth curve for CHO-K1 cells in basal medium (Basal), serum-supplemented medium (SSM) and serum-free medium (H SFM). Results are expressed as average cell number x$10^5$ per well.

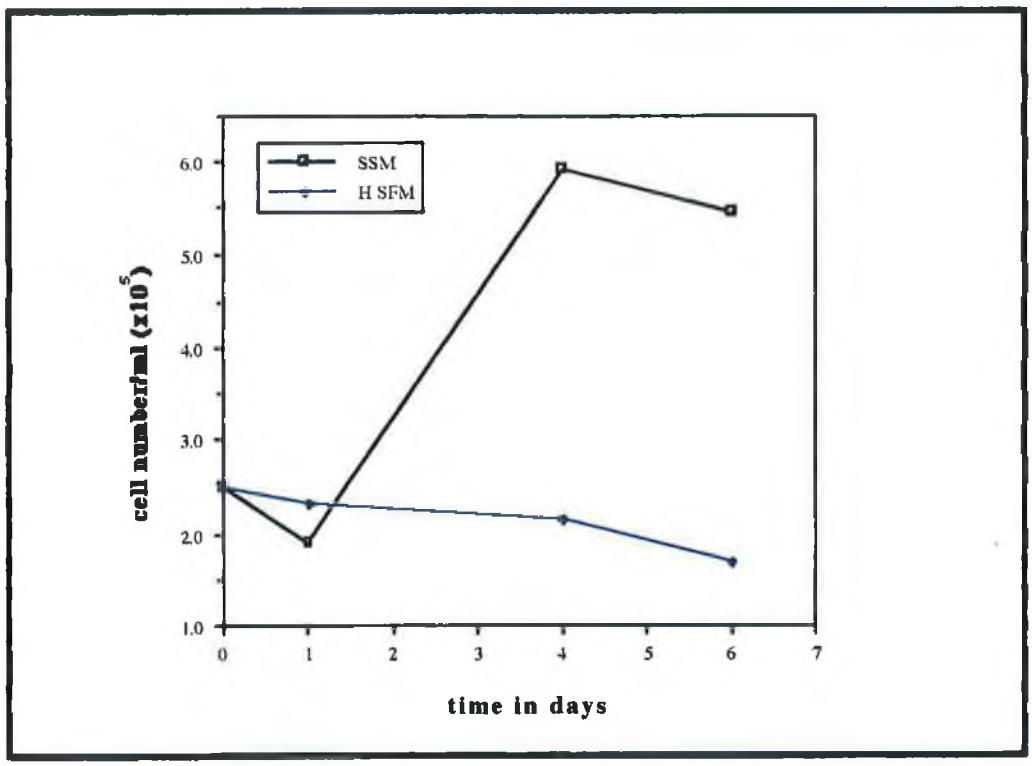


**Fig. 3.1.2** Growth of CHO-K1 cells in Ham's F12 serum-free medium (H SFM) and serum supplemented medium (SSM) in suspension culture. Results are expressed as average cell number x$10^5$ per ml.

### 3.1.1.2 Effect of Pluronic F-68 on growth in SF culture

Pluronic F68 (PF68) is often used in industry as a shear protective agent and also to prevent denaturation of serum proteins during agitation. Bertheussen (1993) suggested the use of PF68 for the SF growth of a number of different cell lines. It was then decided to use this compound in the H SFM (Ham's F12 as basal medium) to see if cell growth was possible in suspension culture. Bertheussen (1993) used a concentration of 20$\mu$g/ml PF68. Good growth of cells was achieved as seen in Fig. 3.1.3.

Addition of Pluronic F68 was found to substantially improve growth over unsupplemented SFM. Viability of these cells was also much higher over the course of the experiment (results not shown).

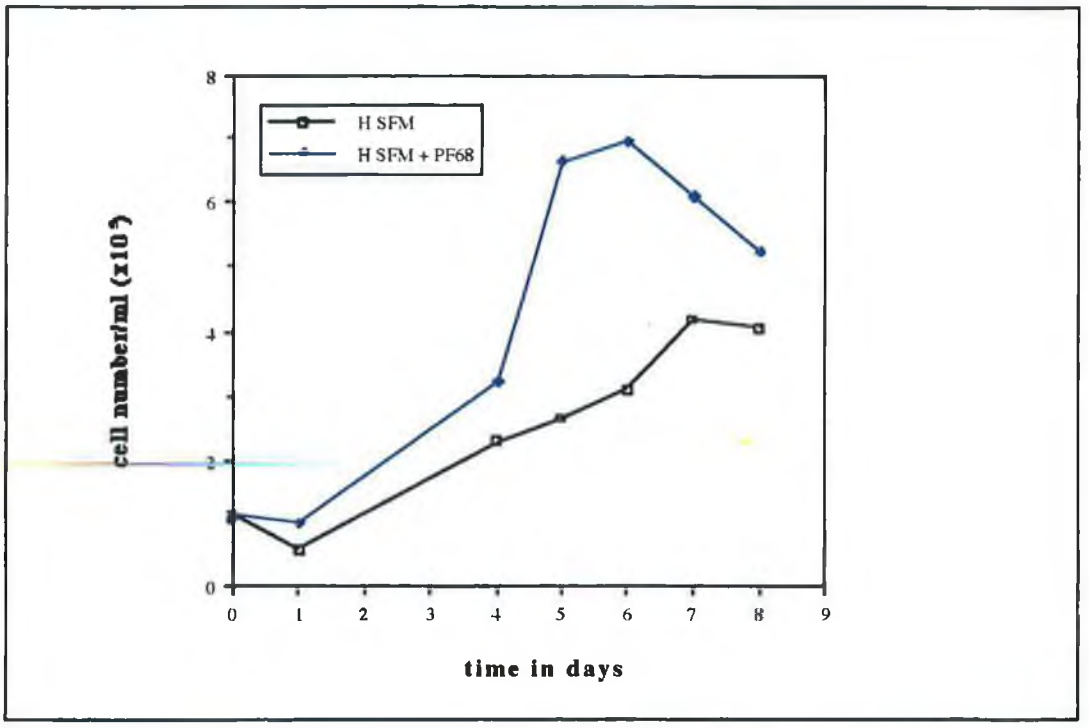


**Fig. 3.1.3** Growth of CHO-K1 cells in SFM with and without Pluronic F68 supplementation. Results are expressed as average cell number x$10^5$ per ml.

Assays (24-well) were then carried out to try to determine a concentration of Pluronic F68 (PF68) in monolayer culture that may give maximum stimulation of growth, or at least to find a high enough concentration that does not result in growth inhibition. Thus maximum protection without growth inhibition could be afforded to the cells.

The first set of assays used concentrations of PF68 between 0 and 2500µg/ml. In the monolayer cultures, 1000 and 2500µg/ml concentrations proved extremely toxic to the cells. Experiments were then carried out to determine the concentration between 0 and 100µg/ml that resulted in the greatest growth stimulation (Fig. 3.1.4). Growth stimulation was seen with a maximum response at 50µg/ml and decreased growth or inhibition beyond this concentration.

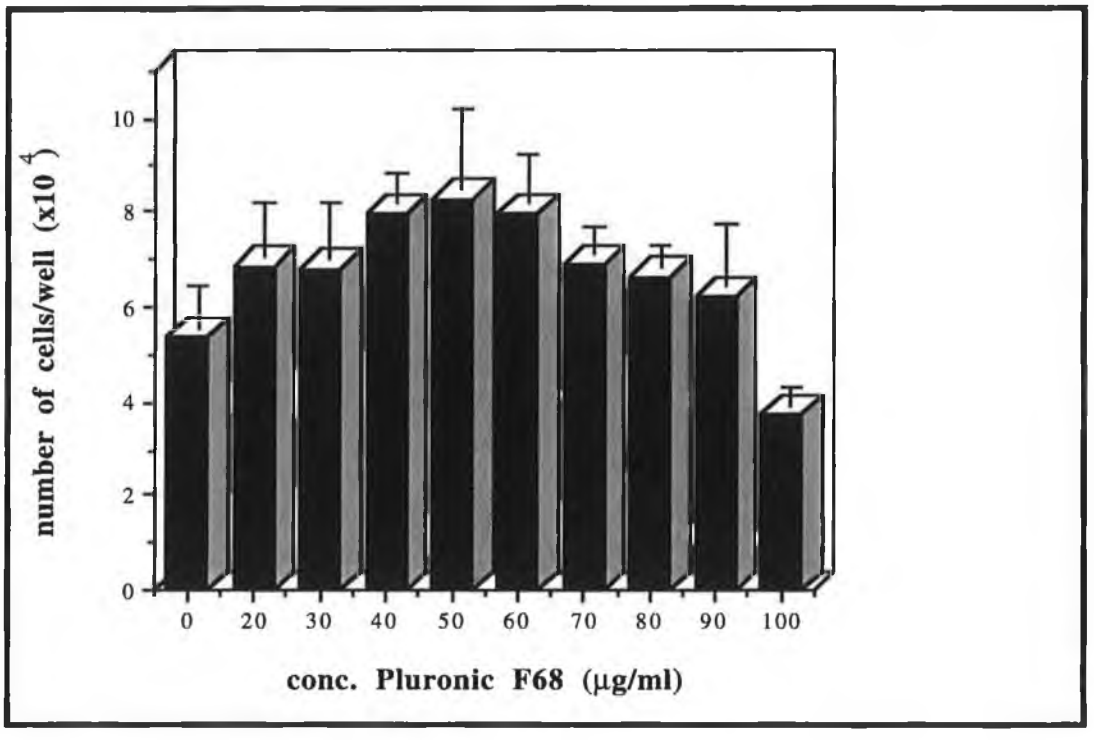


**Fig. 3.1.4** Growth of CHO-K1 cells in different concentrations of Pluronic F68 ranging from 0 to 100µg/ml. Results are expressed as average cell number x$10^4$ per well ± standard deviation (n=3).

On the basis of these results, it was decided to compare the concentration of PF68 used up until now as recommended by Bertheussen, 20µg/ml, with 50µg/ml, to see if the higher concentration of PF68 affords greater protection to the cells from adverse shear forces or actually causes stimualtion of growth. The SFM used was H SFM (Fig. 3.1.5).

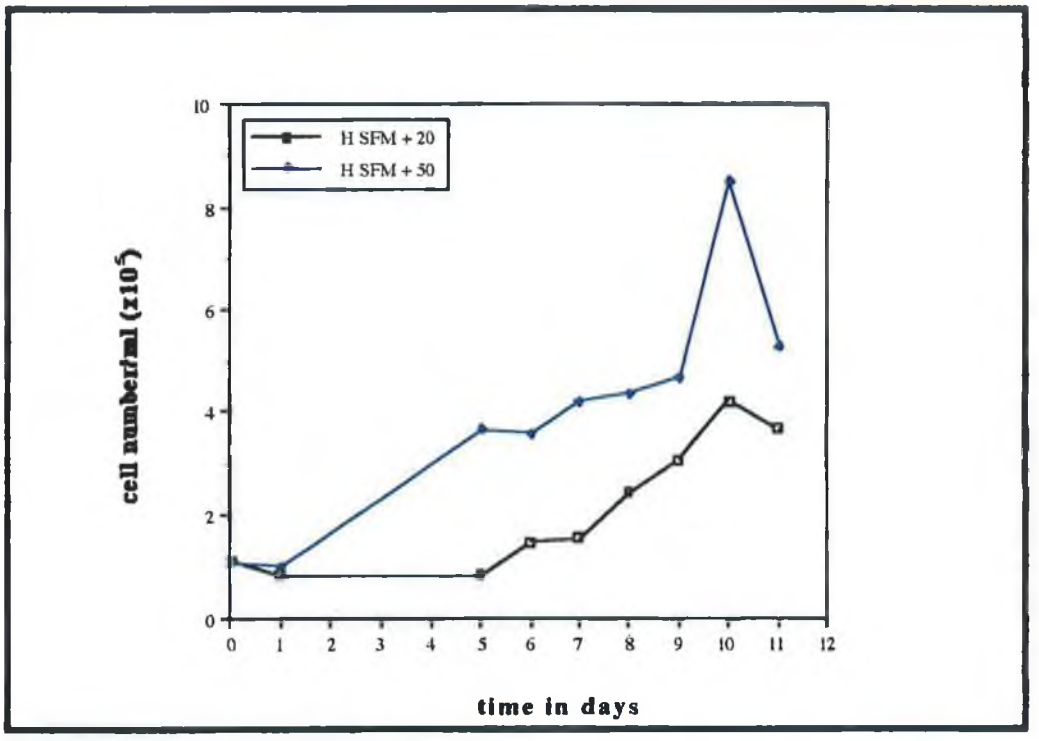


**Fig. 3.1.5** Growth curve for CHO-K1 cells in SFM supplemented with 2 different concentrations of Pluronic F68 (H SFM + 20, supplemented with 20µg/ml; H SFM + 50, supplemented with 50µg/ml Pluronic F68). Results are expressed as average cell number $x10^5$ per ml.

It is clearly evident from the growth curve obtained that 50$\mu$g/ml allows for better growth of the CHO-K1 cell line over a 7 day growth period. Viability was also higher in the 50$\mu$g/ml culture over the 7 day period (results not shown).

### 3.1.1.3 Subcultivation in suspension culture

Experiments were then carried out to determine if the SFM plus Pluronic F68 supplementation was capable of supporting long-term subcultivation of the cell line under SF conditions. Fig. 3.1.6 (a-d) show that this was possible with good growth obtained for 4 passages over a period of about 5 weeks.

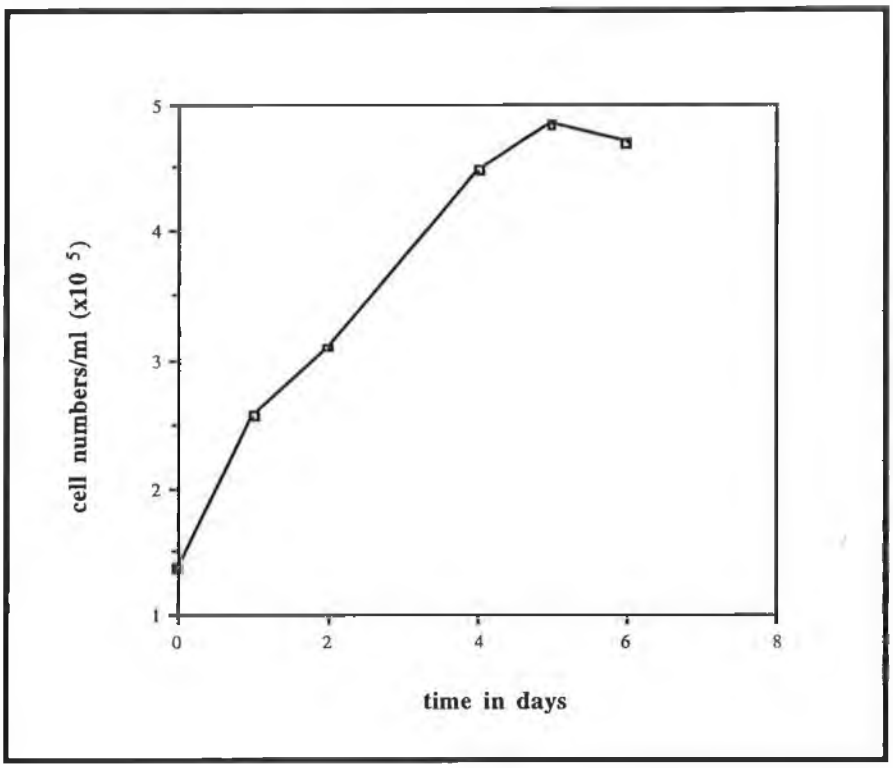


**Fig. 3.1.6(a)** Long-term growth of CHO-K1 cells in H SFM plus 50µg/ml pluronic F68. Growth curve for passage 1 cells. Results are expressed as average cell number x$10^5$ per ml.

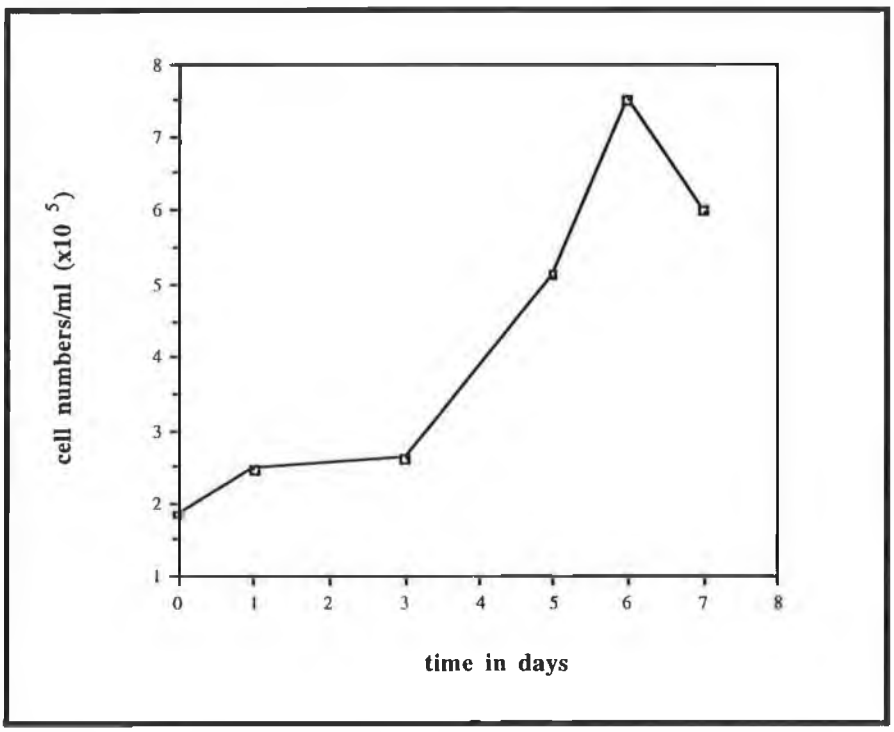


**Fig. 3.1.6(b)** Long-term growth of CHO-K1 cells in H SFM plus 50µg/ml pluronic F68. Growth curve for passage 2 cells. Results are expressed as average cell number x$10^5$ per ml.

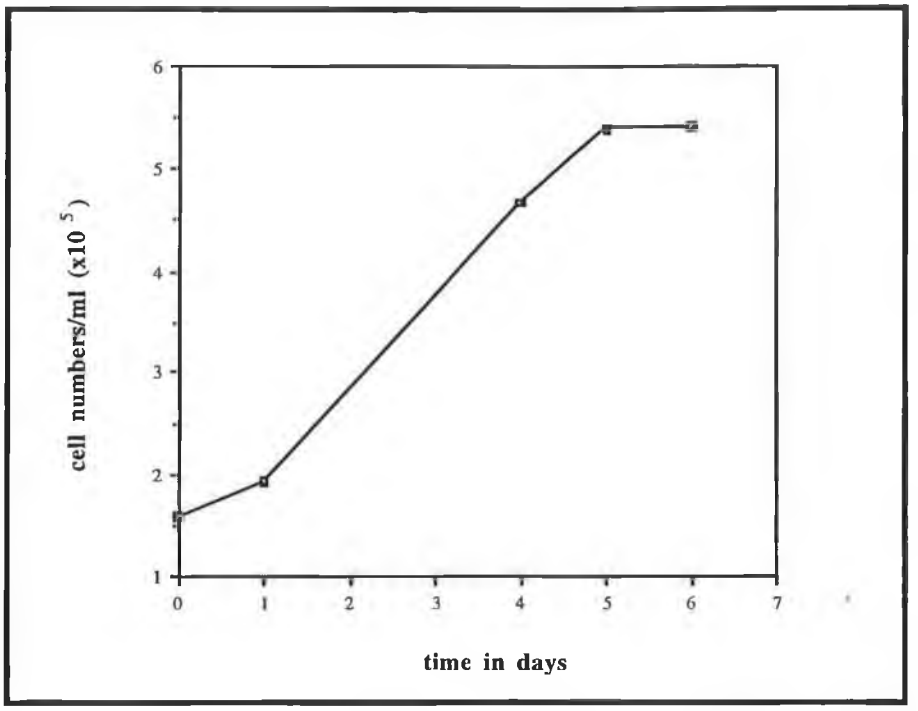


**Fig. 3.1.6(c)** Long-term growth of CHO-K1 cells in H SFM plus 50µg/ml pluronic F68. Growth curve for passage 3 cells. Results are expressed as average cell number x$10^5$ per ml.

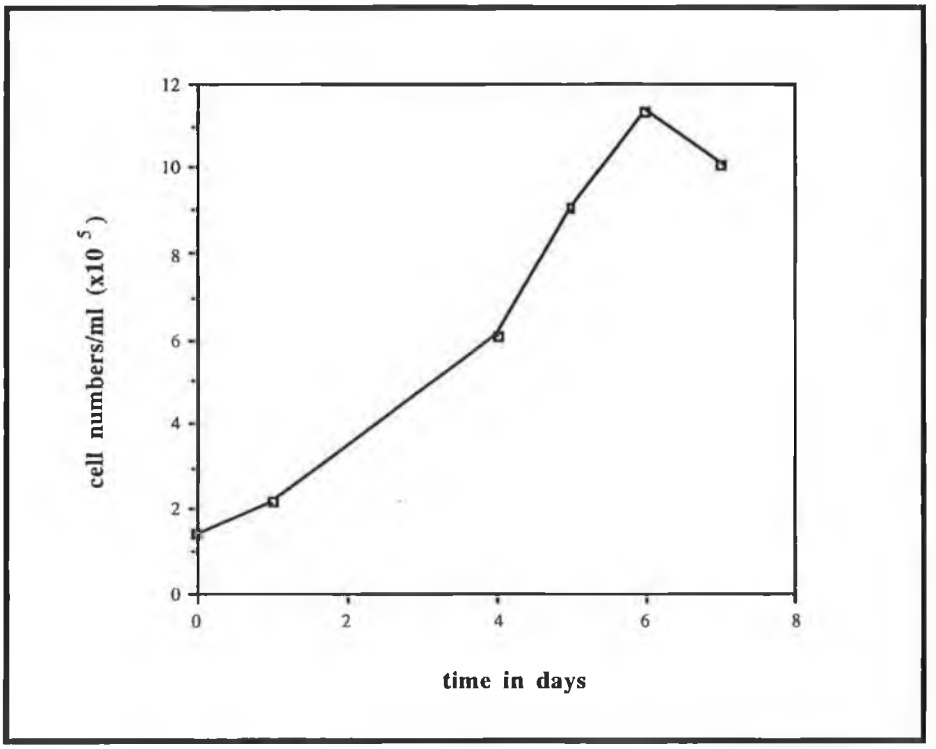


**Fig. 3.1.6(d)** Long-term growth of CHO-K1 cells in H SFM plus 50µg/ml pluronic F68. Growth curve for passage 4 cells. Results are expressed as average cell number x$10^5$ per ml.

### 3.1.1.4 Plasma membrane fluidity measurements in CHO-K1 cells grown in serum-supplemented and serum-free medium

Experiments were undertaken to try and explain the poor growth of CHO-K1 cells in serum-free medium compared to serum-supplemented medium. Plasma membrane fluidity (PMF) appears to be a critical factor in determining the shear sensitivity of cells. An increase in PMF value appears to be associated with an increase in shear sensitivity. Studies were carried set up using the CHO-K1 cell line grown in both SFM and SSM conditions to determine the PMF values in both stationary and agitation culture so as to try and determine if a difference in PMF of the membrane of the SF cells may explain the poor growth of CHO-K1 cells in serum-free suspension culture.

#### 3.1.1.4.1 Determination of PMF for CHO-K1 cells cultured in tissue culture flasks

PMF measurements were carried out as in Section 2.9. The procedure involves labelling the cell membrane with a fluorescent probe (TMA-DPH) which is excited with a polarised monochromatic light. According to the movement of the probe (which depends on PMF) the emitted light will be partially depolarised with respect to the plane of polarisation of the excitation light. The resultant fluorescent anistropy is termed the $r_s$ value. Two parameters are being measured, the local microviscosity and the molecular order which is related to the degree of rotational restriction of the probe in the membrane. Using the steady state fluorescence polarisation method the second parameter is mostly being measured. A change in molecular order is associated with the reciprocal of PMF. Thus an increase in the $r_s$ value is indicative of a decrease in PMF and a decrease in shear sensitivity.

PMF values for CHO-K1 cells growing in serum-supplemented medium and serum-free medium for 3 weeks and 6 months (see Section 3.1.3.3) in tissue culture flasks were determined (see Table 3.1.1).

**Table 3.1.1** PMF values ± standard deviation (n=5) for CHO-K1 growing in tissue culture flasks

| Cell line | Fluorescence Anistropy ($r_s$ value) |
|---|---|
| CHO-K1 SSM | 0.219 ± 0.005 |
| CHO-K1 (3 weeks SFM) | 0.194 ± 0.004 |
| CHO-K1 (6 months SFM) | 0.197 ± 0.007 |

SSM - serum-supplemented medium, SFM - serum-free medium

There is a noted decrease in PMF values for the two serum-free cultures compared to the serum-supplemented control cells. If increased PMF correlates with increased shear sensitivity then these cells should have an increased PMF and thus a decreased $r_s$ value. Thus these cells should be more shear sensitive which has been demonstrated in Section 3.1.1.1.

#### 3.1.1.4.2 Fluorescence anistropy measurement of CHO-K1 cells in serum-free and serum-supplemented suspension culture

PMF values were then determined for CHO-K1 cells in suspension culture over a three day period to try and demonstrate a correlation between cell death in serum-free suspension culture and PMF. Serum-free medium was also supplemented with pluronic F68 at a concentation of 50µg/ml to see if the $r_s$ value is increased, which may explain why cells can survive in serum-free suspension culture with this supplementation.

Cells were seeded into Bellco spinner flasks at a concentration of $1x10^5$/ml and agitated at 50rpm over a 3 day period. Growth curves for viable CHO-K1 in SSM, SFM, SFM plus Pluronic F68 supplementation are shown in Fig. 3.1.7. CHO-K1 grew well at 50rpm in serum-supplemented conditions. However, under serum-free conditions the cells began to die off almost immediately. When pluronic F68 was supplemented to the SFM the cells remained viable in the 3 day assay period. Fluorescence anistropy values were measured at these time points as shown in Fig. 3.1.8.

The $r_s$ value is seen to decrease gradually for CHO-K1 cells in serum supplemented culture. However, the $r_s$ values are significantly higher than for the CHO-K1 serum-free cultures possibly indicating that the cells are more shear sensitive in serum-free conditions. The $r_s$ values were found to be slightly higher for the cells in the PF68 supplemented SFM which may indicate some sort of protective mechanism by this compound. PF68 results in an increase in PMF and a decrease in shear sensitivity.

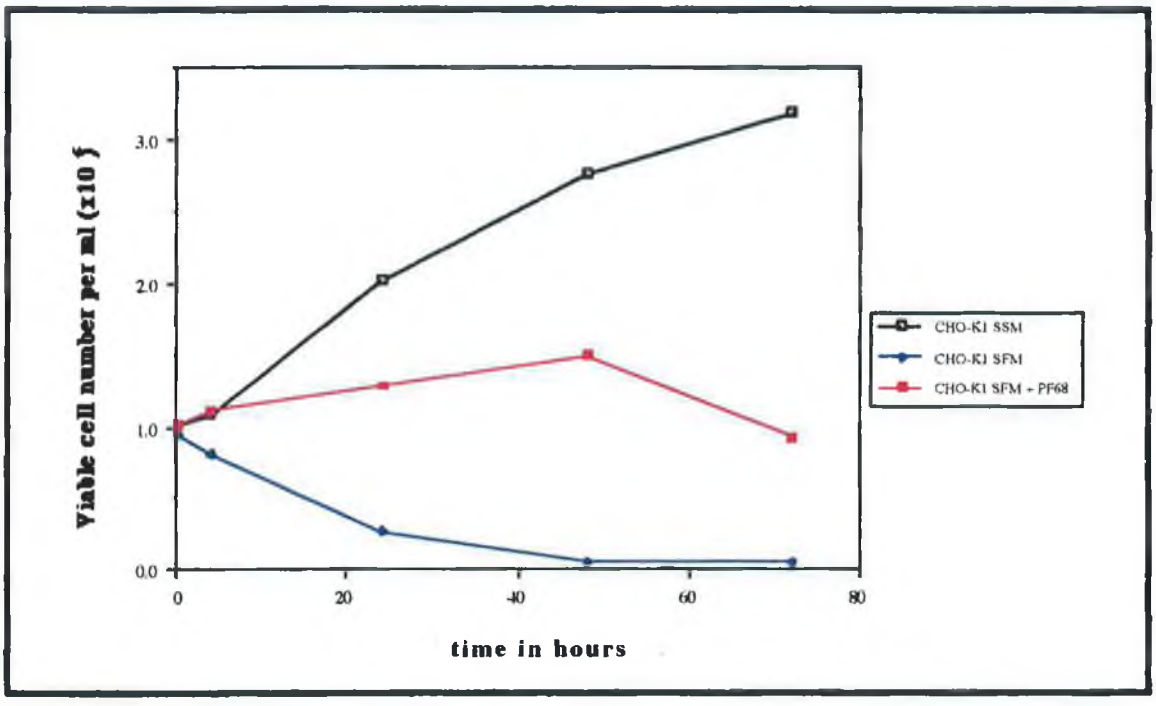


**Fig. 3.1.7** Viable cell number per ml for CHO-K1 cells in serum-supplemented medium (SSM), serum-free medium (SFM) and serum-free medium with pluronic F68 supplementation (SFM + PF68)

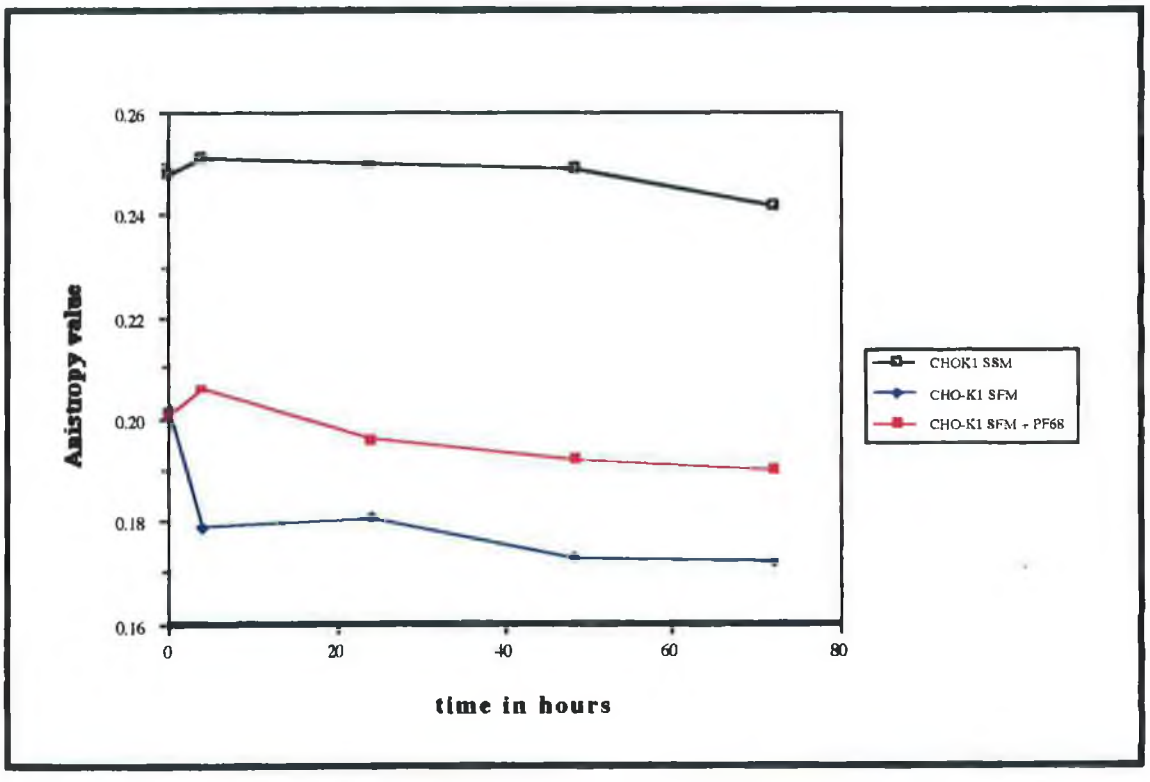


**Fig. 3.1.8** Fluorescence anistropy of CHO-K1 cells in serum-supplemented medium (SSM), serum-free medium (SFM) and serum-free medium supplemented with pluronic F68 (SFM + PF68)

### 3.1.1.5 Growth in SF Microcarrier culture

Microcarriers were introduced into the SFM to see if they allowed for greater protection from shear forces than PF68. Using growth curves, microcarriers were compared to SSM and SFM plus PF68. Growth was compared over a 7 day period in SSM, SFM, SFM plus 50$\mu$g/ml PF68 and SFM plus Cytodex 3 microcarriers at a concentration of 0.3g/100ml (see Section 2.8.5-2.8.7).

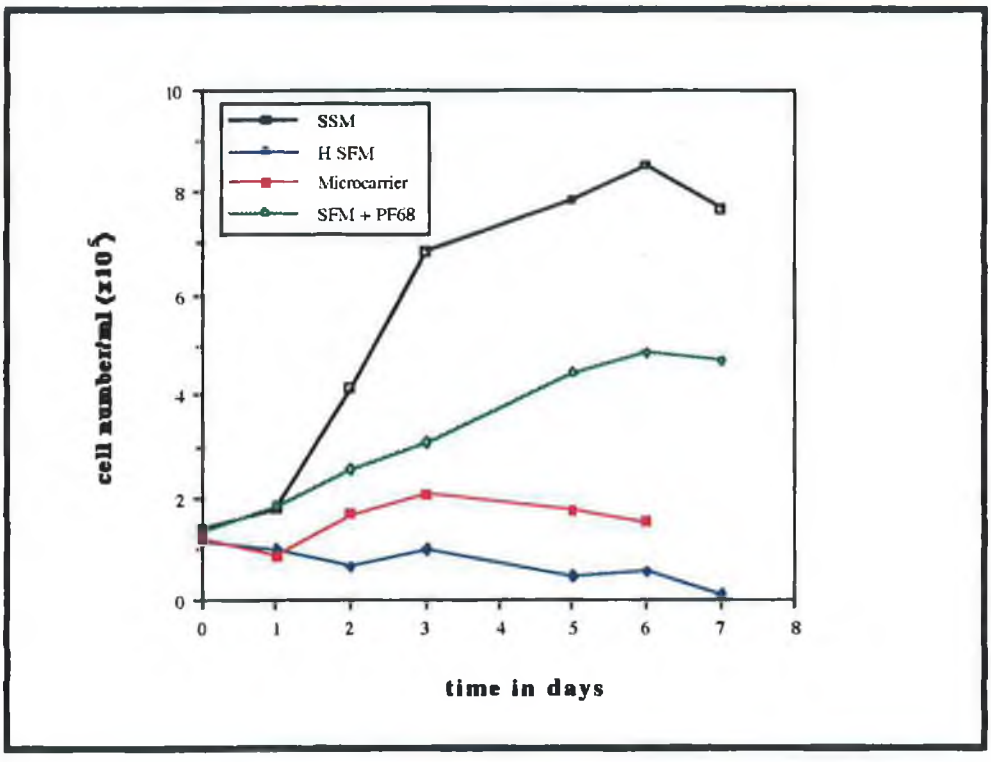


**Fig. 3.1.9** Growth of CHO-K1 cells in serum-free medium (H SFM), H SFM supplemented with Pluronic F68 (H SFM + PF68), H SFM supplemented with microcarriers (Microcarrier) and serum-supplemented medium (SSM). Results are expressed as average cell number x$10^5$ per ml.

As can be seen from Fig. 3.1.9, the microcarriers protected the cells to a certain extent but not as effectively as the serum-free culture plus PF68 supplementation. Again, SF culture without any protective compounds results in cell death.

### 3.1.1.6 Suspension culture using low calcium SFM

Within 24 hours in SF suspension culture, it has been noted that the CHO-K1 cells clump extremely tightly compared to cells growing in serum-supplemented culture. This has also been observed by Renner *et al.* (1993) during the SF growth of another CHO cell line, CHO-SS3. This aggregation appears to affect cell growth with cells at the centre of the aggregate lacking $O_2$ and nutrients, coupled with the adverse shear forces, to cause cell death. $Ca^{2+}$ ions have been thought to play a role in the aggregation of cells which may account for the severe clumping. It was thus decided to try and reduce this clumping by using commercially available basal media that have low $Ca^{2+}$ levels or that are $Ca^{2+}$ free, instead of the basal Ham's F12 being used up until now.

#### 3.1.1.6.1 Growth in low calcium SFM

The first growth curve involved determining whether a low $Ca^{2+}$ (113µM conc.) serum-free media, Ham's F12/ MEM.S (1:1) (H/M) SFM, was even capable of supporting the growth of the CHO-K1 cell line. Cells were grown over a 9 day period in H/M SFM with and without 50µg/ml Pluronic F68 supplementation. Fig. 3.1.10 shows that the medium does support some growth of the cell line when supplemented with PF68.

The second growth curve involved comparing growth of the cell line in H SFM (226µM conc.), Ham's F12/ DMEM (1:1) (H/D) SFM (790µM conc.) and H/M SFM (113µM conc.) and all supplemented with 50µg/ml PF68. Fig. 3.1.11 shows that good growth was obtained in the low $Ca^{2+}$ culture, comparable to that of the high $Ca^{2+}$ cultures, but it was not an improvement. Clumping from microscopic observations was still quite severe.

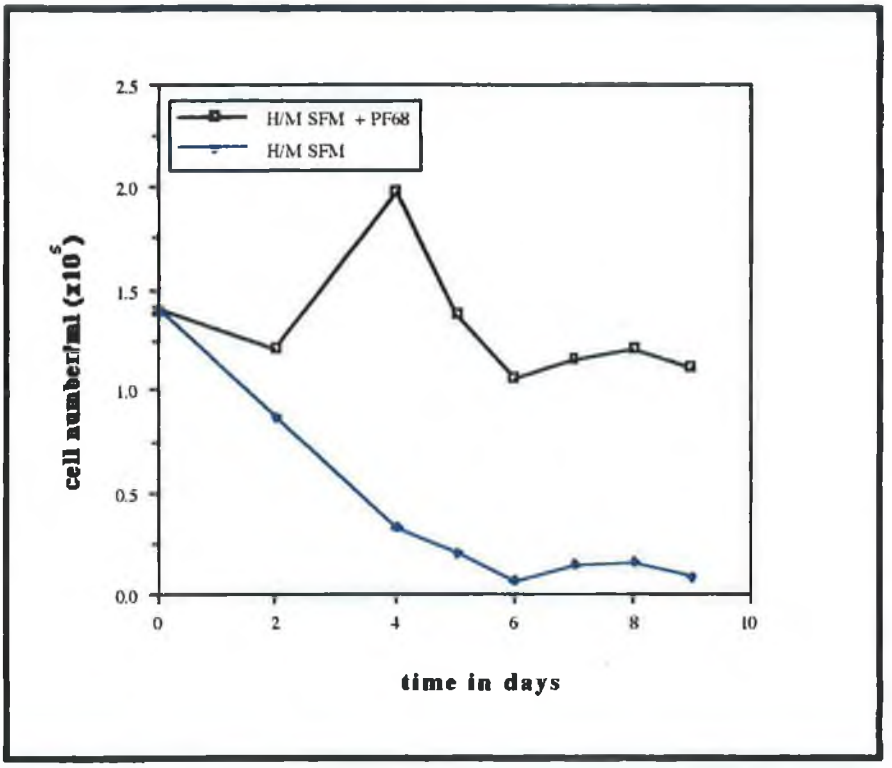


**Fig. 3.1.10** Growth of CHO-K1 in H/M SFM with and without Pluronic F68 at 50µg/ml. Results are expressed as average cell number x$10^5$ per ml.

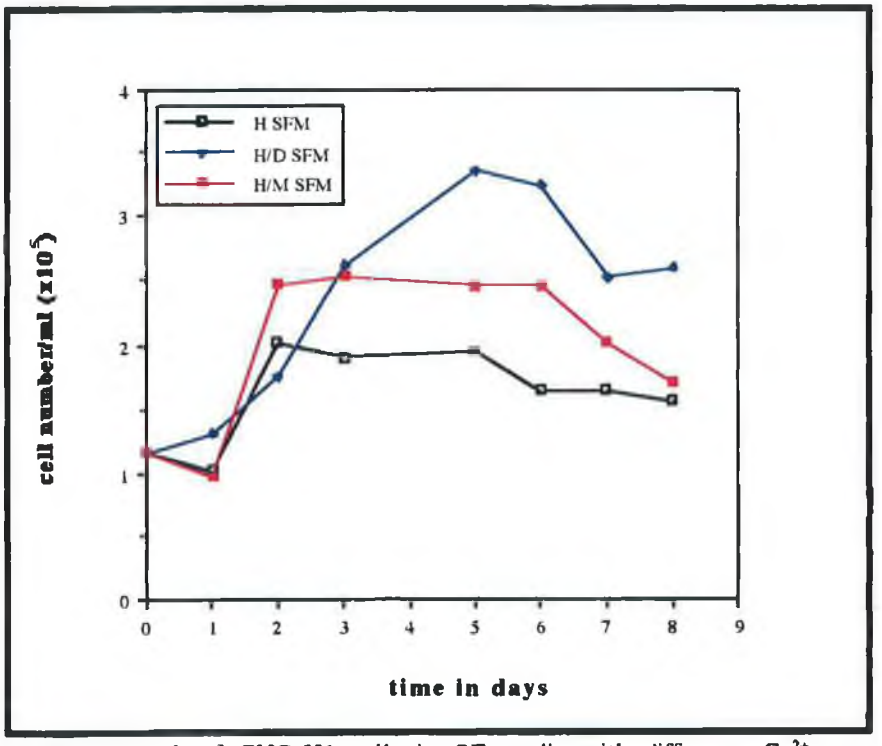


**Fig. 3.1.11** Growth of CHO-K1 cells in SF media with different $Ca^{2+}$ concentrations all supplemented with Pluronic F68 at 50µg/ml. Results are expressed as average cell number x$10^5$ per ml.

### 3.1.1.6.2 Growth in low $Ca^{++}$ and $Ca^{++}$-free SF suspension culture

The next experiment involved comparing growth in high $Ca^{2+}$ (H SFM), low $Ca^{2+}$ (H/M SFM) and $Ca^{2+}$ free (M SFM) over a period of time, all with Pluronic F68 supplementation. Again growth was quite comparable in the high and low $Ca^{2+}$ media, H SFM and H/M SFM respectively (see Fig. 3.1.12). However no growth was observed in the $Ca^{2+}$ free SFM (M SFM). Clumping was still found to be as severe in all serum-free media.

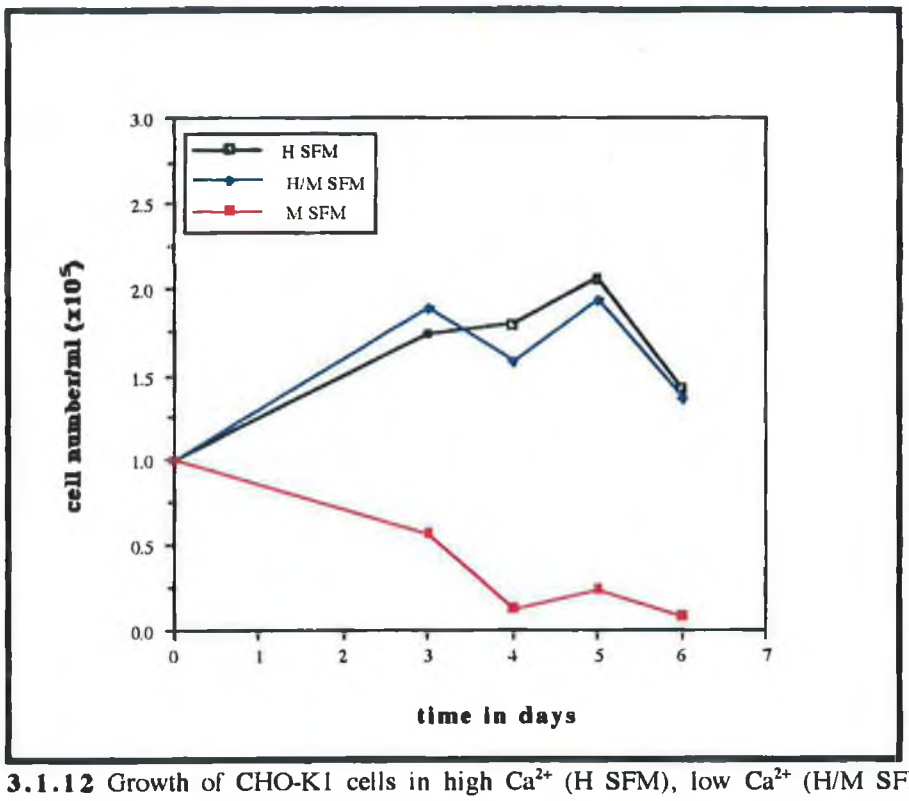


**Fig. 3.1.12** Growth of CHO-K1 cells in high $Ca^{2+}$ (H SFM), low $Ca^{2+}$ (H/M SFM), $Ca^{2+}$-free medium (M SFM) all with Pluronic F68 supplementation. Results are expressed as average cell number $x10^5$ per ml.

### 3.1.2 Long-term growth of CHO-K1 cells in serum-free medium

Long-term subcultivation of a cell line is a good test for a true serum-free medium (SFM). Keenan (1994) found that the SFM developed by Mendiaz *et al.* (1986) for the growth of the CHO-K1 cell line did not support the growth of the cells over a long period of time. Continuous subcultivation was only possible up to 9 passages at most (or 36 days) under SF conditions. After this time, the cells began to detach off the flask surface and usually died.

The aim of this work was to try to extend the cultivation of the cell line over a longer period of time and hopefully determine which substances are being diluted out, or are lacking over long-term subcultivation.

#### 3.1.2.1 Subcultivation of CHO-K1 cells in SFM

A number of subcultivation experiments were carried out on the CHO-K1 cell line. Table 3.1.2 shows the results of two of these experiments.

The results show that the SFM developed by Mendiaz *et al.* (1986) does not support the long-term growth of the cell line. No improvement on the 9 passages (36 days cultivation) achieved by Keenan (1994) was obtained. In the two sets of results shown below, the cells died off after passage number 8 and 7 respectively. Usually a fall off in growth occurred between passages 4 to 6 on average (results not shown).

**Table 3.1.2** Subcultivation of CHO-K1 in SFM

| Passage no. | Experiment 1 | Experiment 2 |
|---|---|---|
| 1 | 1.281 ± 0.119 | 2.260 ± 0.109 |
| 2 | 1.688 ± 0.450 | 1.050 ± 0.060 |
| 3 | 1.850 ± 0.000 | 2.050 ± 0.800 |
| 4 | 1.569 ± 0.106 | 1.650 ± 0.101 |
| 5 | 0.657 ± 0.027 | 1.175 ± 0.027 |
| 6 | 1.448 ± 0.128 | 1.748 ± 0.312 |
| 7 | 0.488 ± 0.050 | 0.145 ± 0.018 |
| 8 | 0.405 ± 0.070 | - |

Results are expressed as average cell number per flask $x10^6$ ± standard deviation (n=3).

Long-term growth of the cell line often resulted in the cells forming aggregates which eventually caused the death of the CHO-K1 cell line after serial cultivation in SFM. Fig. 3.1.13 shows the growth of the CHO-K1 cells at P.1 and P.4 in H SFM showing the formation of aggregates at P.4 in SFM.

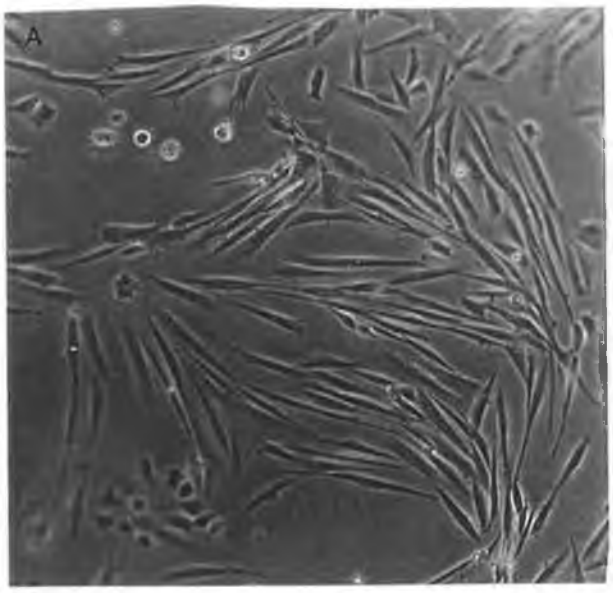


**Fig. 3.1.13(a)** CHO-K1 P.1 in H SFM

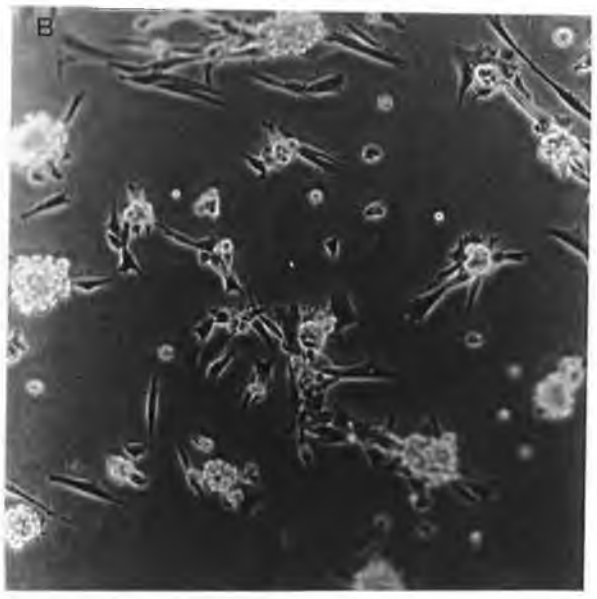


**Fig. 3.1.13(b)** CHO-K1 P.4 in H SFM

### 3.1.2.2 Extension of serum-free subcultivation

Experiments were then carried out to try to extend the subcultivation of CHO-K1 cells by supplementing the SFM with low cost compounds so as to try to avoid the use of expensive cytokines or growth factors.

#### 3.1.2.2.1 Serum-free Briclone

It was decided use SF Briclone, derived from the NCTCC product Briclone, to try to extend the long-term growth of CHO-K1 cells. SF Briclone was used at a 5% concentration supplemented to the Ham's F12 SFM used up until now. Long-term subcultivation experiments were carried out to compare growth in SFM with and without SF Briclone supplementation. As can be seen from Fig. 3.1.14, the SF Briclone allowed extension of the the subcultivation period by approximately 2 passages. Higher cell concentrations were generally achieved during the course of the subcultivations. However, a fall-off in growth in the unsupplemented culture was noted. Growth was really only extended by approximately one week.

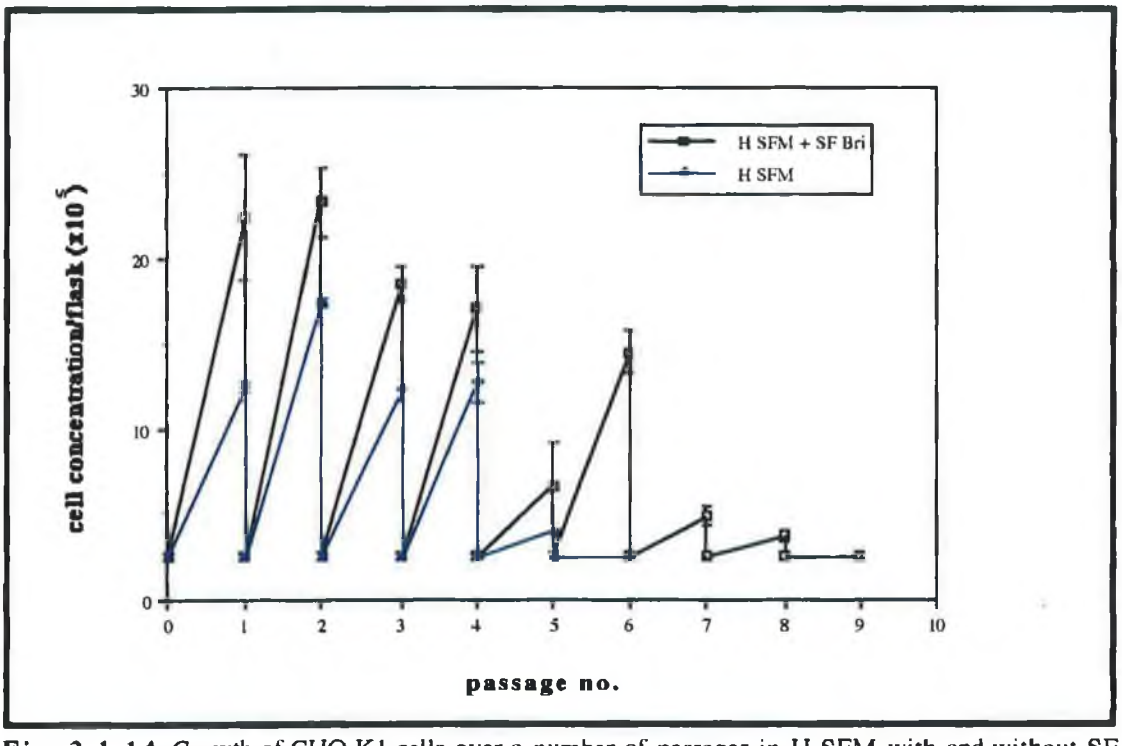


**Fig. 3.1.14** Growth of CHO-K1 cells over a number of passages in H SFM with and without SF Briclone supplementation. Results are expressed as average cell concentration per flask $x10^5$ ± standard deviation (n=3)

As the subcultivations were extended by a week compared to unsupplemented culture, it was decided to attempt to isolate the factors present in the SF Briclone that appeared to be stimulatory to growth and were lost after a number of passages in SFM.

150ml of SF Briclone was fractionated using a YM3 membrane to obtain 15ml of retentate (10x conc.) containing all components with MW > 3000Da and 135ml of filtrate (1x conc.) containing all components with MW < 3000Da. A number of 24-well assays were carried out to compare growth of cells in SFM plus supplemented Briclone fractions - 1x filtrate, 1x retentate, 10x retentate, SF Briclone (unfractionated) - to growth in SFM without supplementation. All the fractions were used at a 5% concentration. Two of the assay results are shown in Fig. 3.1.15(a),(b).

From the two growth curves, it is clear that the 1x filtrate showed the greatest stimulation of growth over the other fractions tried. Generally 1x retentate showed better growth than 10x retentate. However, the SFM and SFM plus 5% SF Briclone results were quite variable between the two assays.

It was then decided to carry out a long-term subcultivation experiment using the various SF Briclone fractions. These assays were carried out in $25cm^2$ vented flasks and passaged every 4 days. The results are shown in Table 3.1.3.

The results in Table 3.1.3 show that again, 5% filtrate supplementation resulted in the greatest stimulation of growth over a four day period, as in passage 1. Cell number reached a total of $40.69x10^5$ from an initial seeding density of $2.5x10^5$/flask. However, the 1x filtrate failed to extend the sub-cultures beyond three passages. The 1x and 10x retentate both failed to stimulate growth beyond 5 passages which is similar to SF Briclone results. It was noted that the 10x retentate showed lower cell number at passage 1 compared to the 1x retentate as was found in the 24-well assays above. However, growth in passages 2, 3 and 4 was much higher in the 10x retentate flasks.

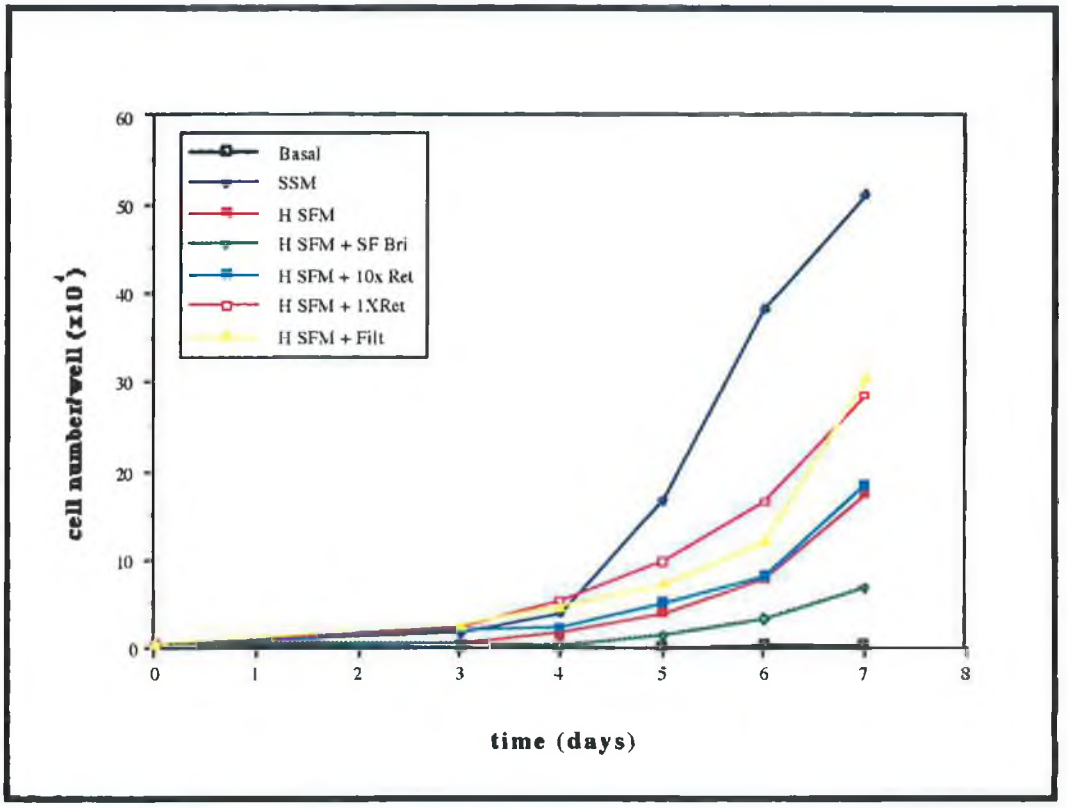


**Fig. 3.1.15** (**a**) above and (**b**) below showing growth of CHO-K1 with SF Briclone fraction supplementation. Results are expressed as average cell number per well x$10^4$ (standard deviation always less than 10%, not shown on graph).

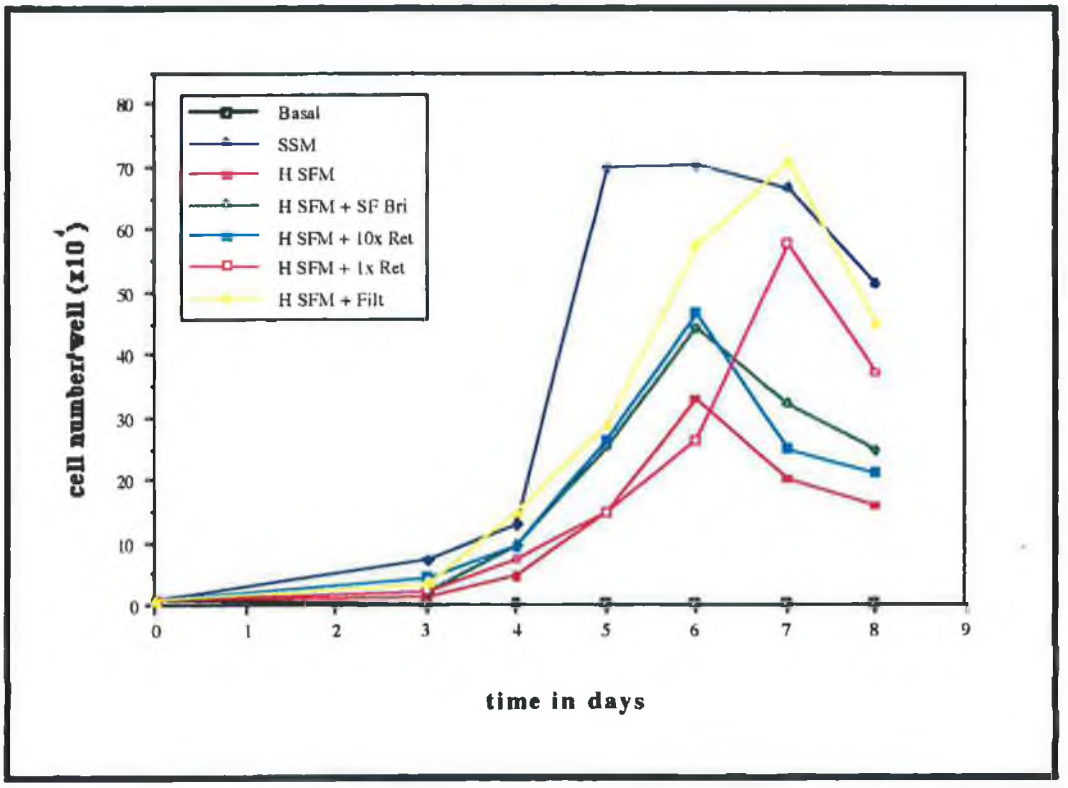

**Table 3.1.3** Long-term subcultivation of CHO-K1 cells using the various SF Briclone fractions

| Passage no. | SFM | + SF Bri | + 5% Filtrate | + 5% Ret 1x | + 5% Ret 10x |
|---|---|---|---|---|---|
| 1 | 21.50 ± 2.22 | 22.44 ± 3.72 | 40.69 ± 2.10 | 34.52 ± 7.39 | 25.46 ± 1.75 |
| 2 | 11.14 ± 0.95 | 33.31 ± 4.90 | 20.31 ± 1.19 | 18.00 ± 0.25 | 37.69 ± 3.94 |
| 3 | 7.65 ± 0.38 | 34.75 ± 4.40 | 1.58 ± 0.30 | 20.75 ± 1.38 | 33.69 ± 0.94 |
| 4 | < 2.5 | 17.06 ± 0.30 | < 2.5 | 12.69 ± 1.81 | 37.63 ± 6.00 |
| 5 | - | < 2.5 | - | < 2.5 | < 2.5 |

Results are expressed as average cell number per flask $x10^5$ ± standard deviation (n=3)

### 3.1.3.2.2 Other components tested in passage number crisis assays

It was then decided to use defined components to try to extend the long-term subcultivation of CHO-K1 cells in SFM, because of the variability in the results associated with the use of the undefined SF Briclone.

The components tested in these assays were as follows - BME vitamins, Pluronic F68 at 20µg/ml, Fibronectin at 10µg/ml, LDL at 100µg/ml, DNase I at 100µg/ml. Conditioned medium (CM) from CHO-K1 cells and SF Briclone were also tested in these assays.

These experiments involved growing CHO-K1 cells in Ham's F12 SFM for 3 passages in 25cm$^2$ flasks. Cells were passaged every 4 days. The cells were then split into 25cm$^2$ flasks containing 8ml of H SFM plus the component to be tested. The positive control was H SFM plus 0.5% FCS and the negative control was H SFM only. The flasks were then passaged every 4 days and seeded at the initial seeding density of 2.5x10$^5$ cells per flask until such a time that the cells had all died or the cell number was less than the initial seeding density. The results of 3 experiments are shown below.

**Table 3.1.4** Effect of different components on extending the subcultivation period for CHO-K1 in SFM

| Exp. no. | BME vitamins | CM CHO-K1 | SF Briclone | Pluronic F68 | Fibro-nectin | LDL | DNase I | +ve control | -ve control |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 5 | 5 | 6 | 6 | - | - | 6 | 6+ | 5 |
| 2 | 4 | 4 | 5 | 5 | 7 | - | - | 7+ | 4 |
| 3 | - | - | - | 6 | 7 | 4 | 4 | 7+ | 5 |

Results are expressed in number of subcultures achieved

Initial results show that fibronectin at a concentration of 10µg/ml allowed the passage number to increase by 2-3 passages over the H F12 SFM, negative control. From microscopic observations, the cells were much more spread out at the higher passages SFM supplemented with fibronectin compared to the H F12 SFM only, where the cells usually started to detach and grow in clumps before growth became inhibited.

### 3.1.2.3 Low Calcium medium - Ham's F12/ MEM.S SFM

Due to the observation of clumping after a number of passages in SF monolayer culture, it was decided to see if the low Calcium medium, H/M SFM, could possibly reduce the clumping of the cells in monolayer culture and extend the long-term subcultivation of the cell line. $Ca^{2+}$ ions have been thought to play a role in the aggregation of cells (Renner *et al.*, 1993) which may explain the clumping noted at the higher passages in H SFM.

Ham's F12/ MEM.S (1:1) SFM, with a $Ca^{++}$ concentration of 113µM (compared to 226µM in H SFM), was used to set up a long-term subcultivation experiment using CHO-K1 cells. The highest number of subcultivations obtained for H SFM was 8 passages (9 obtained by Dr. Joanne Keenan) or 32 days in culture. A subcultivation lasting 52 passages (approximately 11 months) has been successfully obtained using the low Calcium, H/M SFM.

The cells at this stage were frozen under completely serum-free conditions as described in 3.1.4. During the subcultivation experiments shown in Tables 3.1.5 and 3.1.6, the cells appeared to go through a crisis period which lasts between passages 6 and 12 in Experiment 1, between passages 10 and 11 in Experiment 2 and between passages 7 to 10 in Experiment 3. After this crisis, the cells appeared to adapt to the SF conditions with cell counts greatly exceeding counts found initially at passages 1 or 2. Counts for the first 15 to 16 passages are shown for both experiments in Table 3.1.5 and 3.1.6.

From these passage number onwards, very high cell counts have been obtained, usually ranging from 4 to 6 $x10^6$ cells per flask from an initial seeding density of 0.25 $x10^6$ per flask (results not shown). The cells at the higher passages in SF culture appear smaller than earlier passage cells and are not as elongated or as fibroblastic-like. As a result, more space was available for the cell to grow, hence the higher cell number. It appears that the cells adapted to the SFM after going through a crisis period at passages 7 to 11.

The cells in the H/M SFM also appeared to be far less clumped at the higher passages in SFM compared to cells cultured in H SFM. Fig. 3.1.16 shows the morphology of CHO-K1 Passage 4 in serum-free conditions, demonstrating the clumping observed in H SFM.

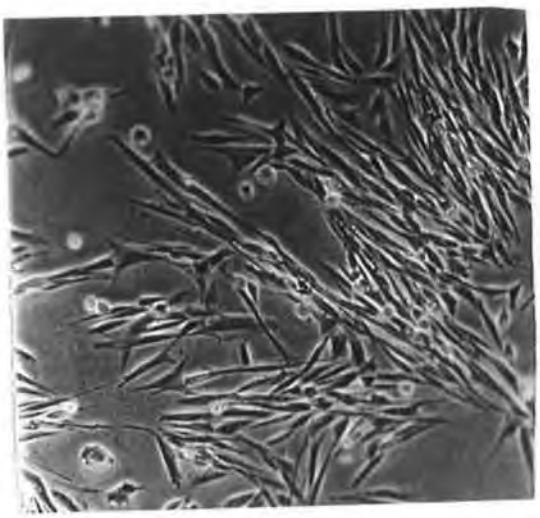

**Fig. 3.1.16(a)** CHO-K1 P.4 in H/M SFM

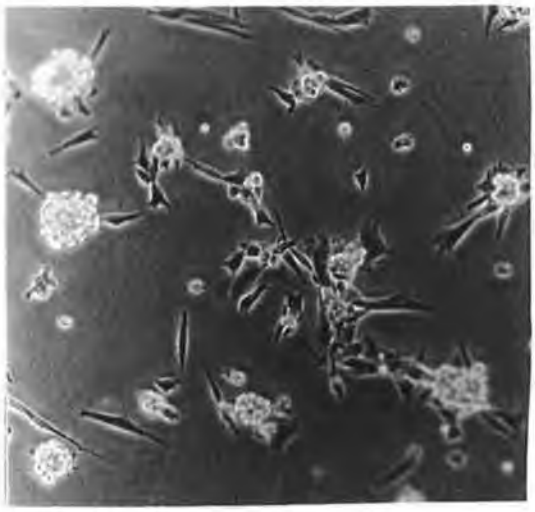

**Fig. 3.1.16(b)** CHO-K1 P.4 IN H SFM

**Table 3.1.5** Long-term subcultivation of CHO-K1 cells in Ham's F12/ MEM.S (1:1) SFM (results are expressed as cell number per flask $x10^5 \pm$ standard deviation (n=2) for experiment 1).

| Passage no. | Experiment 1 | Experiment 2 |
|---|---|---|
| 1 | 16.71 ± 0.710 | 25.25 |
| 2 | 17.31 ± 0.000 | 9.450 |
| 3 | 20.55 ± 4.500 | 29.69 |
| 4 | 14.18 ± 2.930 | 18.70 |
| 5 | 14.29 ± 1.010 | 24.23 |
| 6 | 4.460 ± 0.340 | 11.55 |
| 7 | 3.480 ± 0.530 | 14.93 |
| 8 | 5.160 ± 0.640 | 19.50 |
| 9 | 5.440 ± 0.760 | 17.95 |
| 10 | 5.480 ± 1.400 | 8.210 |
| 11 | 4.440 ± 1.390 | 8.000 |
| 12 | 5.160 ± 1.660 | 48.86 |
| 13 | 11.05 ± 0.330 | 42.70 |
| 14 | 10.30 ± 0.480 | 65.63 |
| 15 | 14.28 ± 1.080 | 45.88 |

**Table 3.1.6** Long-term subcultivation of CHO-K1 cells in H SFM compared to H/M SFM (results are expressed as cell number per flask $x10^5 \pm$ standard deviation (n=2).

| Passage no. | H SFM (EXP. 3) | H/M SFM (EXP. 3) |
|---|---|---|
| 1 | 17.24 ± 1.925 | 17.81 ± 0.481 |
| 2 | 6.250 ± 1.500 | 5.906 ± 0.344 |
| 3 | 10.38 ± 1.875 | 5.938 ± 1.063 |
| 4 | 27.09 ± 3.844 | 13.75 ± 2.375 |
| 5 | 10.41 ± 2.400 | 8.750 ± 1.625 |
| 6 | 5.500 ± 2.063 | 11.31 ± 2.500 |
| 7 | 2.685 ± 0.465 | 5.300 ± 1.550 |
| 8 | 4.050 ± 0.000 | 9.375 ± 0.575 |
| 9 | 9.688 ± 0.000 | 7.656 ± 1.594 |
| 10 | 1.312 ± 0.000 | 4.063 ± 0.938 |
| 11 | - | 11.84 ± 0.094 |
| 12 | - | 7.375 ± 0.188 |
| 13 | - | 7.813 ± 1.813 |
| 14 | - | 12.34 ± 1.031 |
| 15 | - | 17.28 ± 9.156 |
| 16 | - | 6.063 ± 0.188 |

As a consequence of these results it was decided to determine if these cells had adapted to the SFM conditions. An assay was carried out to compare growth of Passage 1 SF to Passage 16 SF CHO-K1 cells over a 7 day period. The assay was carried out in $25cm^2$ flasks containing 8ml of Ham's F12 serum-free medium and seeded at $2.5x10^5$ cells per flask. Three flasks were taken down each day for a seven day period and counted.

As can be seen from the growth curve, Fig. 3.1.17, the cells P.16 SF reached much higher cell numbers, with a maximum cell density of $5.67x10^6$ per flask observed on day 7 compared to a maximum of $2.42x10^6$ per flask for the P.1 SF culture. That the P.1 SF cells showed lower growth than the P.16 cells was surprising as the P.1 cells should still have contained small traces of serum proteins. This seems to suggest that the CHO-K1 P.16 SF cells have adapted to the serum-free conditions in which they have been forced to grow.

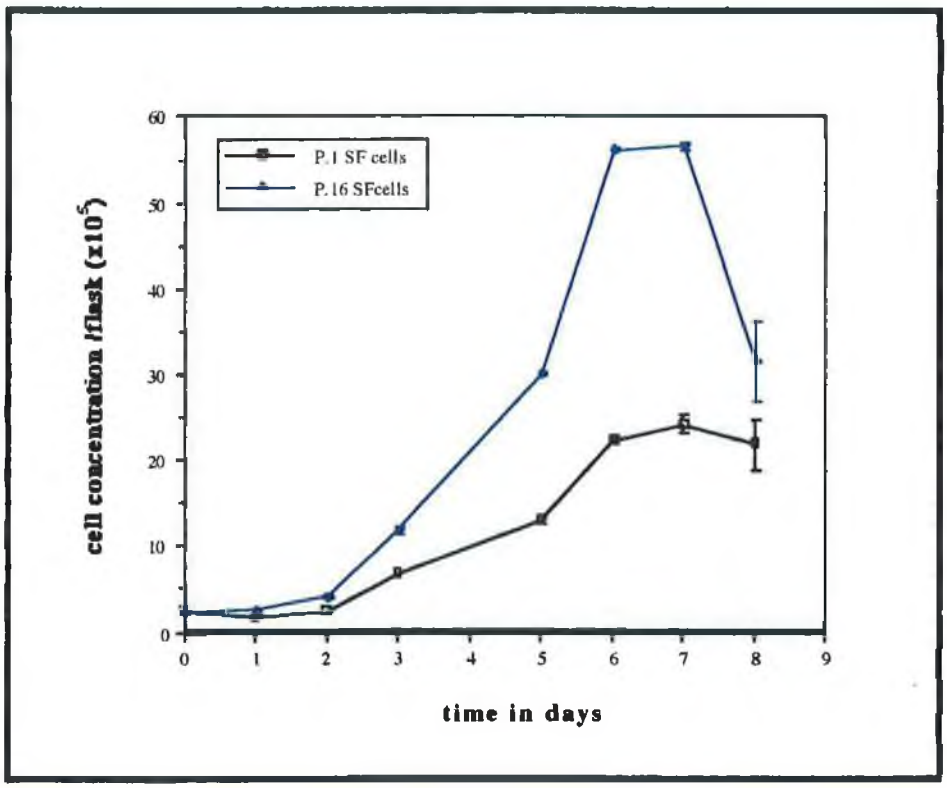


**Fig. 3.1.17** Comparison of growth of CHO-K1 serum-free cells passage 1 and passage 16 in H/M SFM. Results are expressed as average cell concentration per flask $x10^5 \pm$ standard deviation (n=3).

A growth curve was also set up to compare growth of CHO-K1 passage 1 serum-free cells in H SFM and H/M SFM, as a result of the observed differences between the two media types in supporting the long-term growth of the cell line. Assays were set up as previously described, *i.e.* 25cm$^2$ flasks with an initial cell concentration of 2.5x10$^5$ per flask. Two flasks were taken down for the two serum-free media every day over an 8 day period, and counted (Fig. 3.1.18).

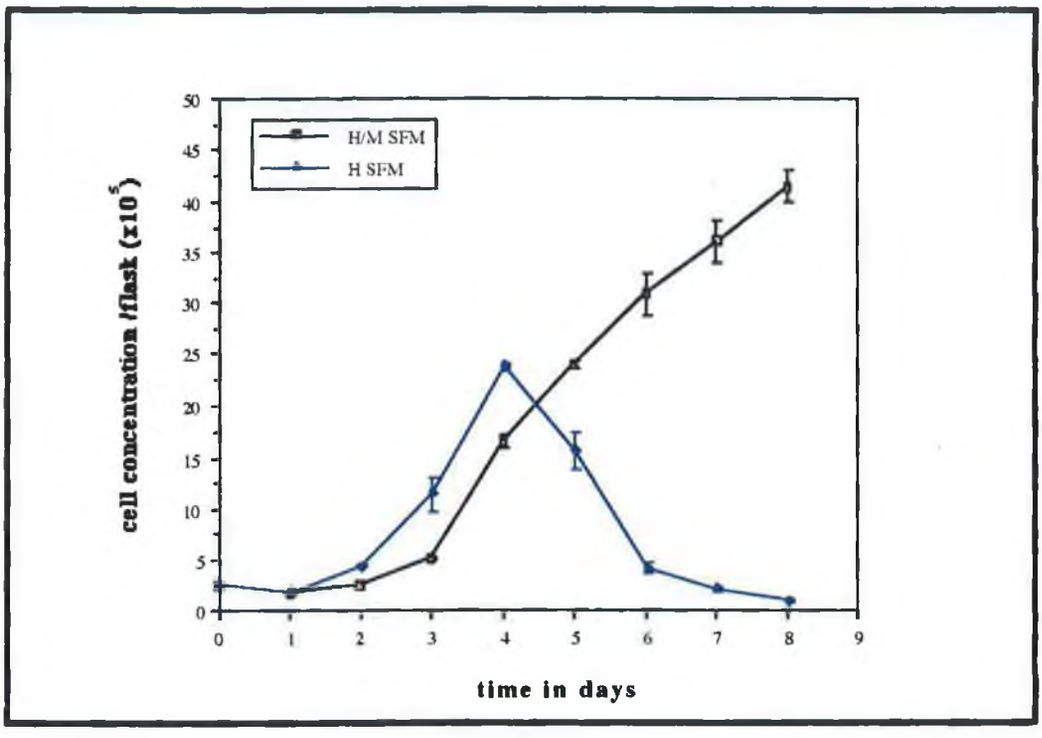


**Fig. 3.1.18** Growth comparison of passage 1 serum-free CHO-K1 cells in H SFM and H/M SFM. Results are expressed as average cell concentration per flask x10$^5$ ± standard deviation (n=2).

The growth curve clearly shows that the low calcium medium, H/M SFM, was capable of supporting better growth of CHO-K1 cells over a longer period of time compared to the high calcium medium, H SFM (greater than 8 days compared to the 4 days for H SFM with much higher cell counts achieved). The cells initially grew faster in the H SFM for the first 4 days. They then started rounding up and detaching from the surface of the flask.

### 3.1.2.4 Calcium addition to low calcium serum-free medium

Experiments were carried out to determine if by adding $Ca^{++}$ to H/M SFM, to a concentration equivalent to H SFM, there is a restriction to the number of passages the cells can grow comparable to that usually obtained for H SFM, where the cells usually die off at passages 4-6 (on average).

Media supplements (Earle's salts, MEM vitamins, amino acids, sodium bicarbonate, phenol red, Table 2.10.1) were bought in to attempt to produce a "home-made" basal medium, to which any concentration of $CaCl_2$ could be added, see Section 2.10.5. When the medium was made up without calcium, the osmolarity was found to be 0.299mOsm/kg and the pH was found to be 7.22 comparable to MEM.S and Ham's F12 basal media.

Subcultivation experiments were carried out to compare growth of CHO-K1 cells in SFM with various concentrations of $Ca^{++}$ ions present.

**Table 3.1.7** Subcultivation experiment for CHO-K1 in SFM with various concentrations of $CaCl_2$ present in addition to the $1x10^{-5}$mg/ml concentration present in the serum-free medium supplements

| Passage no. | H SFM | H/M SFM | M SFM | M SFM + 0.044mg/ml $CaCl_2$ | H/D SFM |
|---|---|---|---|---|---|
| *Conc $CaCl_2$* | *0.044mg/ml* | *0.022mg/ml* | *0mg/ml* | *0.044mg/ml* | *0.158mg/ml* |
| 1 | 9.72 ± 2.34 | 19.9 ± 1.13 | 1.80 ± 0.23 | 12.3 ± 0.16 | 18.1 ± 2.25 |
| 2 | 4.11 ± 0.58 | 14.6 ± 2.98 | - | 0.66 ± 0.02 | 8.91 ± 4.09 |
| 3 | 9.49 ± 0.04 | 8.68 ± 2.11 | - | - | 0.58 ± 0.13 |
| 4 | 4.29 ± 0.47 | 10.4 ± 3.90 | - | - | - |
| 5 | 9.97 ± 0.03 | 3.19 ± 0.44 | - | - | - |

H SFM (Ham's F12 SFM), H/M SFM (Ham's F12/ MEM.S (1:1) SFM), M SFM (MEM.S SFM), H/D SFM (Ham's F12/ DMEM SFM (1:1) SFM. Results are expressed as cell counts per flask $x10^5$ ± standard deviation (n=3).

From this experiment, it is clearly evident that MEM.S SFM medium is not capable of supporting growth of CHO-K1 cells at all. However, supplementation with $CaCl_2$ at concentrations equivalent to HF12 allows good growth for one passage before the cells die off. The very high $Ca^{++}$ SFM (H/D SFM) was capable of supporting growth for only 2 passages, after which time the cells were severely clumped, and then became detached and died off.

Subcultivation experiments were carried out to compare growth of CHO-K1 cells in H/M SFM with $CaCl_2$ supplementation, to that of H SFM (*i.e.* 0.044mg/l). Thus 0.022mg/l of $CaCl_2$ was added to the H/M SFM. If the cells die off at equivalent passage numbers to H SFM, poor growth of the cells at these passage number can possibly be attributed to the high calcium concentrations in the medium. Table 3.1.8 shows the results for two separate experiments.

**Table 3.1.8** Subcultivation of CHO-K1 cells in SFM with different $CaCl_2$ concentrations

| | *Experiment 1* | | | *Experiment 2* | | |
|---|---|---|---|---|---|---|
| **Passage No.** | **H SFM** | **H/M SFM** | **H/M + 0.022mg/l $CaCl_2$** | **H SFM** | **H/M SFM** | **H/M + 0.022mg/l $CaCl_2$** |
| **1** | 7.65 ± 2.10 | 23.48 ± 0.69 | 25.91 ± 1.31 | 9.95 ± 0.00 | 21.26 ± 0.26 | 15.88 ± 1.53 |
| **2** | 7.69 ± 0.39 | 15.75 ± 3.90 | 15.03 ± 0.22 | 3.945 ± 0.08 | 14.69 ± 2.43 | 7.200 ± 0.19 |
| **3** | 0.488 ± 0.10 | 6.13 ± 0.063 | 2.275 ± 0.03 | 3.450 ± 1.36 | 16.45 ± 0.88 | 8.288 ± 0.10 |
| **4** | - | 8.325 ± 0.79 | - | 7.144 ± 0.84 | 8.400 ± 1.35 | 8.700 ± 5.03 |
| **5** | | 15.28 ± 1.21 | | 5.775 ± 1.84 | 8.650 ± 0.15 | 6.675 ± 1.28 |
| **6** | | | | 6.038 ± 0.75 | 8.638 ± 0.79 | 4.500 ± 0.60 |
| **7** | | | | 1.279 ± 1.16 | 5.831 ± 1.11 | 6.413 ± 0.12 |
| **8** | | | | - | 10.29 ± 1.32 | 8.560 ± 0.99 |
| **9** | | | | - | 12.29 ± 2.56 | * |
| **10** | | | | - | 15.26 ± 1.85 | - |
| **11** | | | | - | 14.29 ± 1.55 | - |

Ham's F12 SFM (H SFM), Ham's F12/ MEM.S SFM (H/M SFM) with and without $CaCl_2$ supplementation. Results are expressed in terms of cell counts per flask $x10^5$ ± standard deviation (n=2)

* cells died off at this passage

These results suggest that $CaCl_2$ may be responsible, possibly among other factors, for the fall off in growth in H SFM after a number of passages in SFM. Experiment 1 shows a fall off in growth at passage 3 for both H SFM and $CaCl_2$ supplemented H/M SFM while experiment 2 shows a fall off in growth at P.7 for H SFM and P.8 in H/M SFM supplemented with $CaCl_2$.

### 3.1.3 Cryopreservation of CHO-K1 cells under serum-free conditions

Serum-free cryopreservation of the CHO-K1 cells has been carried out using polyvinylpyrrolidone (PVP) and methylcellulose and comparing to the use of foetal calf serum. Concentrations used were those recommended by Merten *et al.* (1995).

Cells were frozen at concentrations of $5x10^6$ per vial with 0.1% Methylcellulose plus 10% DMSO in SFM, 3.0% PVP plus 10% DMSO in SFM and compared to cells frozen under serum conditions, using 90% FCS + 10% DMSO, as described in Section 2.7.3. Cells were frozen for 7 days and then thawed to assess short term viability when using these compounds during the freezing process. Some vials were also frozen for approximately 300 days and then thawed to assess long term viability of the cells under these conditions.

The first two attempts at freezing using 10% (v/v) DMSO and 0.1% (w/v) MC were unsuccessful possibly due to a number of reasons. Perhaps the cells were not in the exponential phase when they were frozen or the cells were not very healthy when they were frozen. However, two separate attempts were successful and these results are presented in Table 3.1.9.

**Table 3.1.9** Viability and attachment ability of CHO-K1 cells frozen under SF conditions

| | *7 DAYS* | | *300 DAYS* | |
|---|---|---|---|---|
| **Freezing medium** | **% viability on thawing** | **Cells ability to adhere** | **% viability on thawing** | **Cells ability to adhere** |
| MC + DMSO | 78.21 ± 2.87 | most attached < 1 hr | 86.46 ± 1.87 | most attached < 1 hr |
| PVP + DMSO | 84.07 ± 1.38 | most attached < 1 hr | 93.14 ± 2.85 | most attached < 1 hr |
| FCS + DMSO | 84.24 ± 0.91 | most attached < 1 hr | 86.01 ± 1.98 | most attached < 1 hr |

Results are expressed as average percentage viability ± standard deviation (n=6)

This experiment shows that methylcellulose or PVP in combination with DMSO are very effective compounds for serum-free cryopreservation. Thus serum-free conditions can be maintained when freezing cells.

## 3.2 DLKP IN SERUM-FREE MEDIUM

DLKP is a human lung cell line derived in our laboratory from a lymph node biopsy and has been histologically diagnosed as a 'poorly differentiated squamous carcinoma'. Development of a serum-free system for this cell line allows a system that can be used in toxicity profiles and in growth factor and differentiation assays, without serum interference or the variability in results associated with the use of serum.

The serum-free medium developed by Mendiaz *et al.* (1986) and extensively investigated by Keenan (1994) was used as a starting base for the growth of this cell line in SFM. This medium was used because of its simplified nature and the presence of only one mitogen, insulin.

### 3.2.1 Growth of DLKP in H SFM

Initial experiments involved determining whether this simple serum-free medium actually supported growth of the cell line. Cells were set up in 24-well assay plates in SSM, SFM and Basal medium over a 5 day period with one plate taken down every day for five days and cell counts were determined. The following growth curve, Fig. 3.2.1, shows that the serum-free medium is capable of supporting the growth of the cell line, reaching a cell density of $1.6x10^4$ per well from an initial seeding density of $0.5x10^4$ per well. The basal Ham's F12 showed only a very slight increase in cell number, while the SSM showed a high cell density of $6.6x10^4$ per well on day 5. Thus, growth in the serum-free medium is higher than the basal medium but a lot lower than in SSM. However, these initial results looked very promising.

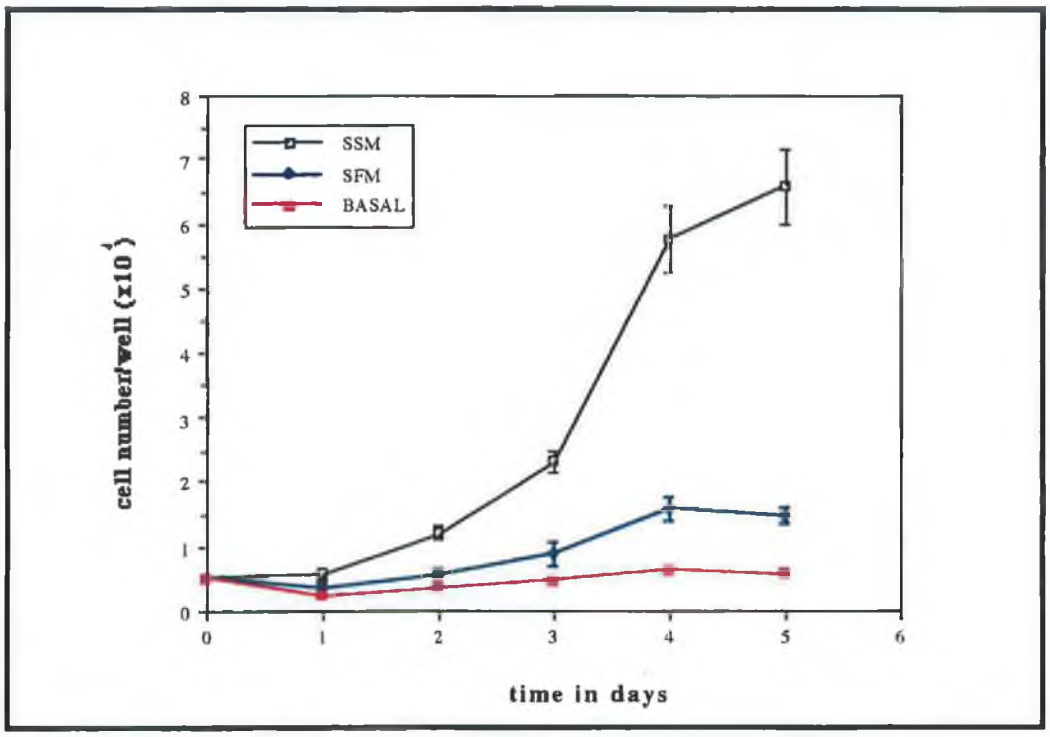


**Fig. 3.2.1** Growth curve for DLKP cells in serum-supplemented medium (SSM), serum-free medium (SFM) and Basal medium (BASAL) over a 5 day period. Results are expressed as average cell number per well $x10^4$ ± standard deviation (n=3).

### 3.2.2 Long-term sub-cultivation and adaptation in SFM

The true test of a serum-free medium, as stated before, is its capability of supporting the long-term sub-cultivation of a cell line. It has been found that the serum-free medium is capable of supporting the continuous cultivation of the DLKP cell line. The medium has been capable of supporting the growth of the cells for at least 2-years in a completely defined system. It has been observed that the cells appear to adapt to the serum-free conditions after a number of passages. Table 3.2.1 shows the growth of DLKP cells over a period of 8 passages in SFM, SSM and basal medium. The cells in basal Ham's F12 die off after 3 passages. Table 3.2.2 shows 3 subcultivation experiments, up to 20 passages, for DLKP cells in SFM. Generally, there is an increase in cell number from about passage 10 onwards showing adaptation of these cells to their SF conditions.

**Table 3.2.1** Subcultivation of DLKP in serum-free medium (SFM), basal medium (Basal) and serum-supplemented medium (SSM)

| | *Experiment 1* | | |
|---|---|---|---|
| **Passage no.** | **SFM** | **Basal** | **SSM** |
| 1 | 4.850 ± 0.050 | 4.200 ± 0.020 | 15.65 ± 0.15 |
| 2 | 9.987 ± 0.513 | 4.175 ± 0.085 | 17.40 ± 1.25 |
| 3 | 8.300 ± 0.800 | 1.750 ± 0.885 | 16.40 ± 1.85 |
| 4 | 4.263 ± 0.262 | - | 17.63 ± 0.57 |
| 5 | 4.850 ± 0.000 | - | 15.10 ± 0.19 |
| 6 | 5.975 ± 0.775 | - | 16.05 ± 1.25 |
| 7 | 4.950 ± 0.475 | - | 21.90 ± 0.99 |
| 8 | 2.075 ± 0.000 | - | 18.20 ± 1.55 |

Results expressed as average cell counts per flask $x10^5$ ± standard deviation (n=3).

**Table 3.2.2** Subcultivation of DLKP in serum-free medium (SFM)

| **Passage no.** | **Experiment 2** | **Experiment 3** | **Experiment 4** |
|---|---|---|---|
| 1 | 3.600 ± 0.050 | 4.850 ± 0.000 | 4.000 |
| 2 | 5.800 ± 1.075 | 4.312 ± 0.487 | 4.481 |
| 3 | 4.700 ± 0.425 | 4.338 ± 0.562 | 6.475 |
| 4 | 2.925 ± 0.150 | 3.400 ± 0.300 | 1.388 |
| 5 | 4.500 ± 0.000 | 3.263 ± 0.588 | 3.787 |
| 6 | 4.219 ± 0.431 | 6.562 ± 0.562 | 2.887 |
| 7 | 6.638 ± 0.000 | 5.213 ± 2.062 | 3.000 |
| 8 | 6.750 ± 0.075 | 4.500 ± 1.012 | 9.188 |
| 9 | 7.312 ± 0.600 | 3.825 ± 0.825 | 8.438 |
| 10 | 6.253 ± 0.403 | 7.669 ± 0.281 | 11.363 |
| 11 | 8.288 ± 1.725 | 5.900 ± 0.150 | 7.013 |
| 12 | 9.381 ± 2.751 | 10.43 ± 0.470 | 9.450 |
| 13 | 9.485 ± 2.520 | 6.463 ± 1.113 | 6.225 |
| 14 | 5.250 ± 0.700 | 9.725 ± 0.575 | 13.25 |
| 15 | 11.275 ± 0.075 | 8.600 ± 0.200 | 8.325 |
| 16 | 13.675 ± 1.075 | 8.375 ± 1.725 | 12.25 |
| 17 | 11.400 ± 2.450 | 10.975 ± 0.008 | 10.406 |
| 18 | 15.175 ± 2.475 | 11.488 ± 1.788 | 11.00 |
| 19 | 9.500 ± 0.100 | 12.675 ± 0.000 | 8.125 |
| 20 | 9.475 ± 0.375 | 10.138 ± 0.138 | 17.125 |

Results expressed as average cell counts per flask $x10^5$ ± standard deviation (n=2) for experiments 2 and 3

Growth assays using high and low passage SF cells were carried out to try to determine whether adaptation has occurred. Assays were carried out in 24-well plates comparing growth of P.1 SF cells to P.13 SF cells over a 6 day period. The following growth curve was obtained.

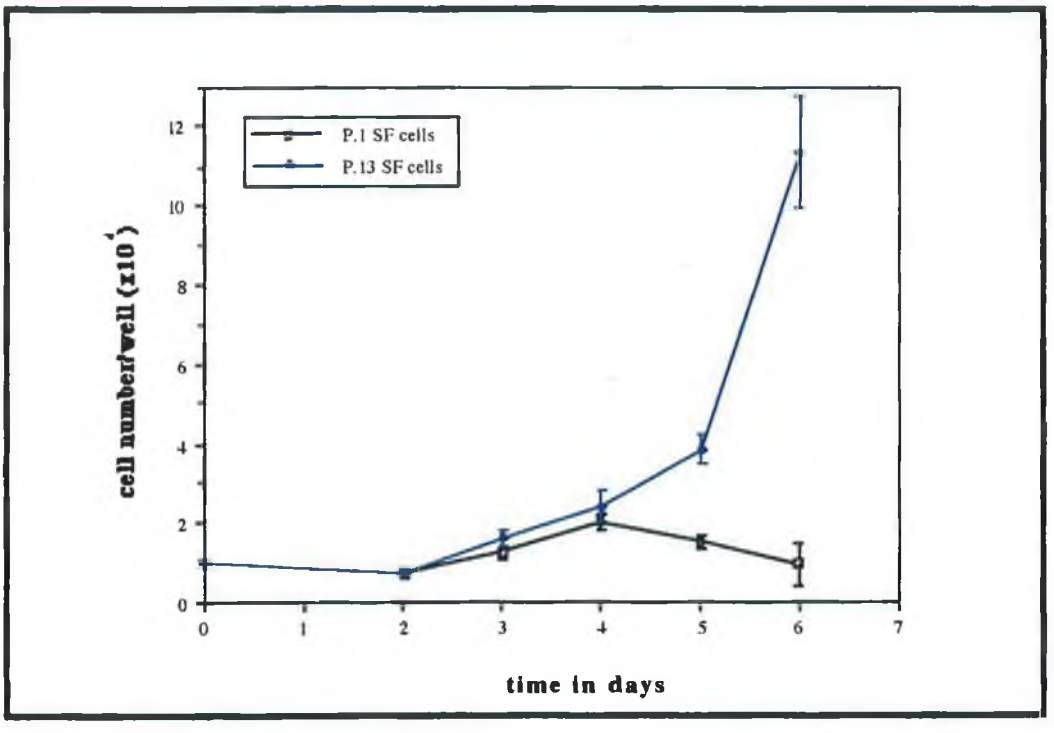


**Fig 3.2.2** Growth curve for passage 1 and passage 13 serum-free DLKP cells in SFM. Results are expressed as average cell number per well $x10^4$ ± standard deviation (n=3).

As can be seen from Fig 3.2.2, the cells P.13 in SFM showed much higher cell number over the 6 day period compared to P.1 cells in SFM.

Experiments were then carried out to investigate changes in the growth properties of the DLKP cells growing for at least two years in this serum-free medium. Growth of the adapted (2-yr) serum-free cells were compared to the parental DLKP cells in serum-free medium, serum-supplemented medium and in basal medium. It was observed that the 2-year SF cells showed much better growth in SFM, see Fig.3.2.3 and Table 3.2.3 compared to the parental cells, suggesting that the cells have adapted well to the conditions in which they were forced to grow over the past two years. It was also observed that the 2-year SF cells showed a restrained growth pace under serum-supplemented conditions compared to the parental cells see Table 3.2.4 and Fig.3.2.4. The 2-year SF cells also showed better poliferation in basal medium compared to the parental cells, see Table 3.2.5 and Fig. 3.2.5.

**Table 3.2.3** Growth of DLKP over a 7 day period in serum-free medium

| | *Experiment 1* | | *Experiment 2* | |
|---|---|---|---|---|
| **Time in days** | **parental DLKP** | **2yr SF DLKP** | **parental DLKP** | **2yr SF DLKP** |
| 1 | 1.350 | 1.275 | 0.950 | 1.800 |
| 2 | - | - | 1.650 | 2.475 |
| 3 | 3.750 | 5.325 | 2.400 | 4.688 |
| 4 | 4.463 | 7.500 | 3.263 | 6.975 |
| 5 | 0.450 | 7.200 | 3.562 | 7.125 |
| 6 | 0.337 | 11.96 | 1.388 | 7.613 |
| 7 | 0.300 | 13.19 | 0.563 | 7.238 |

Results are expressed as cell concentration per flask $x10^5$.

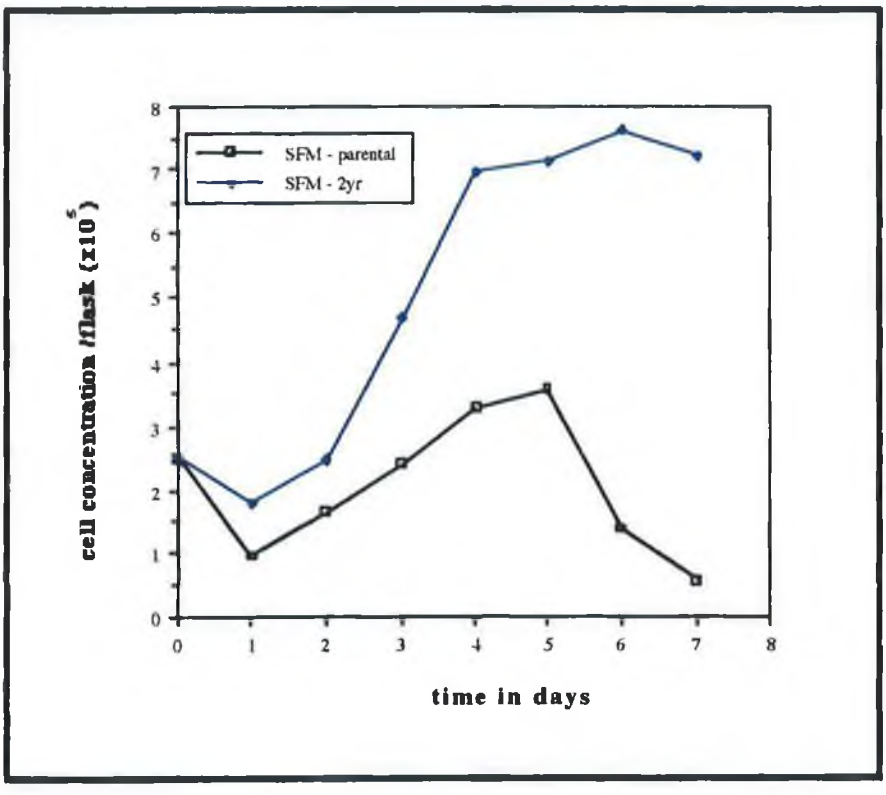


**Fig. 3.2.3** Growth comparison in serum-free medium of DLKP parental cells to cells grown for 2-years in SFM. Results expressed as cell concentration per flask $x10^5$, experiment 2 results shown from Table 3.2.3.

**Table 3.2.4** Growth of DLKP over a 7 day period in serum-supplemented medium

| | *Experiment 1* | | *Experiment 2* | |
|---|---|---|---|---|
| **Time in days** | **parental DLKP** | **2yr SF DLKP** | **parental DLKP** | **2yr SF DLKP** |
| 1 | 3.150 | 2.100 | 3.400 | 3.375 |
| 2 | 5.625 | 5.000 | 4.450 | 3.500 |
| 3 | 18.15 | 7.275 | 8.100 | 5.925 |
| 4 | 35.00 | 11.75 | 18.52 | 10.80 |
| 5 | 33.75 | 15.75 | 19.20 | 14.63 |
| 6 | 37.00 | 20.37 | 26.37 | 14.40 |
| 7 | - | - | 22.63 | 11.50 |

Results are expressed as cell concentration per flask $x10^5$.

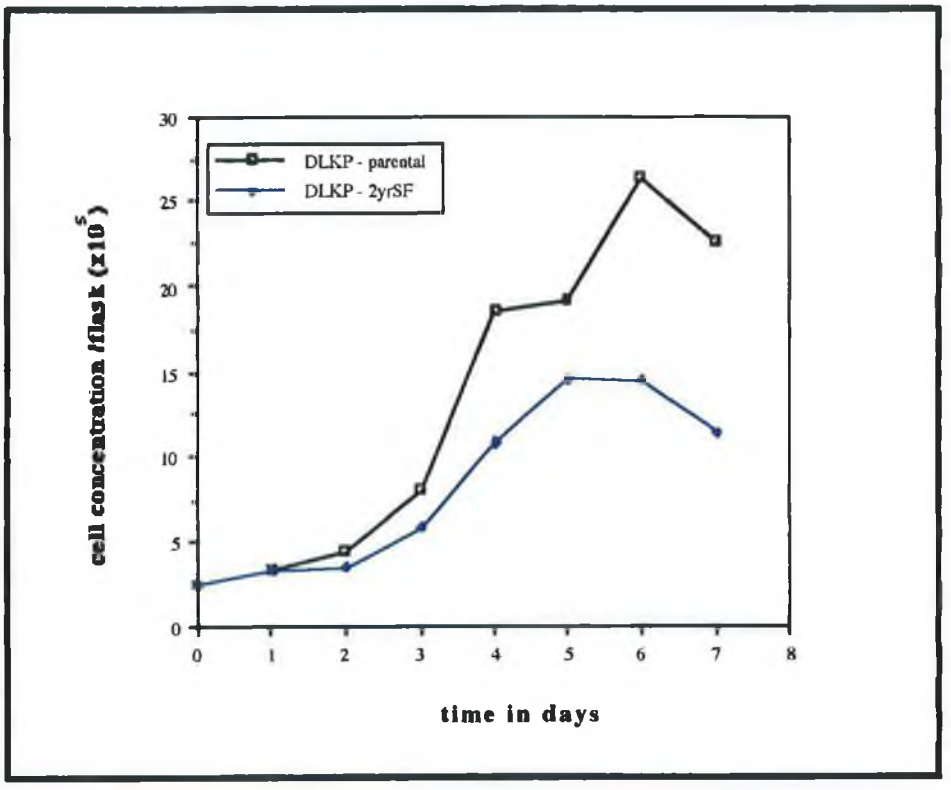


**Fig. 3.2.4** Growth comparison in serum-supplemented medium of DLKP parental cells to cells grown for 2-years in SFM. Results expressed as cell concentration per flask $x10^5$, experiment 2 results shown from Table 3.2.4.

**Table 3.2.5** Growth of DLKP over a 7 day period in basal medium

| Time in days | parental DLKP | 2yr SF DLKP |
|---|---|---|
| 1 | - | - |
| 2 | 0.713 ± 0.128 | 1.950 ± 0.188 |
| 3 | 1.763 ± 0.152 | 4.425 ± 0.563 |
| 4 | 1.988 ± 0.868 | 6.000 ± 0.875 |
| 5 | - | - |
| 6 | 1.613 ± 0.688 | 3.338 ± 0.868 |

Results are expressed as cell concentration per flask $x10^5$.

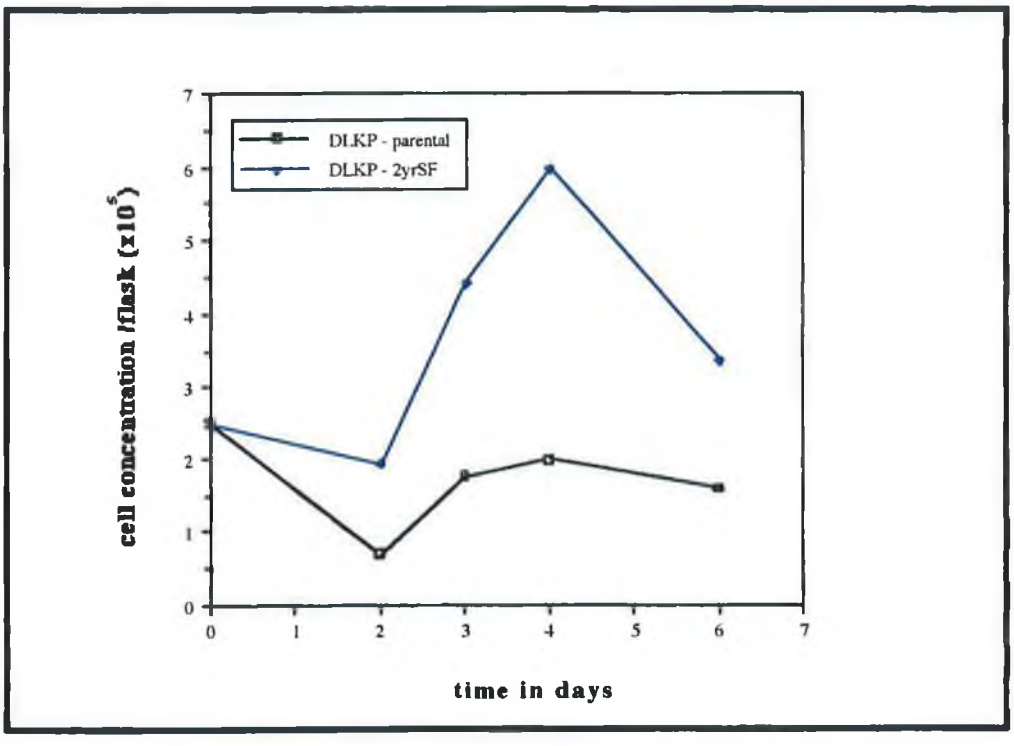


**Fig. 3.2.5** Growth comparison in basal medium of DLKP parental cells to cells grown for 2-years in SFM. Results expressed as cell concentration per flask $x10^5$.

The antigenic phenotype of the SF cells was also investigated using Abs to a variety of normal and tumour cell associated antigens. These 2-year SF cells were compared to the parental cells and SF cells (P20), growing for approximately 3 months under these conditions. The cells were grown on 10-well slides in both serum-free and serum-supplemented medium, and immunocytochemical analysis was carried out using Abs to various antigens as outlined in Table 3.2.6. The epithelial specific markers looked at included pan-cytokeratin, desmosomal protein and epithelial specific antigen; adhesion molecules included $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_5$, and $\alpha_6$ integrins. The NSCLC marker looked at was α-transglutaminase and SCLC markers included NCAM, neurofilaments and serotonin.

It was found that most of these antigens were expressed in similar amounts irrespective of whether the cells were grown in serum or had been kept in serum-free culture for 3 months or 2-years. However, there appears to be an increase in expression of the $\alpha_2$ and $\alpha_3$ integrins as in Fig. 3.2.6.

**Table 3.2.6** Immunocytochemical analysis of DLKP cells grown for periods of time in SFM

| | *Parental DLKP* | | *3 month SF DLKP* | | *2-year SF DLKP* | |
|---|---|---|---|---|---|---|
| **Antigen** | **SFM** | **SSM** | **SFM** | **SSM** | **SFM** | **SSM** |
| pan-CK | -ve | -ve | -ve | -ve | -ve | -ve |
| $\alpha_1$ integrin | -ve | -ve | -ve | -ve | -ve | -ve |
| $\alpha_2$ integrin | weak +ve | weak +ve | weak +ve | weak +ve | strong +ve | strong +ve |
| $\alpha_3$ integrin | +ve | +ve | strong +ve | strong +ve | strong +ve | strong +ve |
| $\alpha_5$ integrin | -ve | -ve | -ve | -ve | -ve | -ve |
| $\alpha_6$ integrin | -ve | -ve | -ve | -ve | -ve | -ve |
| desmo. prot. | -ve | -ve | nd | nd | -ve | -ve |
| ESA | -ve | -ve | nd | nd | -ve | -ve |
| Neurofils. | +ve | +ve | nd | nd | +ve | +ve |
| EGF-R | weak +ve | weak +ve | weak +ve | weak +ve | weak +ve | weak +ve |
| NCAM | -ve | -ve | -ve | -ve | -ve | -ve |
| α-transglut | -ve | -ve | -ve | -ve | -ve | -ve |
| 5HT | -ve | -ve | -ve | -ve | -ve | -ve |

pan-CK = pan cytokeratin, desmo. prot. = desmosomal protein, ESA = epithelial specific antigen, Neurofils. = neurofilaments, EGF-R = epidermal growth factor receptor, NCAM = neural cell adhesion molecule, α-transglut = α-transglutaminase, 5HT = serotonin, n.d. = not determined

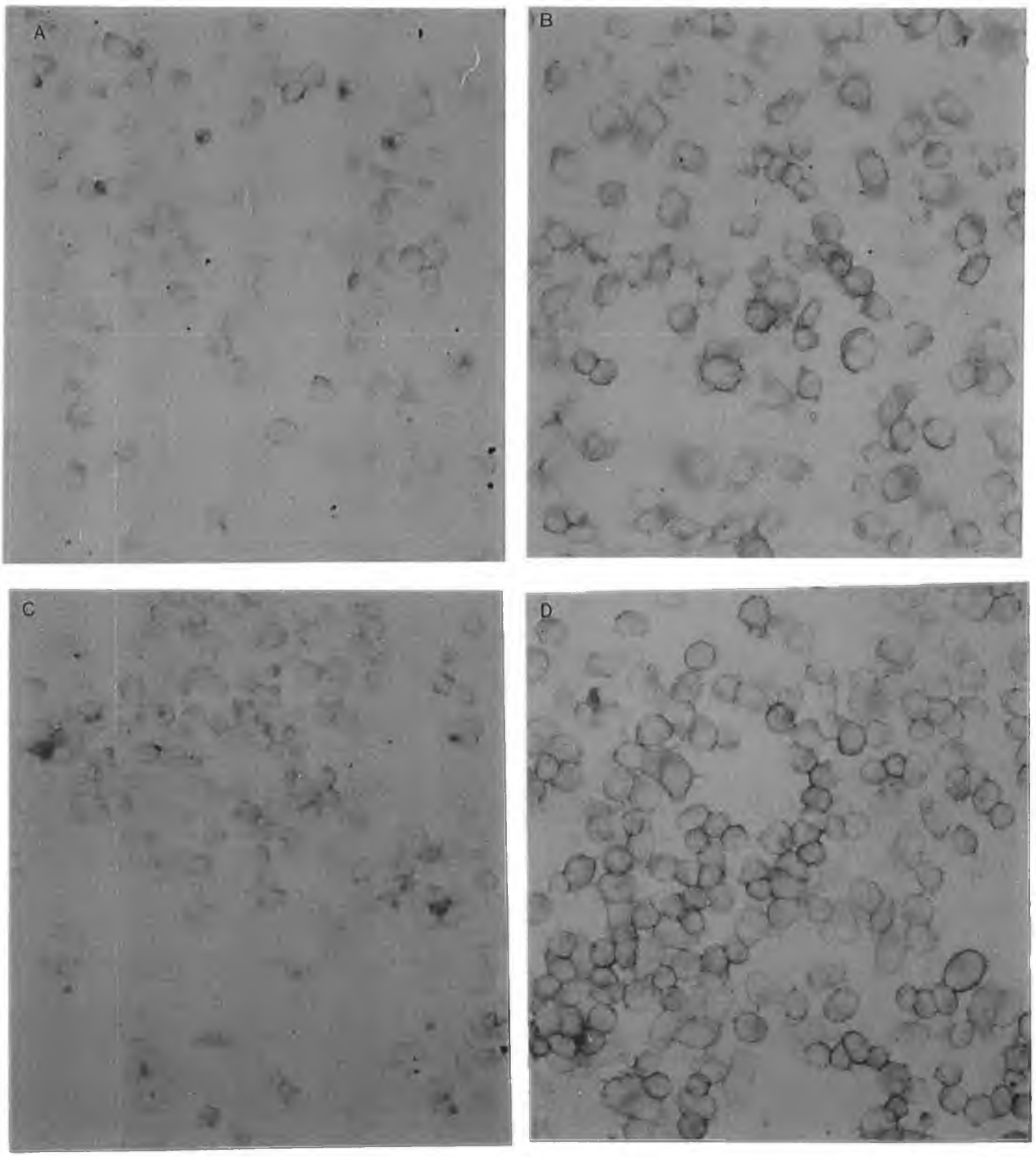


**Fig. 3.2.6** Detection of $\alpha_2$ integrin immunoreactivity in DLKP (a) P.1 SF and (b) 2-year SF. Detection of $\alpha_3$ integrin immunoreactivity in DLKP (c) P.1 SF and (d) 2-year SF.

### 3.2.3 Morphology of DLKP SF cells in culture

When DLKP cells were placed in SFM, usually all the cells were noted to have attached to the flask surface within one hour. However, the cells after 4 days growth were noted not to have spread but remained rounded. This was quite unlike the squamous morphology noted for the cell line in serum-supplemented culture. The cells were quite loosely attached compared to serum-supplemented culture and after 4 days growth many cells were noted to be present in suspension and appeared to be still viable. After long-term growth in serum-free medium (greater than 10 passages), it has been observed that the cells appear to adapt to the SF conditions and show increased growth rates over early passage cells. These cells also appear to be a more homogeneous population than the parental cells and also spread out and are more tightly attached than early passage SF cells, which may explain the increase in expression of $\alpha_2$ and $\alpha_3$ integrins as observed by immunocytochemical analysis in Table 3.2.6. Samples of these high passage SF cells were put into serum-supplemented medium to compare morphology to parental serum-supplemented cultures. Changes in morphology were apparent, with the cells becoming squamous in appearance similar to the DLKP-SQ morphology. The cell population appeared to be almost totally homogeneous.

Fig. 3.2.7 shows morphology of DLKP in serum-free medium both short term SF (P.1) and long-term SF (P.30). The morphology of the long-term SF cells in serum-supplemented medium is also shown (DLKP SFS cells).

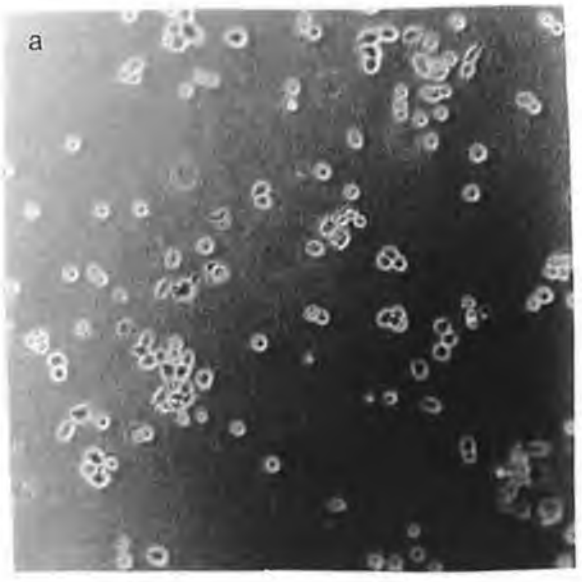

**Fig. 3.2.7(a)** P.1 SF DLKP cells

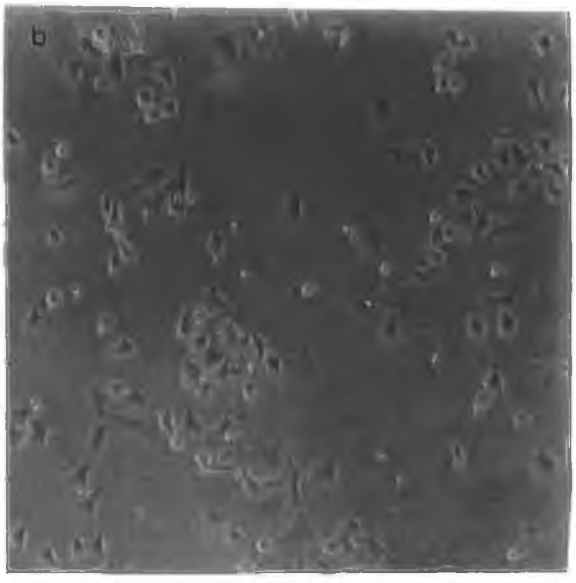

**Fig. 3.2.7(b)** P.30 SF cells

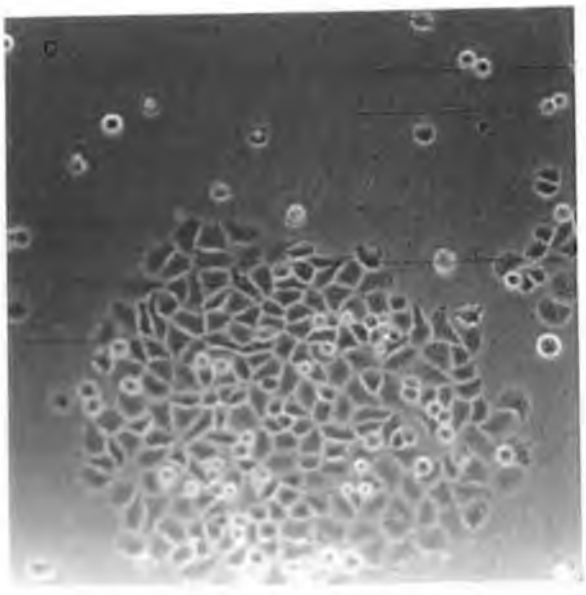

**Fig. 3.2.7(c)** DLKP SS cells

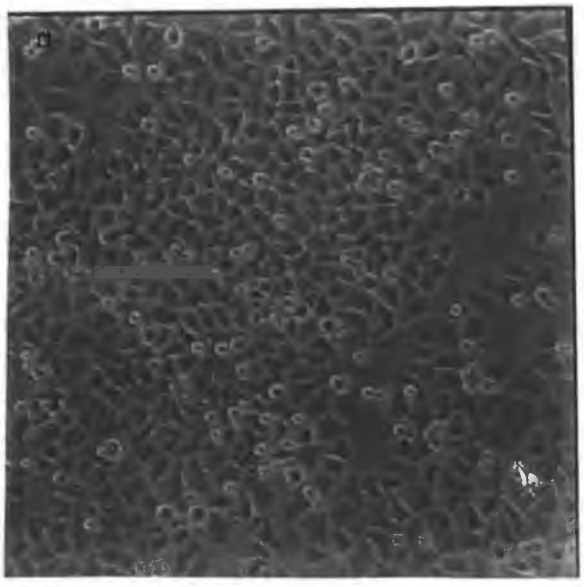

**Fig. 3.2.7(d)** DLKP SFS cells (grown 25 passages in SFM and now P.1 in SSM)

### 3.2.4 Cryopreservation of DLKP cells in SFM

To obtain a true serum-free system, it is essential that the cell line can be cryopreserved under serum-free conditions. The results of two simple experiments are shown in Table 3.2.7. The cells were frozen for 2 weeks before being thawed and assessed for viability. Cells were frozen in a mixture of 10% DMSO and 0.1% Methylcellulose as outlined in Section 2.7.4.

Cells were noted to attach to the flasks within 30 minutes after thawing and successful serial cultivation of the cell was achieved there afterwards. Thus, cryopreservation of the cell line under SF conditions is possible.

**Table 3.2.7** Serum-free freezing of DLKP serum-free cells, both long-term and short term serum-free

| SF Cells used | Viability on thawing (%) |
|---|---|
| Passage 1 SF | 75.00 ± 5.20 |
| Passage 22 SF | 80.94 ± 7.22 |

Cell viability on thawing ± standard deviation (n=6)

### 3.2.5 Development of miniaturised assay system for DLKP SF cells

A miniaturised assay system was developed for DLKP cells in SFM to aid in the investigations into the effects of different basal media and mitogenic components on the growth of the cell line under defined conditions. The assay system would also be beneficial for future toxicity and differentiation assays to be carried out under SF conditions.

5 miniaturised assay systems have extensively investigated for quantification of growth and use in toxicity testing (Martin and Clynes, 1993). The CDVE assay which is routinely used in our laboratory was investigated under SF conditions. Cells were plated onto 96-well plates and left to grow for the required amount of time. The media was then removed and the cells rinsed with PBS but due to the loose attachment of SF DLKP cells most of the cells were lost during this stage of the assay even with applying the utmost care. It was then decided to use the Sulforhodamine B (SRB) assay (see Section 2.10.2) investigated by Martin (1992) which is an effective assay for cells that grow loosely attached or completely in suspension. Again the cells were plated onto 96-well plates in SFM and allowed to grow for the required amount of time. In this assay, the cells were fixed before the waste medium was removed by layering 50µl of 50% cold TCA onto the media in the wells and leaving the plates at 4°C for 1 hour. The media was then removed and the wells rinsed 5 times with tap water and allowed to dry completely. No significant amounts of cells were lost at this step. The wells were then stained with SRB stain for 30 minutes. Staining for 5 minutes with crystal violet was also found to be effective.

### 3.2.5.1 Cell concentration optimisation for miniaturised SF assay system

To use crystal violet (CV) or sulforhodamine B (SRB) as an end point for experiments using DLKP cells in SFM, it was necessary to establish a linear relationship between cell number and absorbance at 620nm for CV and SRB.

A number of different cell concentrations were used ranging from $5x10^2$ to $1x10^4$ per well. Cells were seeded into 96-well plates at these concentrations and allowed to grow for 7 days. The plates were then fixed as described and stained with SRB or CV. From the graphs Fig. 3.2.8(a) and 3.2.8(b), it is clear that there is a linear relationship between SRB or CV and absorbance at 620nm and that a cell concentration of $3x10^3$ cells per well is ideal for setting up 96-well SF assays for the time period of 7 days for both end-points used.

Thus an assay system has been developed which allows DLKP cells to be used under complete SF conditions for a variety of different assays - toxicity, growth factor and differentiation.

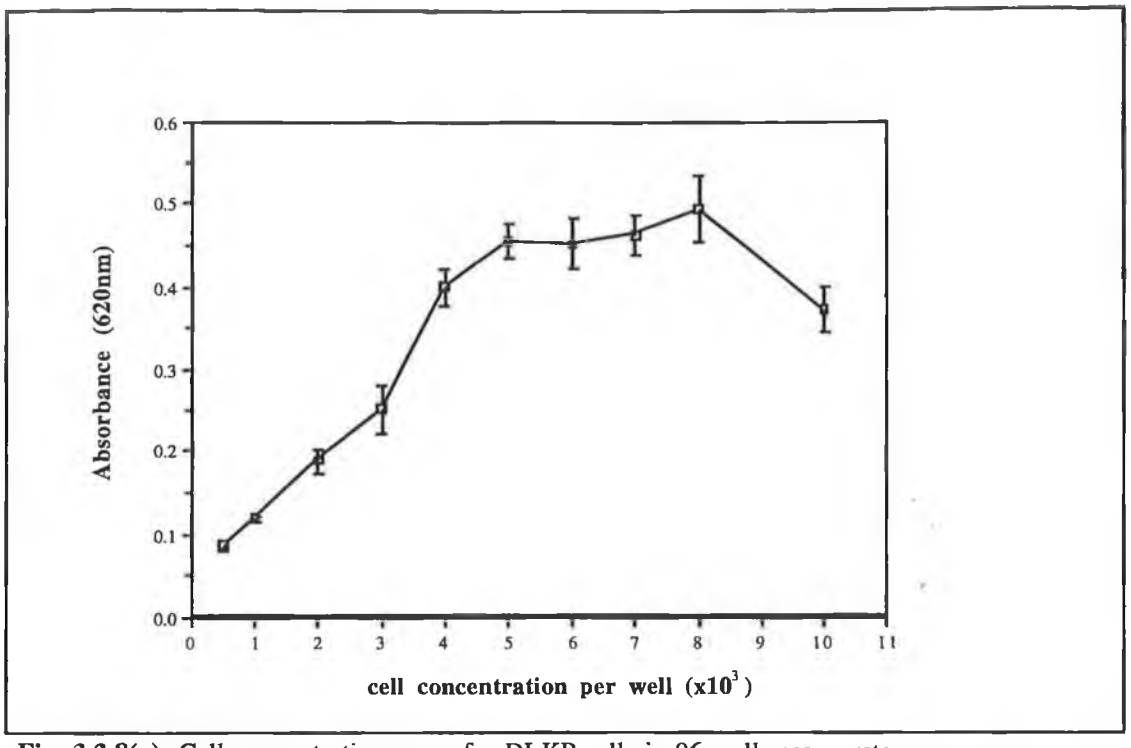


**Fig. 3.3.8(a)** Cell concentration assay for DLKP cells in 96-well assay system.
*Crystal violet staining.* Results expressed as average absorbance at 620nm ± standard deviation (n=8)

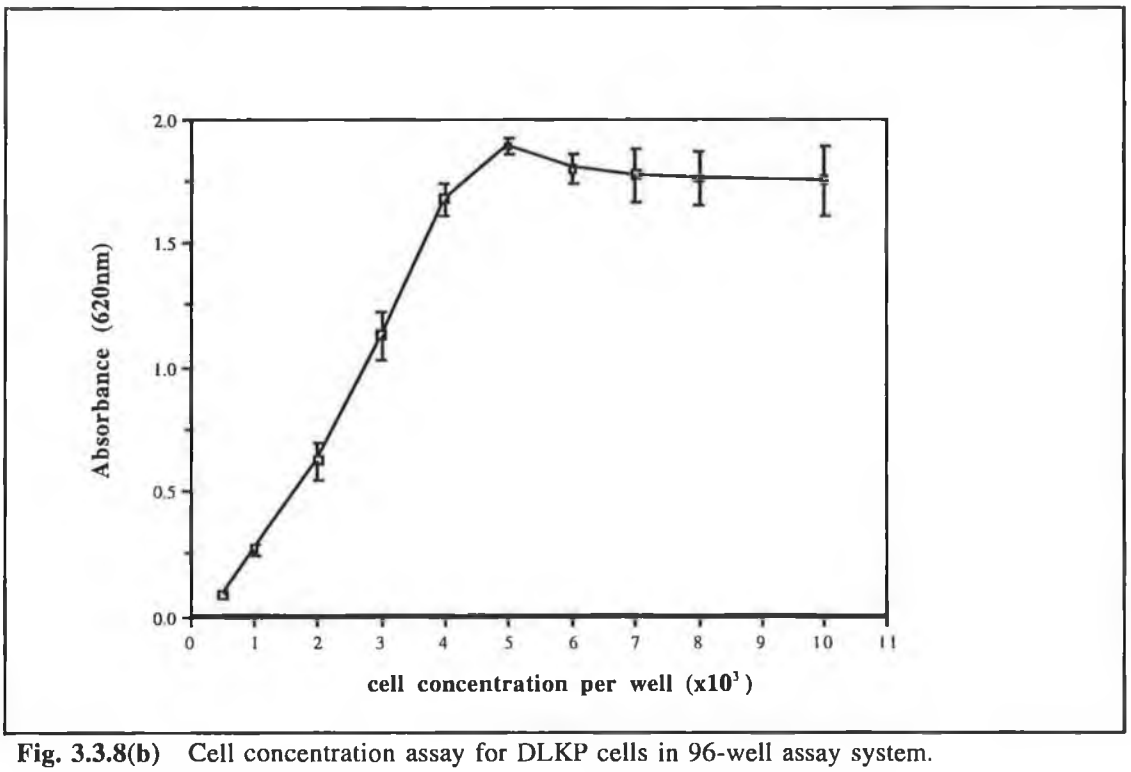


**Fig. 3.3.8(b)** Cell concentration assay for DLKP cells in 96-well assay system.
*SRB staining.* Results expressed as average absorbance at 620nm ± standard deviation (n=8).

### 3.2.6 Improvements in the SFM for the growth of early passage DLKP

The majority of experimental assays that will usually be carried out on the DLKP cell line will be concerned with using cells that are grown for a short time only in SFM. The only mitogen present in the serum-free medium is insulin, so it appears that the DLKP cell line is capable of autocrine regulation in order for the cells to be able to grow in such a simple defined medium. A number of different experiments have been carried out to try and improve the SFM.

#### 3.2.6.1 Optimisation of basal medium for SF growth of DLKP cells

The basal medium provides many soluble, low molecular weight nutrients to the cell such as ions, carbohydrates, vitamins, amino acids and nucleosides., Many cell lines have been grown in SFM using a 50/50 mix of DME/F12 as the basal medium. This was compared to the Ham's F12 SFM being used up until now. Growth performance of the DLKP cells was assessed after 4 days growth in 25cm$^2$ flasks from an initial seeding density of 2.5x10$^5$cells/ flask in the two serum-free media. The results of three separate assays are shown in Table 3.2.8.

Overall, a slight increase in cell number was observed using Ham's F12 as the basal medium in the serum-free medium formulation.

**Table 3.2.8** Optimisation of basal medium for DLKP growth

| Experiment no. | Ham's F12 SFM (H SFM) | Ham's F12/ DME SFM |
|---|---|---|
| 1 | 5.220 ± 0.472 | 4.330 ± 0.437 |
| 2 | 4.317 ± 0.591 | 3.883 ± 0.249 |
| 3 | 5.025 ± 0.801 | 4.375 ± 0.459 |

Results are expressed as average cell concentration per flask x10$^5$ ± standard deviation (n=3).

### 3.2.6.2 Optimisation of insulin concentration in SFM

Insulin is a polypeptide of 5700 molecular weight that affects the metabolism of the cell in a number of different ways. It is thought that its principle effect is on glucose metabolism. Mendiaz *et al.* (1986) found that insulin is mitogenic for the growth of CHO-K1 cells in SFM but also exerts a mitogenic response from many cell lines most commonly through the IGF-1 receptor (Conover *et al.*, 1989). Many other investigators use insulin at a concentration between 1 and 10µg/ml in SF formulations. It was thus decided to try and determine if insulin is mitogenic to the growth of DLKP cells and to optimise this concentration.

96-well assays have been carried out using 9 different insulin concentrations ranging from 0 to 20µg/ml in the defined SFM. This assay has been repeated twice with 3 repeat plates and 8 repeat wells per plate per assay. The same result has been found in both cases with 2.5µg/ml insulin proving to give the greatest stimulation of growth. The concentration of insulin used up until now, 10µg/ml, proved to be slightly inhibitory to growth. See Fig.3.2.9.

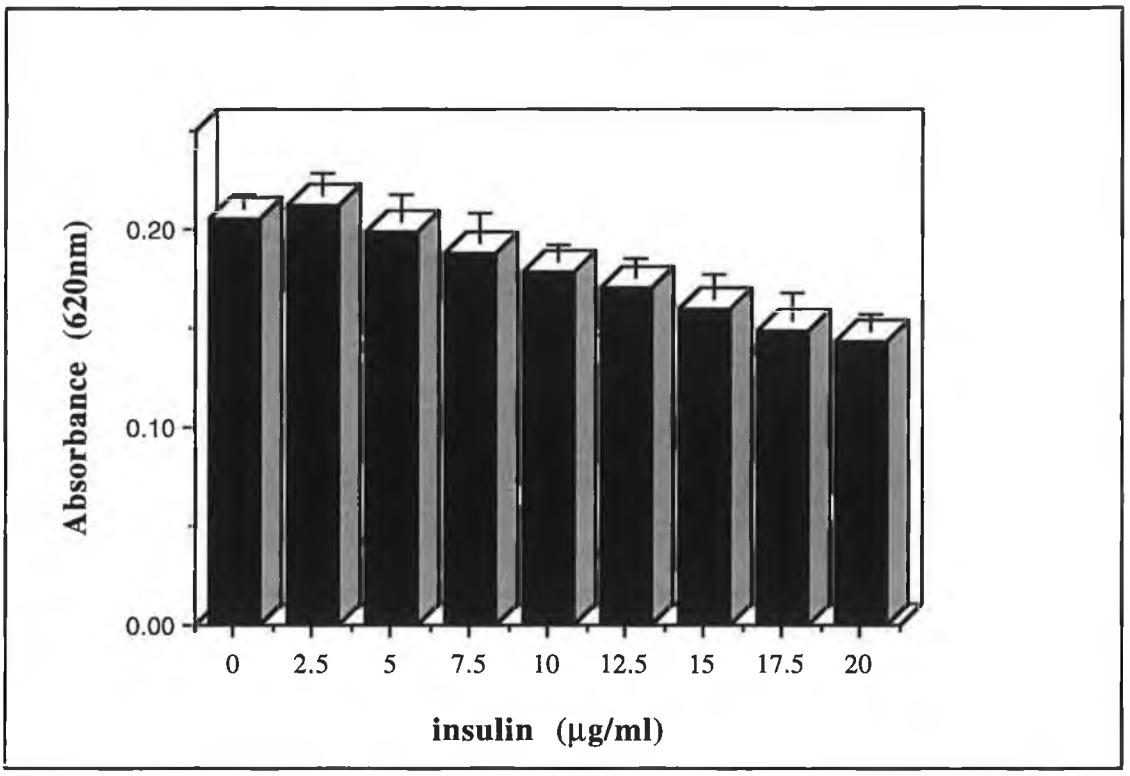


**Fig. 3.2.9** Effect of different insulin concentrations on the growth of DLKP cell in SFM. Results are expressed as average absorbance at 620nm ± standard deviation (n=8).

### 3.2.6.3 Optimisation of transferrin concentration in SFM

Transferrin is an iron-transport protein that is also capable of chelating toxic metals. These properties make it one of the most universally used components in serum-free media formulations and most cell lines show a positive response to its presence in SFM. Similar assays to those described for insulin optimisation were carried out. Fig. 3.2.10 shows that over the range of concentrations used, holo-transferrin (iron saturated) was found to be slightly inhibitory to growth.

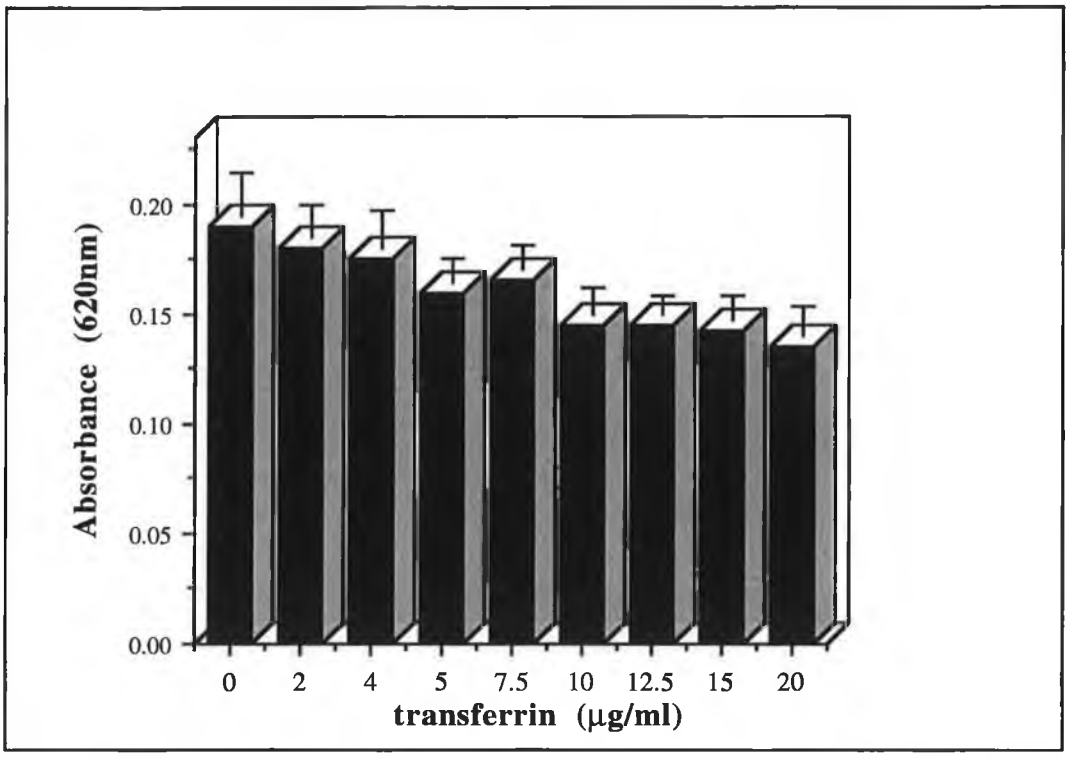


**Fig. 3.2.10** Effect of different concentrations of transferrin on the growth of DLKP cells in SFM. Results are expressed as average absorbance at 620nm ± standard deviation (n=8).

### 3.2.6.4 Optimisation of fibronectin concentration in SFM

A number of assays were carried out to assess the effect of fibronectin on cellular growth.

Assays were carried out in 6-well plates using two different concentrations of fibronectin commonly cited in the literature. Cells were seeded at a concentration of $5x10^4$ per well and counted four days later.

**Table 3.2.9** Effect of different fibronectin concentrations on DLKP cell growth

| Fibronectin conc. (μg/ml) | cell counts ($x10^4$/well) | fold increase in cell nos. |
|---|---|---|
| 0 | 6.680 ± 0.42 | 1.34 |
| 5 | 10.50 ± 0.81 | 2.10 |
| 10 | 10.25 ± 2.05 | 2.05 |

Results are expressed as average cell concentration per flask ± standard deviation (n=3). Fold increase in cell number refers to the increase in number from the initial seeding density of $2.5x10^5$/flask on Day 1

From these results, it is clear that fibronectin has a definite stimulatory effect on cell growth, with an almost 2-fold increase in cell number over unsupplemented SFM. The cells are also much more spread out and tightly attached compared to unsupplemented SFM.

Fig. 3.2.13 shows the morphology of DLKP cells in fibronectin supplemented SFM compared to unsupplemented SFM.

A growth assay was set up to compare growth of the DLKP cells in SFM with and without fibronectin supplementation. Again, when fibronectin was added at a concentration of 5μg/ml, DLKP showed on average a 2-fold increase in cell number over a 4-5 day growth period.

Fig. 3.2.12 and Table 3.2.10 shows a growth curve for DLKP cells in SFM with and without fibronectin supplementation.

**Table 3.2.10** Growth of DLKP in serum-free medium (SFM), serum-free medium supplemented with fibronectin (SFM + FBN), basal medium (Basal) and serum-supplemented medium (SSM)

| Time in days | SFM | SFM + FBN | Basal | SSM |
|---|---|---|---|---|
| 1 | 1.125 | 1.650 | 1.280 | 3.400 |
| 2 | 2.775 | 4.950 | 0.712 | 4.450 |
| 3 | 3.975 | 6.375 | 1.762 | 8.100 |
| 4 | 6.150 | 6.675 | 1.988 | 15.525 |
| 5 | 5.188 | 9.387 | 3.221 | 26.375 |

Results are expressed as cell concentration per flask ($x10^5$).

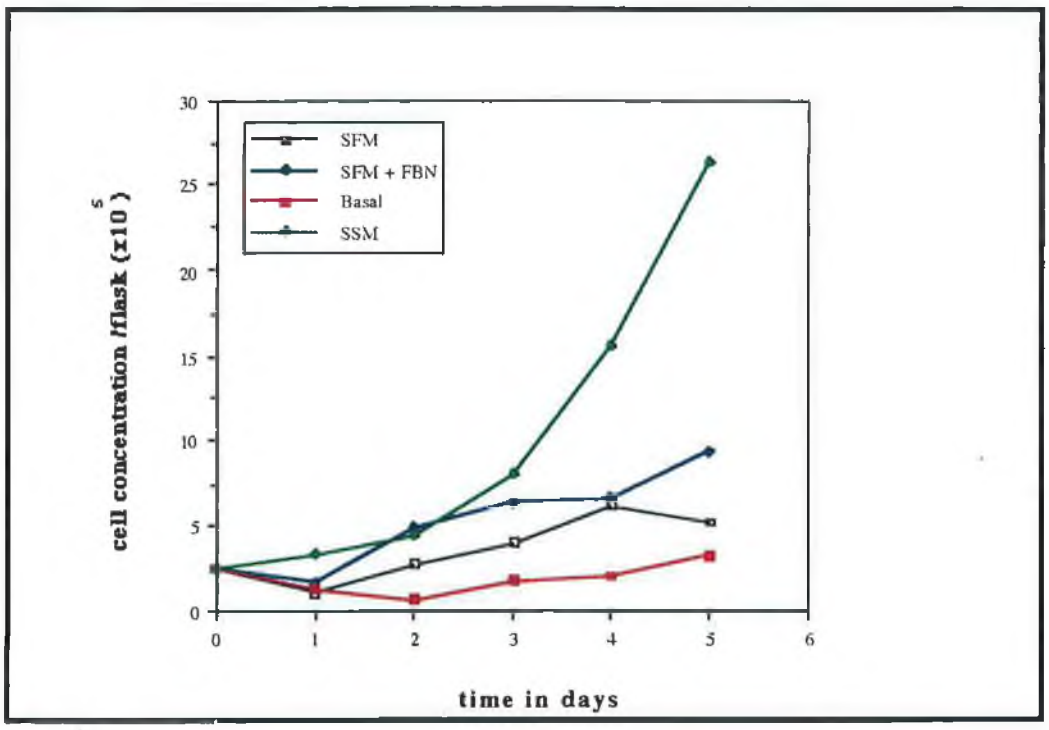


**Fig. 3.2.11** Growth curve for DLKP in serum-free medium (SFM), serum-free medium supplemented with fibronectin (SFM + FBN), basal medium (Basal) and serum-supplemented medium (SSM). Results are expressed as cell number per flask ($x10^5$).

DLKP cells in SFM without fibronectin supplementation appear to adapt to the SF conditions over a long period of time with cell number much lower in early passage SF compared to Passage 10 SF onwards (see Table 3.2.11(b) and Fig. 3.2.12). Sub-cultivation experiments were carried out with fibronectin supplementation to try to improve the growth of the cells in these early passages in the SFM.

Table 3.2.11 and Fig 3.2.12 show that fibronectin definitely improves the growth of early passage SF cells, but as the cells adapt to the SF conditions little difference in cell number was observed from about Passage 9 onwards. Table 3.2.11 shows this response to fibronectin up to 3 passages in Assay 1, and up to 4 passages in Assay 2.

**Table 3.2.11(a)** Subcultivation of DLKP in SFM with and without fibronectin supplementation

| | *Experiment 1* | | *Experiment 2* | |
|---|---|---|---|---|
| **Passage no.** | **SFM** | **SFM + FBN** | **SFM** | **SFM + FBN** |
| 1 | 5.188 ± 0.413 | 9.388 ± 0.688 | 4.200 ± 0.100 | 6.775 ± 0.075 |
| 2 | 4.275 ± 0.400 | 3.787 ± 0.012 | 2.625 ± 0.063 | 7.469 ± 0.219 |
| 3 | 2.762 ± 0.213 | 5.383 ± 0.018 | 1.950 ± 0.150 | 4.031 ± 0.919 |
| 4 | | | 6.375 ± 0.638 | 8.231 ± 0.994 |

Results are expressed as average cell concentration per flask $x10^5$ ± standard deviation (n=2).

**Table 3.2.11(b)** Subcultivation of DLKP in SFM with and without fibronectin supplementation

| | *Experiment 3* | |
|---|---|---|
| **Passage no.** | **SFM** | **SFM + FBN** |
| 1 | 4.000 | 6.169 |
| 2 | 4.481 | 3.806 |
| 3 | 3.475 | 4.700 |
| 4 | 1.388 | 3.263 |
| 5 | 3.788 | 2.737 |
| 6 | 2.888 | 2.812 |
| 7 | 3.000 | 6.263 |
| 8 | 9.188 | 10.275 |
| 9 | 8.438 | 8.288 |
| 10 | 11.363 | 8.588 |
| 11 | 7.013 | 8.025 |
| 12 | 9.450 | 7.575 |
| 13 | 6.225 | 6.450 |

Results are expressed as average cell concentration per flask $x10^5$

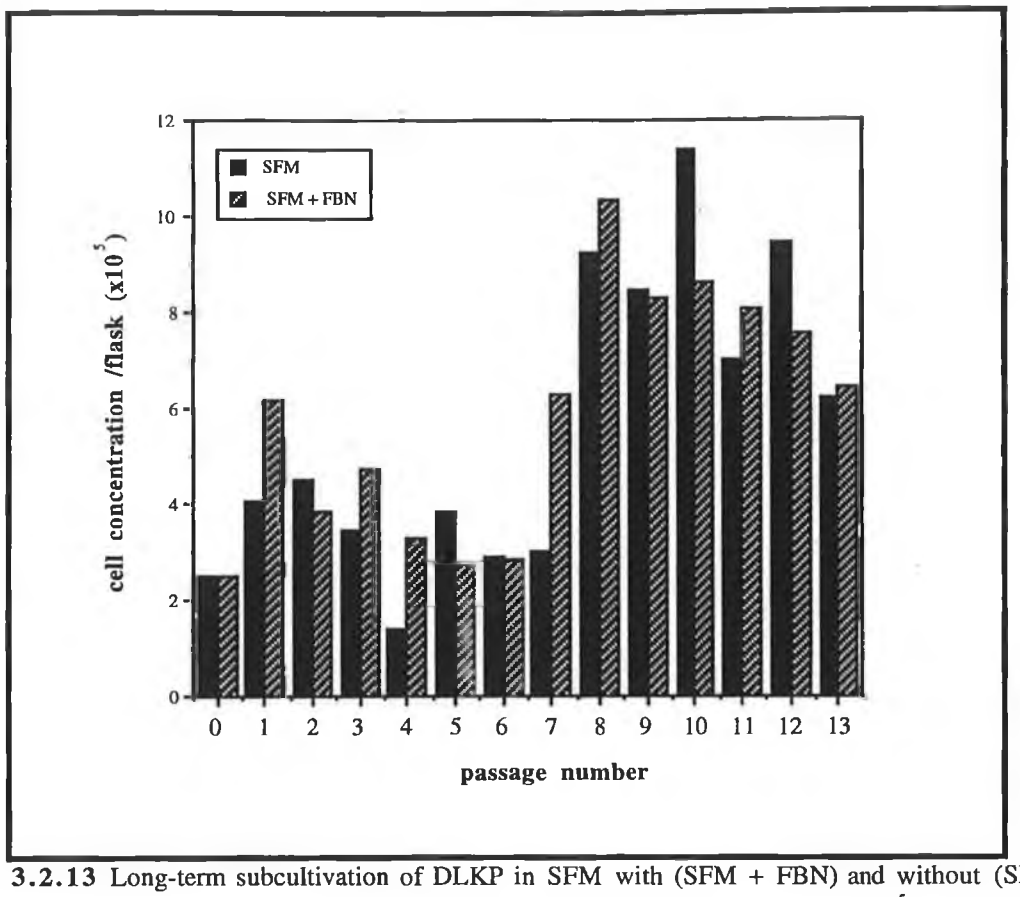


**Fig. 3.2.13** Long-term subcultivation of DLKP in SFM with (SFM + FBN) and without (SFM) fibronectin supplementation. Results are expressed as cell concentration per flask x$10^5$; experiment 3 results shown to demonstrate the adaptation of cells to serum-free medium over long-term growth.

### 3.2.6.5 Response of DLKP to Laminin

Laminin is found exclusively in the basement membrane *in vivo* (Timpl *et al*, 1989), and very little is actually found in serum.

However, DLKP growing in SFM were found to be extremely responsive to laminin, supplemented to the medium at 5μg/ml, a concentration commonly used in the literature. Table 3.2.12 shows similar if not slightly higher cell number for laminin supplemented SFM compared to fibronectin supplemented SFM, with at least a 2 to 2.5 fold increase in cell number, over the control SFM flasks without supplementation. Subcultivation experiments have shown that laminin supplementation significantly improveD the cell number that can be obtained during early passages in SFM.

When laminin was added to the SFM, DLKP cells were noted to become more spread out, but there was also a very definite increase in "neurite-like" processes coming from the cells. Fig 3.2.13 shows growth of DLKP cells in laminin supplemented SFM.

**Table 3.2.12** Subcultivation of DLKP in SFM with (SFM + Ln) and without laminin supplementation at 5μg/ml

| Passage no. | SFM | SFM + Ln (exp.1) | SFM + Ln (exp 2) |
|---|---|---|---|
| 1 | 3.375 ± 0.025 | 9.9375 ± 0.300 | 9.275 ± 1.975 |
| 2 | 3.938 ± 0.225 | 12.063 ± 2.125 | 7.050 ± 0.525 |
| 3 | 8.083 ± 0.581 | 10.306 ± 0.413 | 8.281 ± 0.098 |
| 4 | 5.285 ± 0.987 | 12.813 ± 0.063 | 9.931 ± 1.006 |
| 5 | 7.875 ± 0.875 | 12.789 ± 1.275 | 10.50 ± 0.975 |

Results are expressed in terms of cell concentration per flask $x10^5$ ± standard deviation (n=2).

### 3.2.6.6 Response of DLKP to Type IV Collagen

Collagen is the most abundant protein in the basement membrane of the cell. Type IV collagen has been found to be involved in the binding of epithelial carcinoma cells (Palm and Furcht, 1982). It is the most commonly used collagen in SFM.

Supplementing the SFM with type IV collagen at a concentration of 5µg/ml was found not to improve the growth of the cells at all during early passages in SFM. Similar growth of cells was obtained for collagen supplemented SFM when compared to the control, unsupplemented SFM. See Table 3.2.13.

No apparent change in the morphology was observed when cultured in collagen containing SFM (Fig. 3.2.13).

**Table 3.2.13** Subcultivation of DLKP in SFM with (SFM + Coll) and without Type IV Collagen supplementation at 5µg/ml

| Passage no. | SFM | SFM + Coll (exp.1) | SFM + Coll (exp.2) |
|---|---|---|---|
| 1 | 3.375 ± 0.025 | 3.994 ± 0.019 | 4.450 ± 0.550 |
| 2 | 3.938 ± 0.225 | 3.969 ± 0.406 | 4.144 ± 0.206 |
| 3 | 8.013 ± 0.581 | 3.581 ± 0.356 | 3.713 ± 0.000 |
| 4 | 5.825 ± 0.987 | 4.639 ± 1.181 | 4.050 ± 0.975 |
| 5 | 7.875 ± 0.875 | 6.506 ± 0.281 | 5.287 ± 0.150 |

Results are expressed in terms of cell concentration per flask $x10^5$ ± standard deviation (n=2)

**Fig. 3.2.13(a)** Morphology of DLKP in fibronectin supplemented SFM

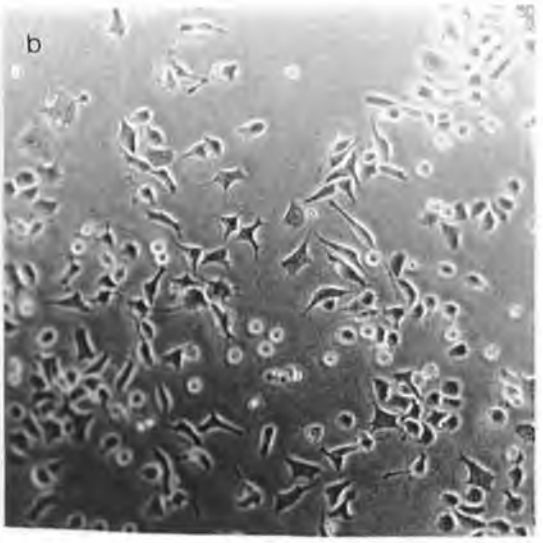

**Fig. 3.2.14(b)** Morphology of DLKP in laminin supplemented SFM

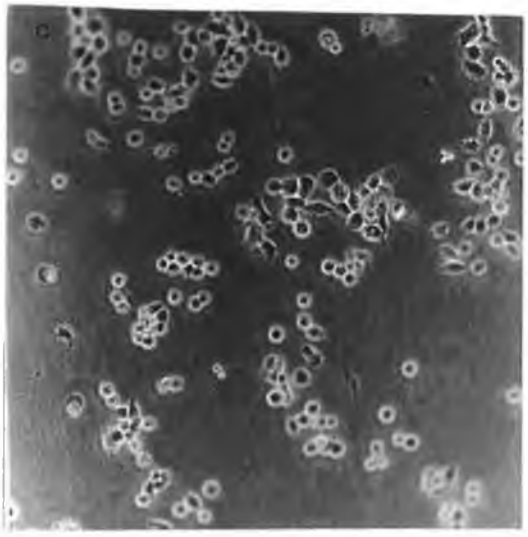

**Fig. 3.2.14(c)** Morphology of DLKP in Collagen supplemented SFM

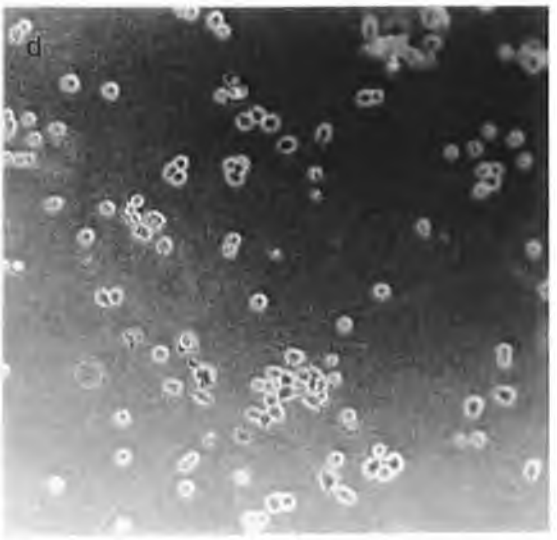

**Fig. 3.2.14(d)** Morphology of DLKP in SFM

### 3.2.7 Sensitivity of DLKP cells to chemotherapeutic drugs in SFM

The serum-free medium can be used as a tool to study the effects of chemotherapy drugs on the DLKP cell line without the variability in results associated with the use of serum. Serum proteins have been reported to possibly have a slight protective effect against cytotoxic drugs and other serum components, such as prostaglandins and cytokines, and thereby can also interfere with the performance of a drug. The objective of this study was to assess the effect of three structurally different chemotherapeutic drugs, Adriamycin, Vincristine and VP16, on the DLKP cells under serum-free conditions and to compare results obtained to serum-supplemented assays.

Chemotherapy drug profiles were obtained for early passage (P.1 SF DLKP) and late passage (P.22 SF DLKP) in both serum-supplemented and serum-free medium.

#### 3.2.7.1 Sensitivity to Adriamycin

DLKP P.1 and P.22 SF cells were exposed to increasing concentrations of Adriamycin ranging from 1.25ng/ml to 80ng/ml in both serum-free and serum-supplemented media. The results in Table 3.2.14 show the mean results of 3 separate experiments with 8 replicates per experiment.

**Table. 3.2.14** Sensitivity of DLKP P.1 and P.22 to Adriamycin in both SFM and SSM

| DLKP cells used | Adriamycin ($IC_{50}$ ng/ml) | Adriamycin (fold w.r.t. DLKP in SSM) |
|---|---|---|
| P.1 SF | 20.63 ± 3.40 | 1.5 |
| SSM | 13.74 ± 2.60 | 1.0 |
| P.22 SF | 39.90 ± 13.4 | 2.9 |
| P.22 SF in SSM | 21.10 ± 2.25 | 1.53 |

The fold difference in $IC_{50}$ value for each with respect to (wrt) DLKP SSM is shown for the drug.

The $IC_{50}$ value to Adriamycin was found to increase by almost 3-fold in long-term serum-free cells when the assay was performed in SFM, and by 1.5-fold when carried out in serum-supplemented medium.

### 3.2.7.2 Sensitivity to Vincristine

DLKP P.1 and P.22 SF cells were exposed to increasing concentrations of Vincristine in the range 0.094ng/ml to 6.0ng/ml in both serum-free and serum-supplemented media. The results in Table 3.2.15 show the mean results from 2 separate experiments with 8 replicates per experiment.

**Table. 3.2.15** Sensitivity of DLKP P.1 and P.22 to Vincristine in both SFM and SSM

| DLKP cells used | Vincristine ($IC_{50}$ ng/ml) | Vincristine (fold w.r.t. DLKP in SSM) |
|---|---|---|
| P.1 SF | 0.266 ± 0.093 | 1.06 |
| SSM | 0.251 ± 0.029 | 1.00 |
| P.22 SF | 0.453 ± 0.068 | 1.80 |
| P.22 SF in SSM | 0.317 ± 0.073 | 1.26 |

The fold difference in $IC_{50}$ value for each with respect to (wrt) DLKP SSM is shown for the drug.

No difference in $IC_{50}$ value was observed whether assayed in SFM or SSM. However, P.22 SF cells showed a slight increase in $IC_{50}$ in serum-free medium.

### 3.2.7.3 Sensitivity to VP16

DLKP P.1 and P.22 SF cells were exposed to increasing concentrations of VP16 ranging from 9.4ng/ml to 600ng/ml in both serum-free and serum-supplemented media. Again the results are the mean of three separate experiments with 8 replicates per experiment and are shown in Table 3.2.16.

**Table. 3.2.16** Sensitivity of DLKP P.1 and P.22 to VP16 in both SFM and SSM

| DLKP cells used | VP16 ($IC_{50}$ ng/ml) | VP16 (fold w.r.t. DLKP in SSM) |
|---|---|---|
| P.1 SF | 15.75 ± 5.98 | 0.58 |
| SSM | 27.38 ± 9.09 | 1.00 |
| P.22 SF | 16.00 ± 2.81 | 0.58 |
| P.22 SF in SSM | 21.95 ± 0.84 | 0.80 |

The fold difference in $IC_{50}$ value for each with respect to (wrt) DLKP SSM is shown for the drug.

The cells were found to be extremely vulnerable to VP16 when assayed in serum-free medium with a resultant decrease in $IC_{50}$ value.

## 3.3 DLKP CLONES AND MDR VARIANT IN SERUM-FREE MEDIUM

Cultivation of the DLKP cell line has been found to be possible in serum-free medium. This allows a system to be used so that experiments can be carried out in conditions free from serum interference and variability in results that can be associated with the presence of undefined components in the medium.

This SFM was applied to the growth of a number of variant DLKP cell lines. The parental DLKP cell line contains three morphologically distinct populations during normal cultivation in serum-supplemented conditions. These 3 clones have been established from the original parental population (McBride, 1995) and may represent tumour heterogeneity *in vivo*. The SFM was also used to try and develop a system for the growth of the MDR variant, DLKPA, in a totally defined environment. This would then allow a system to be in place that can be used for toxicity assays to be carried out in conditions where a totally defined environment is more beneficial than using serum-supplemented conditions.

### 3.3.1 Growth of DLKP Clones in SFM

DLKP contains three morphologically distinct populations. Dr. Shirley McBride established three clones corresponding to these populations from the parental DLKP cells. These clones were designated -M (mesenchymal - like), -I (intermediate - like) and SQ (squamous - like). On prolonged subculture, -SQ and -M can each interconvert with -I, but -SQ and -M do not interconvert. This may represent tumour heterogeneity *in vivo*. The development of a SFM for the growth of these clonal cell lines would be an extremely useful tool to study this interconversion process.

Investigations were carried out into the growth of these clones in the SFM developed initially for DLKP. When cultured in SFM, the number of DLKP-SQ and -M cells always failed to increase above seeding density levels at any stage, regardless of frequency of media change or the length of time in culture. However, in contrast, DLKP-I grew very well under SF conditions, even better than the parental DLKP population and were capable of being cultured up to at least 10 passages at a time (McBride, 1995).

In this thesis, investigations were carried out to try to find a SFM that would allow the proliferation of each of the clones. Growth experiments were carried out using fibronectin supplementation to the SFM, to evaluate its effect on the growth of each of the clonal cell lines. DLKP has already been found to be extremely responsive to fibronectin at early passages in SFM.

### 3.3.1.1 DLKP-SQ

The DLKP-SQ 'squamous-like' clone appears to make up the largest proportion of the DLKP heterogeneous parental population of cells. Approximately 65% of the parental population appear to have this morphology by microscopical observations. However, this clonal cell line does not proliferate in the SFM used for the growth of the parental cell line. The cells always failed to proliferate above the initial seeding density. However, addition of fibronectin to the SFM at a concentration of 5μg/ml permitted growth of DLKP-SQ.

#### 3.3.1.1.1 Morphology of DLKP-SQ in fibronectin supplemented SFM

DLKP-SQ, when placed in SFM, failed to form colonies and remained rounded in appearance. However when fibronectin was added at a concentration of 5μg/ml, the cells began to spread and resemble the squamous-like phenotype of their serum-supplemented counterparts. Figure 3.3.1 shows morphology of DLKP-SQ in unsupplemented and fibronectin-supplemented SFM.

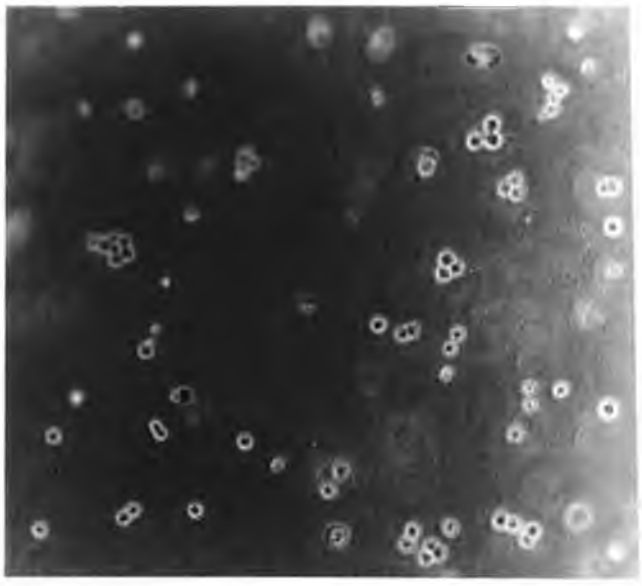

**Fig. 3.3.1(a)** Morphology of DLKP-SQ in SFM

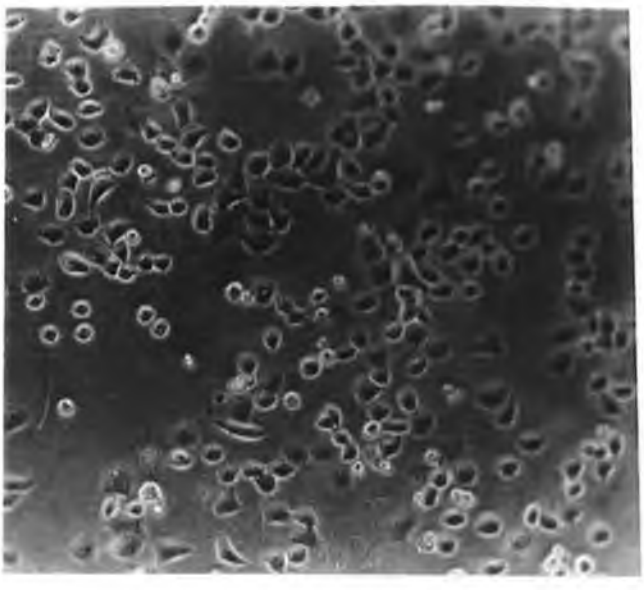

**Fig.3.3.1(b)** Morphology of DLKP-SQ in SFM plus 5μg/ml fibronectin

### 3.3.1.1.2 Growth curve for DLKP-SQ in fibronectin supplemented SFM

Table 3.3.1 shows growth of DLKP-SQ over a 7 day growth period in SFM and fibronectin supplemented SFM (results for three separate experiments are shown). Fig. 3.3.2 shows a growth curve to compare growth of DLKP-SQ cells in fibronectin supplemented and unsupplemented SFM.

**Table 3.3.1(a)** Growth curves for DLKP-SQ over a 7 day growth period

| | *Experiment 1* | | | |
|---|---|---|---|---|
| **Time in days** | **SFM** | **SFM + FBN** | **Basal** | **SSM** |
| 1 | - | - | - | - |
| 2 | 0.600 | 1.462 | 0.975 | 5.00 |
| 3 | 0.525 | 3.375 | 0.975 | 8.600 |
| 4 | 1.725 | 5.025 | 1.125 | 15.13 |
| 5 | 0.9 | 8.325 | 1.763 | 18.37 |
| 6 | - | - | - | - |
| 7 | 1.65 | 6.338 | 0.938 | 25.75 |

Serum-free medium (SFM), fibronectin supplemented SFM (SFM + FBN), basal medium (Basal) and serum-supplemented medium (SSM). Results are expressed as cell concentration per flask $x10^5$.

**Table 3.3.1(b)** Growth curves for DLKP-SQ over 5 day growth periods

| | *Experiment 2* | | *Experiment 3* | |
|---|---|---|---|---|
| **Time in days** | **SFM** | **SFM + FBN** | **SFM** | **SFM + FBN** |
| 1 | 1.088 | 2.213 | - | - |
| 2 | 1.350 | 1.463 | 2.388 ± 0.238 | 6.388 ± 0.113 |
| 3 | 1.725 | 3.263 | 2.525 ± 0.000 | 8.150 ± 0.650 |
| 4 | 0.975 | 3.713 | 2.425 ± 0.175 | 6.375 ± 1.175 |
| 5 | 1.950 | 3.488 | 1.513 ± 0.038 | 6.038 ± 0.063 |

Serum-free medium (SFM), fibronectin supplemented SFM (SFM + FBN). Results are expressed as cell concentration per flask $x10^5$ ± standard deviation (n=3) for experiment 3

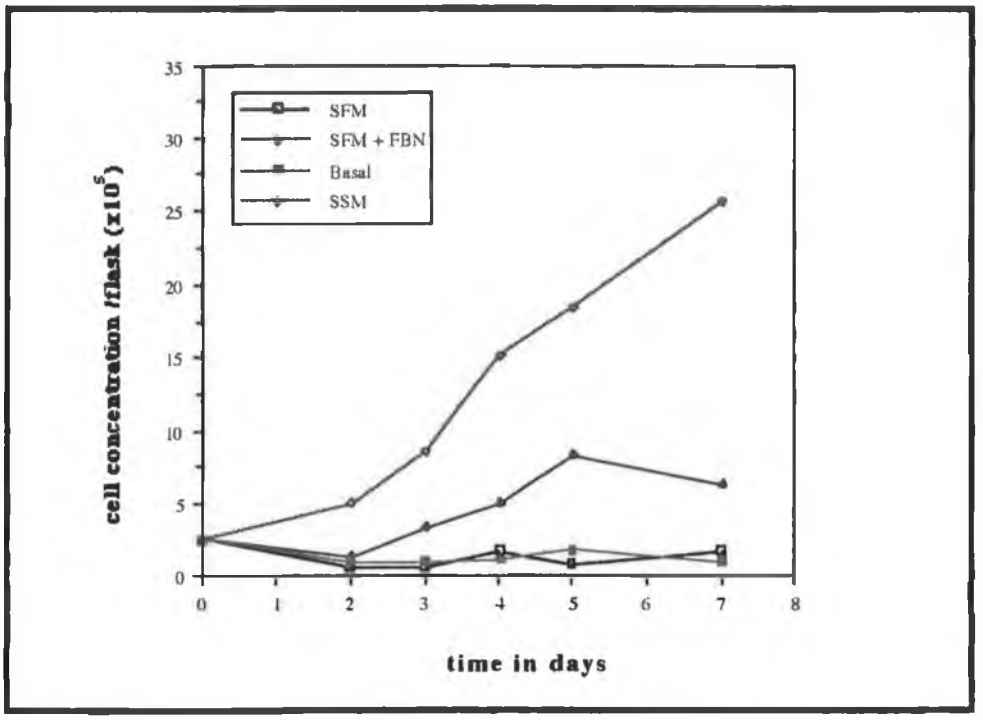


**Fig. 3.3.2** Growth curve for DLKP-SQ in serum-free medium (SFM), fibronectin supplemented serum-free medium (SFM + FBN), basal medium (Basal) and serum-supplemented medium (SSM), Results are expressed as cell concentration per flask $x10^5$, results for experiment 1 are shown.

### 3.3.1.1.3 Subcultivation of DLKP-SQ in fibronectin supplemented SFM

Subcultivation of DLKP-SQ was found to be possible once fibronectin was added to the SFM. Cells were passaged once every 5-7 days and counted before seeding new flasks at a density of $2.5x10^5$ per flask.

Table 3.3.2 shows two separate subcultivation experiments up to 4 passages for DLKP-SQ.

**Table 3.3.2** Subcultivation of DLKP-SQ in fibronectin supplemented SFM (SFM + FBN)

| | *Experiment 1* | | *Experiment 2* | |
|---|---|---|---|---|
| **Passage no.** | **SFM** | **SFM + FBN** | **SFM** | **SFM + FBN** |
| 1 | 1.313 ± 0.038 | 7.625 ± 0.175 | 0.575 ± 0.025 | 6.875 ± 0.312 |
| 2 | - | 5.125 ± 0.500 | - | 6.338 ± 0.000 |
| 3 | - | 2.175 ± 0.150 | - | 2.812 ± 0.188 |
| 4 | - | 3.169 ± 0.094 | - | 3.150 ± 0.188 |

Results are expressed in terms of cell concentration per flask $x10^5$ ± standard deviation (n=3)

### 3.3.1.2 DLKP-I

The DLKP-I clone appears in the parental population as small colonies with indistinct cell boundaries. They make up about 30% of the heterogeneous parental population. These cells have been found to grow very well in the SFM developed for the growth of the parental cell line, with cell counts often exceeding the parental population in SFM (McBride, 1995).

Addition of fibronectin to the SFM generally allows better growth of the cells, especially during earlier passages in SF conditions.

#### 3.3.1.2.1 Morphology of DLKP-I in serum-free medium

The morphology of DLKP-I in unsupplemented SFM resembles that of DLKP-I in serum-supplemented medium, where the cells form colonies with indistinct cell boundaries. Addition of fibronectin to the medium causes the cells to spread out a lot more, while the morphology appears to resemble the DLKP-M cells in fibronectin supplemented SFM. Fig. 3.3.3 shows morphology of DLKP-I in SFM and SFM supplemented with 5µg/ml fibronectin.

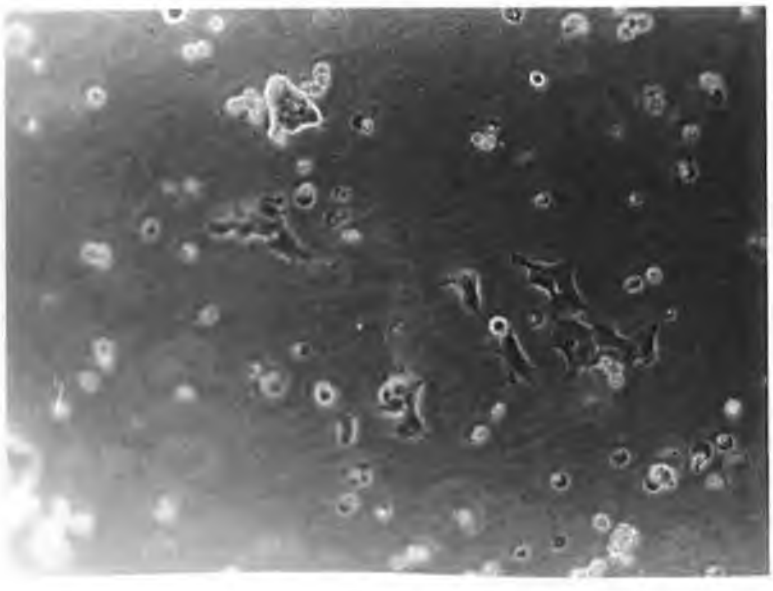

**Fig. 3.3.3(a)** Morphology of DLKP-I in SFM

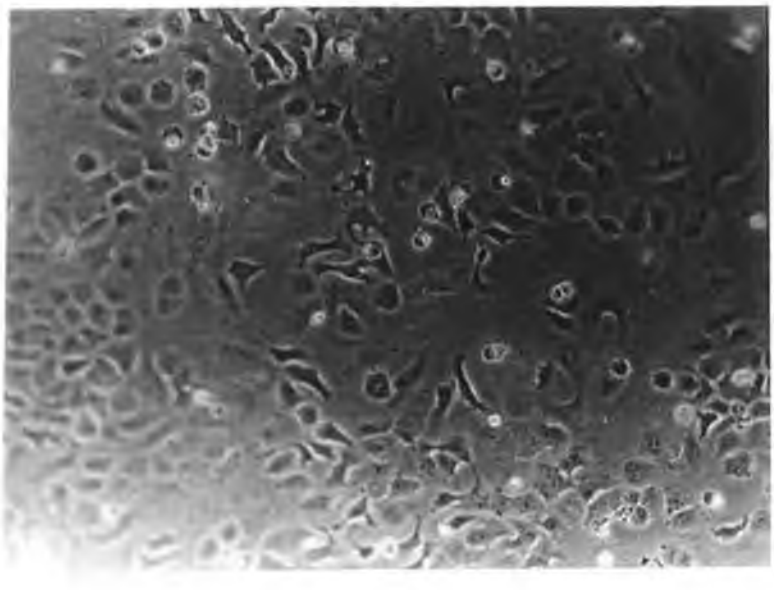

**Fig.3.3.3(b)** Morphology of DLKP-I in SFM plus 5μg/ml fibronectin

### 3.3.1.2.2 Growth curves for DLKP-I in serum-free medium

Growth assays were set up to compare growth of DLKP-I in unsupplemented and fibronectin-supplemented SFM over 5 to 7 day growth periods.

Table 3.3.3 shows the results for 3 separate experiments. Fig 3.3.4 shows the growth curves obtained in experiment 1.

**Table 3.3.3(a)** Growth curves for DLKP-I over a 7 day growth period

| | *Experiment 1* | | | |
|---|---|---|---|---|
| **Time in days** | **SFM** | **SFM + FBN** | **Basal** | **SSM** |
| 1 | 0.863 | 2.400 | 0.450 | 2.200 |
| 2 | 1.838 | 5.213 | 0.750 | 4.350 |
| 3 | 2.362 | 8.750 | 1.088 | 8.625 |
| 4 | 2.850 | 8.500 | 1.350 | 20.13 |
| 5 | 5.175 | 6.375 | 2.288 | 28.50 |
| 6 | - | - | - | - |
| 7 | 2.438 | 3.038 | 1.463 | 32.63 |

Serum-free medium (SFM), fibronectin supplemented SFM (SFM + FBN), basal medium (Basal) and serum-supplemented medium (SSM). Results are expressed as cell concentration per flask $x10^5$.

**Table 3.3.3(b)** Growth curves for DLKP-I over 5 day growth periods

| | *Experiment 2* | | *Experiment 3* | |
|---|---|---|---|---|
| **Time in days** | **SFM** | **SFM + FBN** | **SFM** | **SFM + FBN** |
| 1 | 0.413 | 1.238 | - | - |
| 2 | 0.900 | 2.288 | 5.238 ± 0.138 | 9.225 ± 0.050 |
| 3 | 1.688 | 4.575 | 5.525 ± 0.100 | 7.037 ± 0.688 |
| 4 | 2.438 | 7.988 | 4.825 ± 0.425 | 6.850 ± 0.100 |
| 5 | 4.050 | 7.425 | 3.850 ± 0.325 | 4.713 ± 0.237 |

Serum-free medium (SFM), fibronectin supplemented SFM (SFM + FBN). Results are expressed as cell counts per flask $x10^5$ ± standard deviation (n=3) for experiment 3.

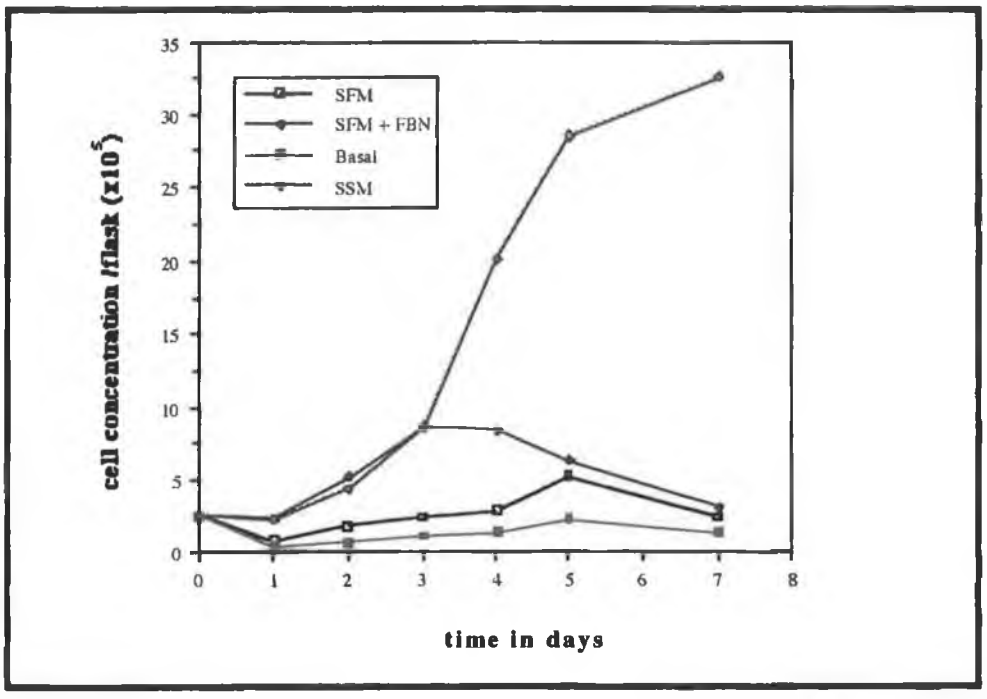


**Fig. 3.3.4** Growth curve for DLKP-I in serum-free medium (SFM), fibronectin supplemented serum-free medium (SFM + FBN), basal medium (Basal) and serum-supplemented medium (SSM). Results are expressed as cell concentration per flask x$10^5$, results for experiment 1 are shown.

### 3.3.1.2.3 Subcultivation of DLKP-I in serum-free medium

Subcultivation of DLKP-I was found to be possible in both supplemented and unsupplemented SFM. Generally fibronectin addition increased cell counts over unsupplemented SFM during the early passages in SF conditions. However, after this time (exp.1), cell counts were found to be quite similar irrespective of whether fibronectin was supplemented to the medium or not (Table 3.3.4).

**Table 3.3.4** Subcultivation of DLKP-I

| | *Experiment 1* | | *Experiment 2* | |
|---|---|---|---|---|
| **Passage no.** | **SFM** | **SFM + FBN** | **SFM** | **SFM + FBN** |
| 1 | 4.675 ± 0.175 | 8.700 ± 0.000 | 3.075 ± 0.750 | 7.613 ± 0.450 |
| 2 | 7.250 ± 2.625 | 5.590 ± 1.720 | 5.756 ± 0.281 | 4.725 ± 0.600 |
| 3 | 7.439 ± 1.189 | 9.563 ± 1.313 | 4.050 ± 7.425 | 6.150 ± 1.238 |
| 4 | 7.144 ± 0.431 | 12.08 ± 1.538 | 4.825 ± 0.425 | 8.250 ± 0.250 |
| 5 | 15.79 ± 0.413 | 10.95 ± 0.000 | 5.525 ± 0.100 | 6.219 ± 0.469 |
| 6 | 8.494 ± 0.244 | 7.238 ± 0.072 | | |
| 7 | 4.444 ± 0.581 | 4.650 ± 0.510 | | |
| 8 | 8.981 ± 0.956 | 9.600 ± 0.185 | | |
| 9 | 11.00 ± 0.938 | * | | |
| 10 | 11.34 ± 0.019 | | | |
| 11 | 5.688 ± 2.875 | | | |
| 12 | 7.188 ± 1.313 | | | |
| 13 | 12.75 ± 0.688 | | | |

Serum-free medium (SFM), fibronectin supplemented SFM (SFM + FBN). Results are expressed in terms of average cell counts per flask $x10^5$ ± standard deviation (n=2).
* fungal contamination at this passage

### 3.3.1.3 DLKP-M

The DLKP-M clone appears in the parental population as irregular, fibroblastoid cells that do not appear to form colonies. They make up approximately 5% of the parental population.
Like DLKP-SQ, this cell line fails to proliferate in the SFM investigated for DLKP. However, addition of fibronectin at a concentation of 5µg/ml had a profound on the growth of the cell line under SF conditions.

#### 3.3.1.3.1 Morphology of DLKP-M in serum-free medium

The morphology of DLKP-M in unsupplemented SFM resembles their serum-supplemented counterparts. The cells appear elongated with neurite-like processes present in some of the cells. No colony formation occurred. However, on addition of fibronectin to the SFM, the cells became very spread out and appeared to grow to confluence (Fig. 3.3.5)

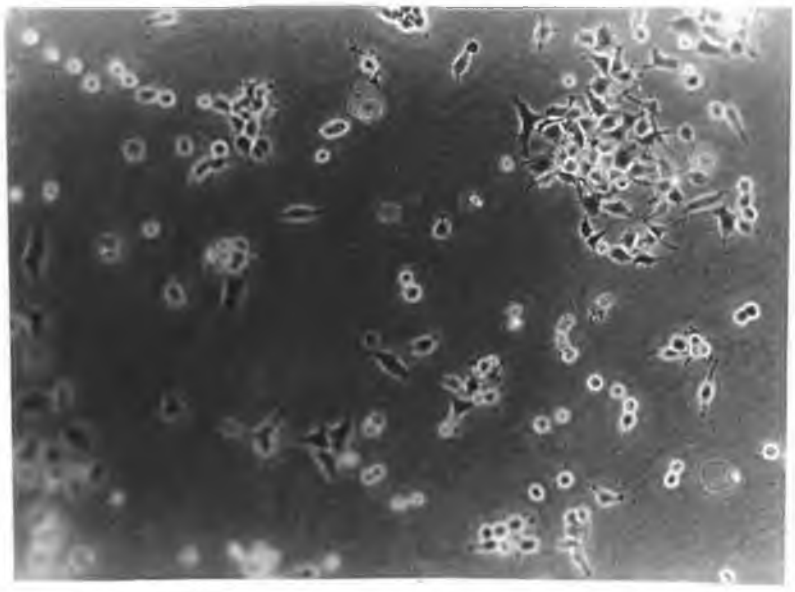

**Fig. 3.3.5(a)** Morphology of DLKP-M in SFM

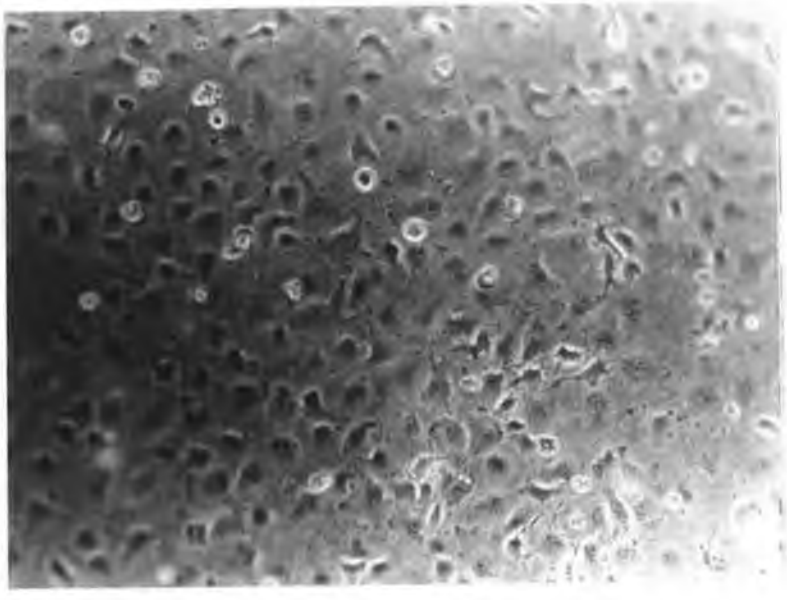

**Fig.3.3.5(b)** Morphology of DLKP-M in SFM plus 5μg/ml fibronectin

### 3.3.1.3.2 Growth curves for DLKP-M in serum-free medium

Table 3.3.5 shows growth of DLKP-M over 4-5 day periods in SFM with and without fibronectin supplementation (results for 3 separate experiments shown). Fig. 3.3.6 shows a growth curve to compare growth of DLKP-M in supplemented and unsupplemented SFM.

**Table 3.3.5(a)** Growth curves for DLKP-M over a 5 day growth period

| | *Experiment 1* | | | |
|---|---|---|---|---|
| **Time in days** | **SFM** | **SFM + FBN** | **Basal** | **SSM** |
| 1 | 1.125 | 1.650 | 0.413 | 2.663 |
| 2 | 1.500 | 4.350 | 0.525 | 5.363 |
| 3 | 1.800 | 6.188 | 1.163 | 11.70 |
| 4 | 2.0625 | 10.24 | 1.575 | 14.85 |
| 5 | 2.0625 | 6.338 | 1.500 | 31.05 |

Serum-free medium (SFM), fibronectin supplemented SFM (SFM + FBN), basal medium (Basal) and serum-supplemented medium (SSM). Results are expressed as cell concentration per flask $x10^5$.

**Table 3.3.5(b)** Growth curves for DLKP-M over a 4 day growth period

| | *Experiment 2* | | *Experiment 3* | |
|---|---|---|---|---|
| **Time in days** | **SFM** | **SFM + FBN** | **SFM** | **SFM + FBN** |
| 1 | 0.275 | 0.788 | 0.675 | 1.688 |
| 2 | 1.125 | 2.625 | 2.138 | 5.925 |
| 3 | 2.588 | 5.550 | 2.424 | 9.900 |
| 4 | 2.513 | 6.788 | 2.100 | 3.300 |

Serum-free medium (SFM), fibronectin supplemented SFM (SFM + FBN). Results are expressed as cell concentration per flask $x10^5$.

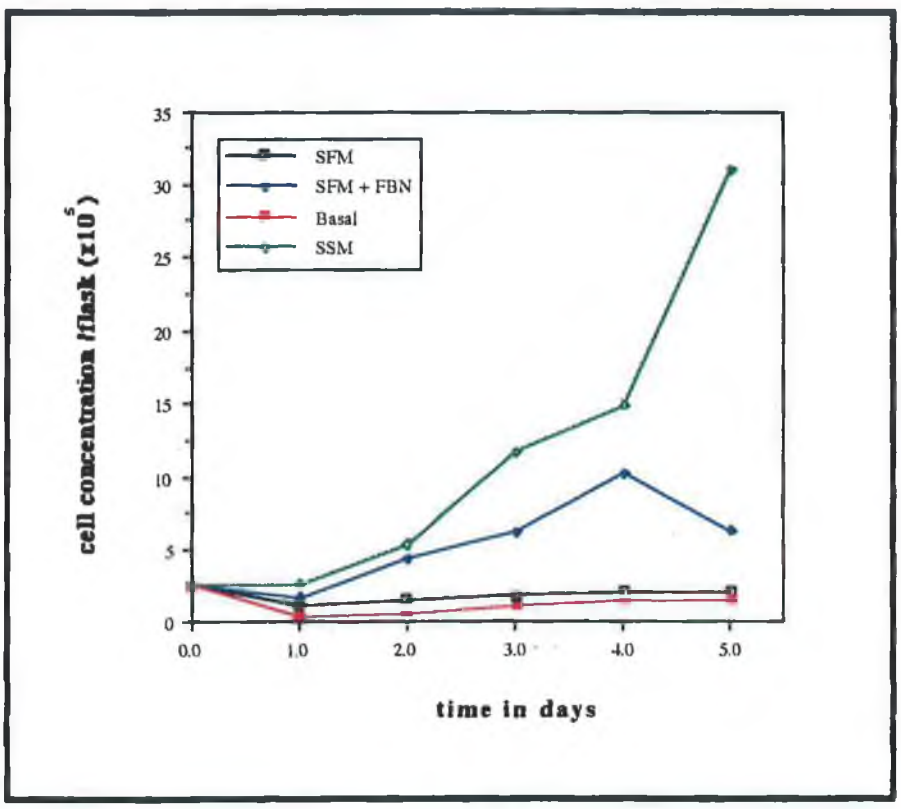


**Fig. 3.3.6** Growth curve for DLKP-M in serum-free medium (SFM), fibronectin-supplemented serum-free medium (SFM + FBN), basal medium (Basal) and serum-supplemented medium (SSM), Results are expressed as cell concentration per flask $x10^5$, results for experiment 1 are shown.

### 3.3.1.3.3 Subcultivation of DLKP-M in serum-free medium

Subcultivation of DLKP-M was found to be possible in fibronectin supplemented SFM. Table 3.3.6 shows two separate subcultivation experiments up to 3 passages for DLKP-M.

**Table 3.3.6** Subcultivation of DLKP-M

| | *Experiment 1* | | *Experiment 2* | |
|---|---|---|---|---|
| **Passage no.** | **SFM** | **SFM + FBN** | **SFM** | **SFM + FBN** |
| 1 | 1.650 ± 0.025 | 10.65 ± 0.300 | 0.694 ± 0.169 | 7.781 ± 0.344 |
| 2 | - | 7.550 ± 1.200 | - | 3.875 ± 0.688 |
| 3 | - | 9.950 ± 0.860 | - | 8.750 ± 1.312 |

Serum-free medium (SFM) and fibronectin supplemented SFM (SFM + FBN). Results are expressed in terms of average cell concentration per flask $x10^5$ ± standard deviation (n=3).

### 3.3.2 DLKPA IN SERUM-FREE MEDIUM

DLKPA is a multiple drug resistant (MDR) variant of DLKP which was selected to increasing concentrations of the chemotherapeutic drug, Adriamycin. It was decided to try to develop a system for the growth of this MDR model cell line in SFM that would allow extensive toxicity work to be carried out under defined, serum interference-free conditions. A system would then be available that allows the effects of different chemotherapeutic in combination with growth factors, prostaglandins *etc.* to be tested without the risk of serum contamination.

#### 3.3.2.1 Morphology of DLKPA in serum-free medium

In SF DLKPA culture, the cells grew much more spread out and tightly attached to the flask surface as compared to early passage DLKP cells. Confluency is usually reached in SF DLKPA (see Fig. 3.3.7) culture which did not occur in DLKP culture. The morphology of the DLKPA cell line in SFM was quite similar to SSM.

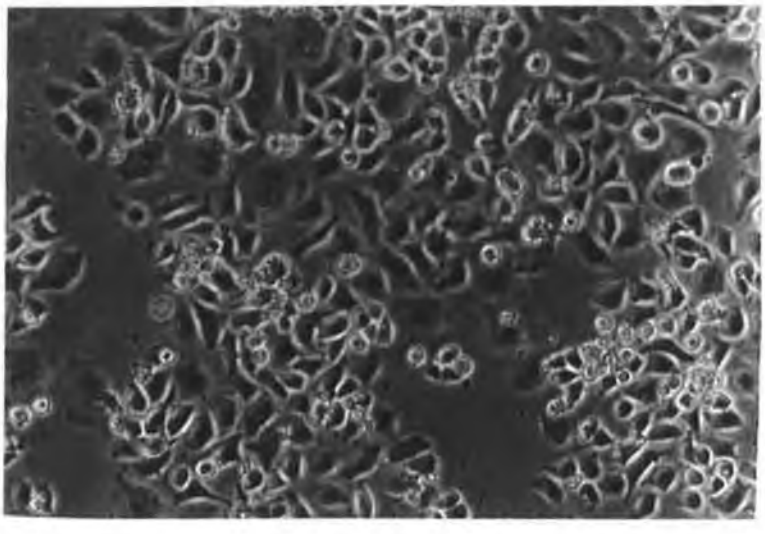

**Fig. 3.3.7** Growth of DLKPA cells in SFM - P.3 SF cells

### 3.3.2.2 Growth response of DLKPA in SFM

The SFM used initially for the growth of CHO-K1 and then DLKP cells, H SFM, was used as a basis for the SF growth of DLKPA cells. A growth curve was obtained over a 10 day period for DLKPA cells in SFM, SSM and Basal medium (see Fig. 3.3.8)

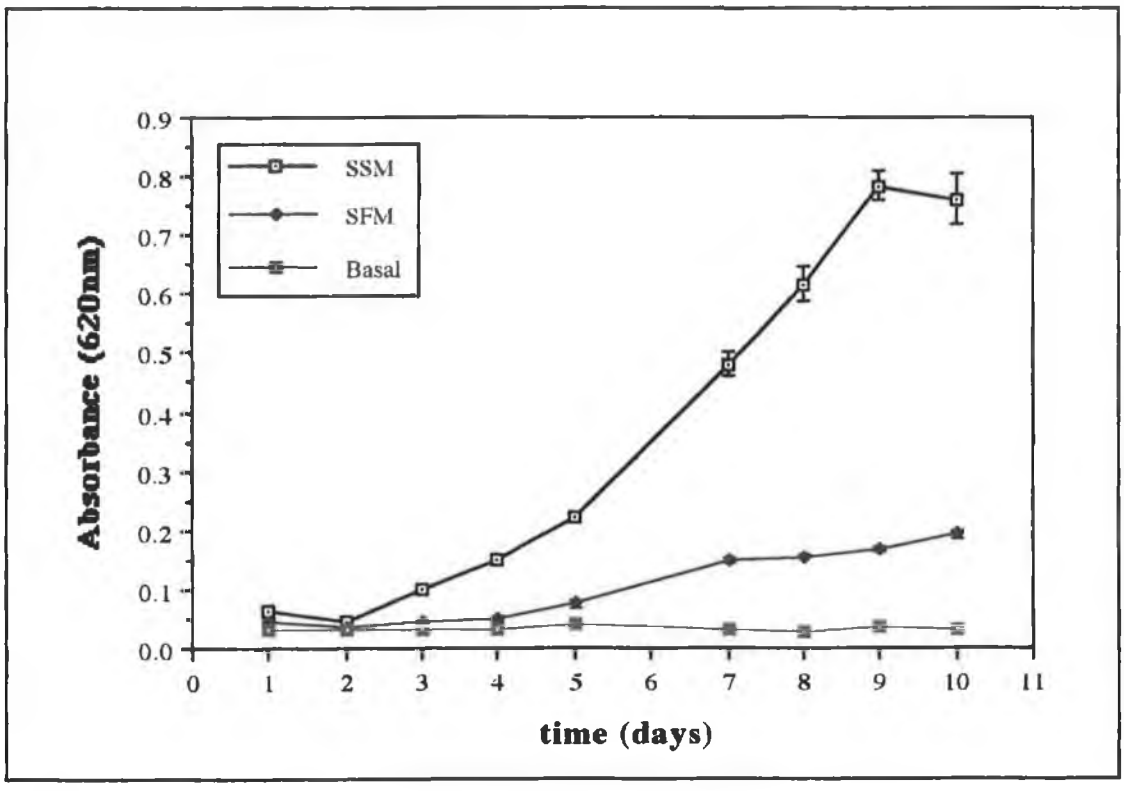


**Fig. 3.3.8** Growth curve for DLKPA cells in basal medium (Basal), serum-free medium (SFM) and serum-supplemented (SSM). Results are expressed as average absorbance at 620nm ± standard deviation (n=8).

### 3.3.2.3 Long-term cultivation in serum-free medium

The ability of this cell line to grow for long periods of time in SFM was then investigated. Passaging of the cell line was carried out approximately once every 7 to 10 days. Long-term growth has been found to be possible with two sets of experimental results shown in Table 3.3.7 where one experiment was carried out to P.15 SF (7 months in culture) and another was carried out to P.14 SF (6 months in culture).

Trypsinisation times were found to take on average between 10 to 15 minutes compared to the 3 to 5 minutes in DLKP SF culture. This seems to tie in with the microscopic observation that the DLKPA cells attach to the substratum much more tightly in SFM compared to DLKP SF cells.

**Table 3.3.7** Long-term growth of DLKPA cells in Ham's F12 SFM

| Passage no. | Experiment 1 | Experiment 2 |
|---|---|---|
| 1 | 23.375 | 15.488 ± 0.175 |
| 2 | 8.875 | 14.000 ± 1.575 |
| 3 | 16.400 | 17.006 ± 2.381 |
| 4 | 13.625 | 11.375 ± 0.438 |
| 5 | 14.525 | 15.375 ± 0.075 |
| 6 | 21.600 | 18.375 ± 2.925 |
| 7 | 22.188 | 16.406 ± 1.344 |
| 8 | 18.188 | 21.650 ± 6.500 |
| 9 | 11.400 | 12.825 ± 0.900 |
| 10 | 21.188 | 13.969 ± 1.656 |
| 11 | 12.313 | 14.325 ± 0.300 |
| 12 | 11.563 | 8.883 ± 0.275 |
| 13 | 18.188 | 17.563 ± 0.063 |
| 14 | 13.625 | 25.188 ± 0.000 |
| 15 | 17.550 | - |

Results are expressed in terms of cell concentration per flask $x10^5$ ± standard deviation (n=3) for experiment 2.

### 3.3.2.4 Cryopreservation of DLKPA cells in SFM

The same procedure was carried out as for DLKP SF cells. The cells were frozen in 10% DMSO and 0.1% Methylcellulose in SFM as before. Results from two separate experiments (Table 3.3.8) show that this SF freezing medium is capable of supporting good viability of the cells. The freezing technique appears to have no adverse long-term effect on the cell line in SF conditions. The cells were also noted to have attached to the flask surfaces within one hour of thawing.

**Table 3.3.8** Serum-free cryopreservation of DLKPA

| Cells used | % Viability |
|---|---|
| P.1 SF | 78.66 ± 3.03 |
| P.9 SF | 81.23 ± 4.44 |

Viability of cells after thawing ± standard deviation (n=6)

### 3.3.2.5 Development of assay system for miniaturised *in vitro* toxicity assays

The principal aim of the DLKPA SF work was to develop a system which enables chemotherapeutic toxicity assays to be carried out under defined conditions. A similar set of growth concentration assays were carried out for DLKPA cells as for DLKP cells. A range of cell concentrations were set up in 96-well plates as before ranging from $5x10^2$ to $1x10^4$ cells/ well over a 7 day and 10 day assay period. The cells were fixed using the method outlined for DLKP cells, using 50% cold TCA, and stained at the end point of the assay using crystal violet. The following concentration curves were obtained for the 7 and 10 day period (Fig. 3.3.9) and show that a cell concentration of $3x10^3$ cells per well over a 10 day period is the most effective concentration of cells to lie well within the linear part of the crystal violet absorbance curve.

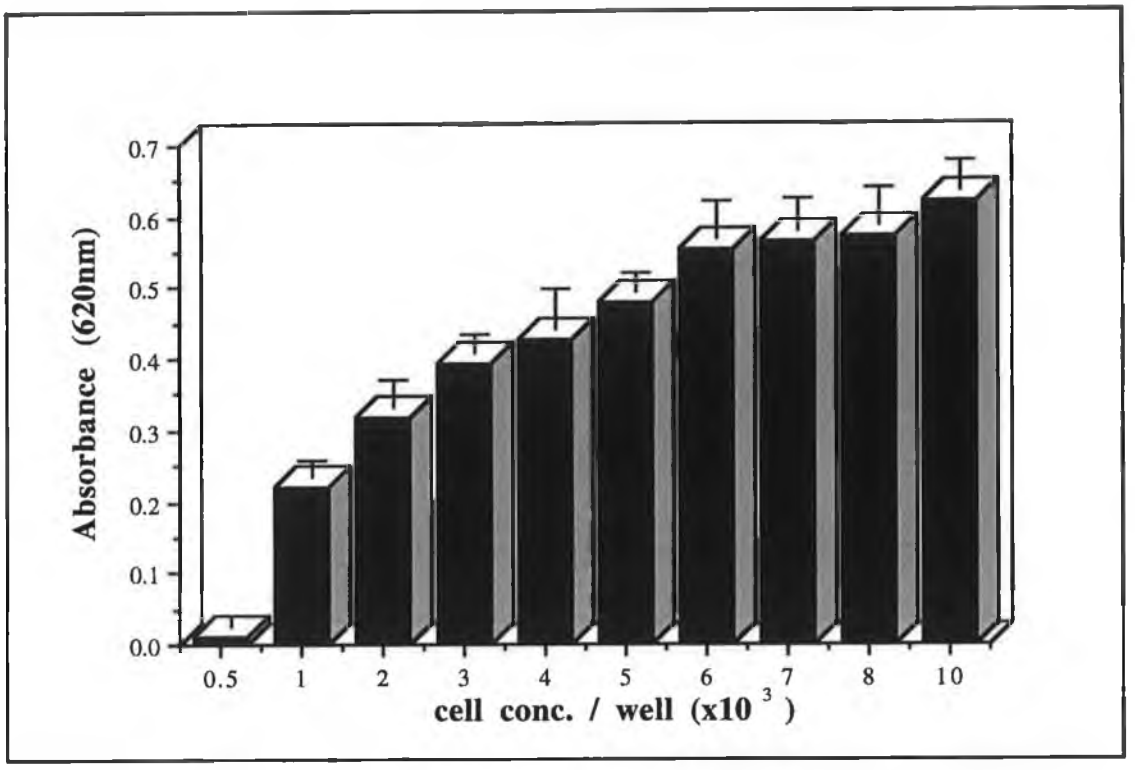


**Fig. 3.3.9(a)** Cell concentration curve for DLKPA cells over a 7 day growth period

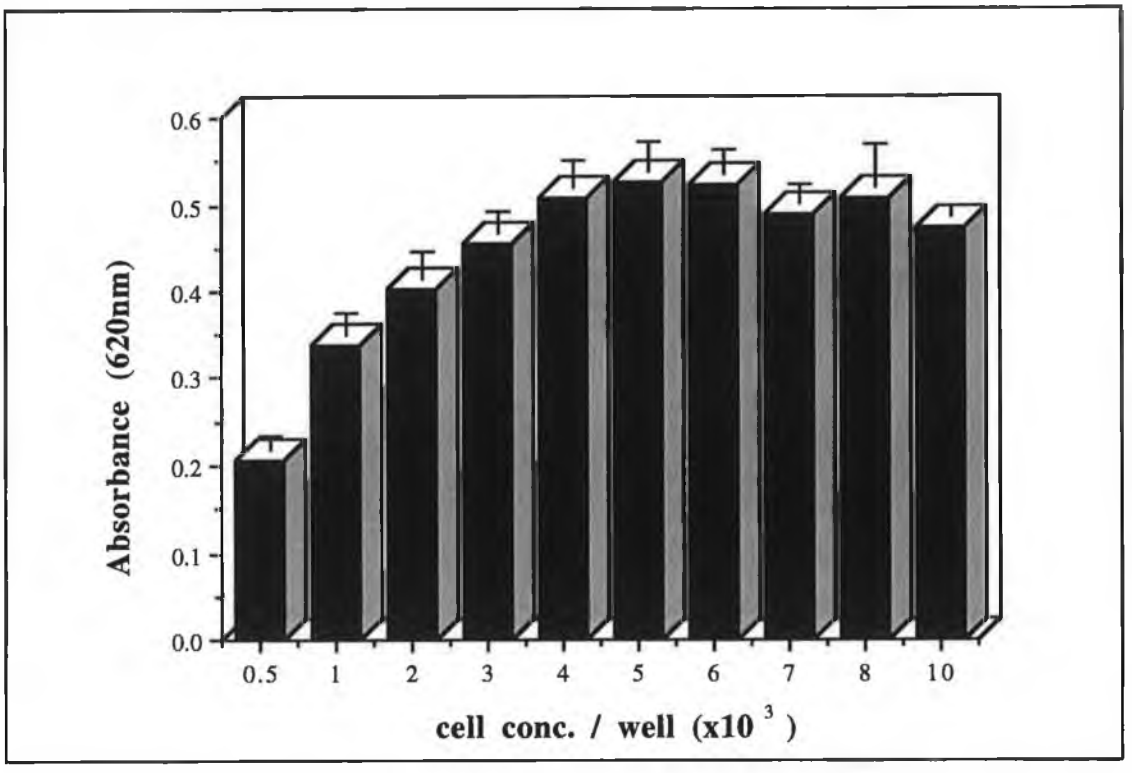


**Fig. 3.3.9(b)** Cell concentration growth curve for DLKPA cells over a 10 day period

## 3.4 INVESTIGATIONS INTO KERATIN 8 AND 18 EXPRESSION IN DLKP AND DLKPA

The DLKP cell line has been described pathologically as a poorly differentiated human lung carcinoma. Keratin intermediate filaments are expressed in epithelial cells in a cell- and tissue-specific manner. DLKP is normally keratin negative. However, treatment of this cell line with the differentiating agent, 5-bromodeoxyuridine (BrdU) over a 5-day period, induces expression of K8 and K18, shown by immunocytochemical methods (McBride and Clynes, manuscript submitted). BrdU treatment of a similar keratin negative cell line, H82, also results in induction of K8 and K18. These cells normally grow in suspension but on treatment with BrdU the cells begin to attach to the flask surface. BrdU treatment of a keratin positive cell line, A549, results in an increase in the expression of K8 and K18. However, BrdU treatment of a non-epithelial leukaemic cell line, HL60, which are normally keratin negative shows no induction of K8 or K18 filaments.

Studies were carried out to determine if BrdU can induce similar expression of keratin 8 and 18 proteins in DLKPA. The effect of the highly toxic chemotherapeutic agent, Adriamycin, on keratin expression in these cell lines was investigated.

Northern blot analysis has revealed that the levels of K8 and K18 mRNA are the same before and after BrdU treatment. These results seem to suggest that BrdU is acting at a post-transcriptional level to reverse the block in keratin 8 and 18 expression in keratin-negative DLKP cells. This suggests that BrdU may be exerting its effect directly or indirectly on protein translation, or stabilisation of translation products already present! Studies were carried out using the protein synthesis inhibitors, cycloheximide and Actinomycin D to determine if there might be some evidence for a protease causing a de-stabilisation or rapid degradation of a translation product for keratin 8 and 18.

### 3.4.1 Effect of BrdU on keratin 8 and 18 expression in DLKPA

DLKPA is a multiple drug resistant (MDR) variant of DLKP, which was selected to increasing concentrations of the chemotherapeutic drug, Actinomycin. There have been varying reports in the literature regarding relationships between multiple drug resistance and keratin expression (see Section 4.6.1). Experiments were carried out to determine if keratin filaments are expressed in DLKPA, and if BrdU could induce keratin expression in a manner similar to DLKP cells. All experiments were carried out in 6-well tissue culture plates, including immunocytochemical analyses, using concentrations of BrdU at 0, 1 and 10μM over a 5-7 day period (as described in Section 2.13-2.14).

The BrdU-treated cells showed similar morphology to BrdU-treated DLKP cells were found at 1μM and 10μM concentrations of BrdU. A 1μM concentration had little effect on morphology, allowing 70-80% survival similar to DLKP. However, 10μM BrdU had a much more pronounced effect on morphology, where the cells were much larger in appearance and their cytoplasm was stretched and spread out (Fig. 3.4.1). Similar toxicity levels to DLKP were noted at this concentration, with about 20-30% survival.

Table 3.4.1 lists a brief description of the effect of BrdU concentrations on the morphology of DLKPA and DLKP cell lines after 7 days exposure to the compound.

**Table 3.4.1** Summary of morphological effects of bromodeoxyuridine (BrdU) on DLKP and DLKPA

| Cell Line | Control | 1 μM BrdU | 10 μM BrdU |
|---|---|---|---|
| DLKP | ~ 70% confluent | ~ 70-80% control similar morphology to control | ~ 20-30% of control - larger cells and stretched |
| DLKPA | ~ 60% confluent | ~ 70-80% control. Similar morphology to control | ~ 20-30% of control - larger cells and stretched |

Immunocytochemical analysis was carried out on the BrdU-treated DLKPA cells. An anti-pan cytokeratin Ab was first used, which can detect the following cocktail of keratin filaments; cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19.

Negative staining for the pan-cytokeratin Ab was noted in the control, untreated wells. However, approximately 10% and 20-30% of cells were found to react positively with those Ab's following treatment with 1μM and 10μM BrdU respectively (Fig. 3.4.2). Similar results were observed using Abs to the individual keratins 8 and 18. Therefore, a similar effect to DLKP is noted on BrdU treatment of DLKPA.

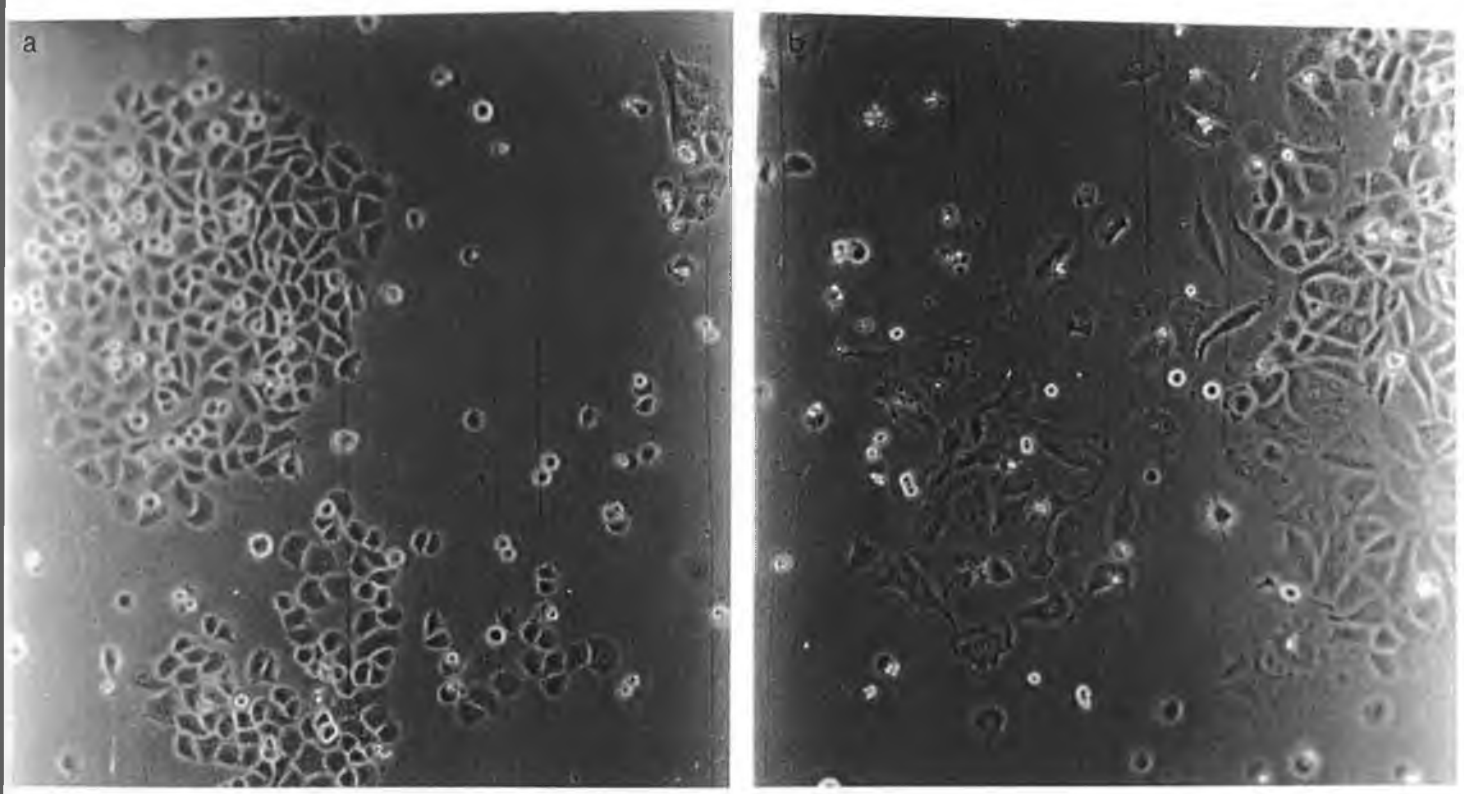


**Fig. 3.4.1** Morphology of DLKP without (**a**) and (**b**) with 10μM BrdU treatment for 7 days

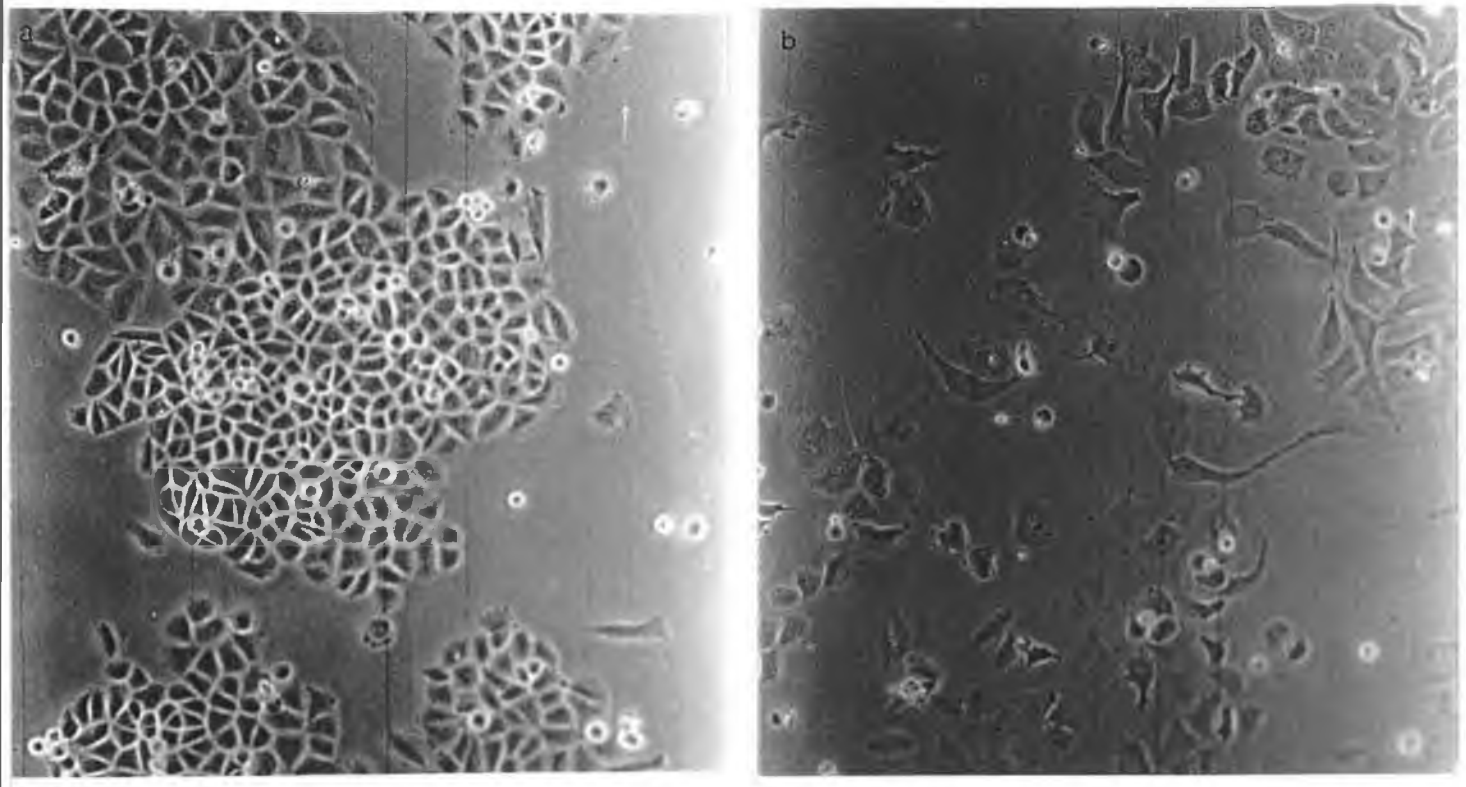


**Fig. 3.4.1** Morphology of DLKPA without (**a**) and (**b**) with 10μM BrdU treatment for 7 days

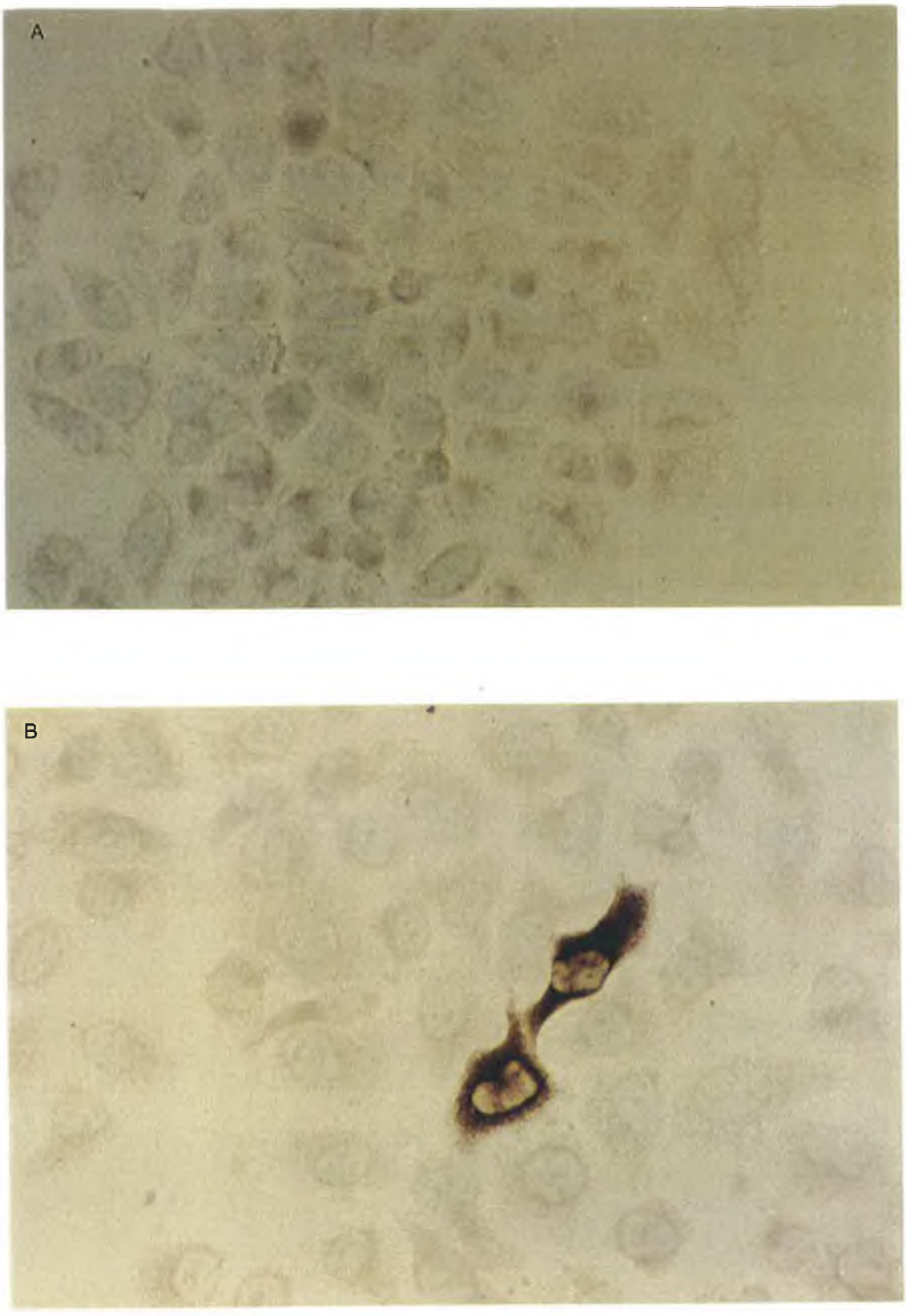


**Fig. 3.4.2** BrdU-induced keratin expression in DLKPA cells. (a) No immunoreactivity with anti-pan keratin Ab was detected in untreated cells. (b) Positive reactivity in 10μM BrdU-treated cells with anti-pan Ab. (x100)

### 3.4.2 Effect of Adriamycin on DLKP cells - to try to rule out a toxic effect from BrdU resulting in keratin expression

Bromodeoxyuridine at the concentration used in the study was found to be quite toxic to the cells ($IC_{50}$ of DLKP to BrdU is 2.6μM, $IC_{50}$ of DLKPA to BrdU is 1.7μM, Dr. Shirley McBride, PhD 1995). Experiments were carried out using the highly toxic chemotherapeutic drug Adriamycin, at concentrations which would result in similar levels of toxicity to the cells ($IC_{50}$ of DLKP to Adr is 0.01μg/ml approx; while the $IC_{50}$ of DLKPA to Adr is 2.5μg/ml approx.) to try to rule out the possibility that keratin expression was induced as a result of cytotoxic stress to the cells rather than the differentiating effect of BrdU.

**Table 3.4.2** Concentrations of Adriamycin chosen for assay

| Cell Line | equivalent to 1μM BrdU | equivalent to 10μM BrdU |
|---|---|---|
| DLKP | 0.002μg/ml Adr | 0.02μg/ml Adr |
| DLKPA | 0.4μg/ml Adr | 0.4μg/ml Adr |

The concentrations of Adriamycin chosen for DLKP were also used to treat HL60, A549 and H82 cells. Experiments were carried out in 6-well tissue culture plates as before. Cells were treated with Adriamycin for 5 days, the plates fixed and immunocytochemistry performed using Abs to cytokeratins 8 and 18.

No induction of cytokeratins 8 and 18 was observed in DLKP or DLKPA with increasing concentrations of Adriamycin. In A549 cells, there was no apparent increase in intensity of staining as is usual for BrdU-treated A549 cells. HL60 cells remained negative for cytokeratin expession while H82 cells showed no positive reactivity to cytokeratins 8 and 18. No adherent cells were observed in the treated wells compared to BrdU-treated H82 cells.

### 3.4.3 Long-Term Cytokeratin Expression in DLKP Cells

The ability of DLKP cells to retain cytokeratin expression following removal of BrdU from the medium was examined. Cells were treated with BrdU at 0, 5 and 10μM concentrations for 10 days. After this time, one plate was taken down and fixed (PLATE 1), while the second and third plates were fed with growth medium without BrdU for 5 days; after which one of the plates was taken down and fixed (PLATE 2), while the third plate was passaged - setting up a further two 6-well plates in BrdU-free medium. These plates were allowed to grow for a further 7 days, after which one of the plates was fixed (PLATE 3), while the other plate was passaged as before, setting up two 6-well plates. This serial passaging was carried out twice more (PLATE 4 and PLATE 5). Immunocytochemistry was then carried out using Abs to cytokeratins 8 and 18, with the results shown in Table 3.4.3.

**Table 3.4.3** Long-term keratin 8 and 18 expression in DLKP BrdU-treated cells

| | BrdU conc. | CK 8 | CK 18 |
|---|---|---|---|
| **PLATE 1** | 5μM | 5-10% +ve | 10 % +ve |
| | 10μM | 30% +ve | 30% +ve |
| **PLATE 2** | 5μM | 5% +ve | 10% +ve |
| | 10μM | 20% +ve | 15-20% +ve |
| **PLATE 3** | 5μM | -ve | 5% +ve |
| | 10μM | 5-10% +ve | 5-10% +ve |
| **PLATE 4** | 5μM | -ve | -ve |
| | 10μM | -ve | 5% +ve |
| **PLATE 5** | 5μM | -ve | -ve |
| | 10μM | -ve | -ve |

The results suggest that the cytokeratin positive cells are gradually being "diluted" out of the system. However, the cells appear to retain their cytokeratin positivity for at least two weeks on the removal of BrdU from the medium.

### 3.4.4 Protein Synthesis Inhibitor Studies on DLKP Cells

All experiments (including exposure to cycloheximide and Actinomycin D & immunocytochemical analysis) were carried out in 6-well tissue culture plates, as outlined in Section 2.13.

#### 3.4.4.1 Cycloheximide

Cycloheximide was added to the cells at concentrations ranging from 500ng to 100µg/ml for various lengths of time to try and determine if it can allow the induction of keratin expression in DLKP cells.

*Exp.1:* used concentrations of cycloheximide used in the literature (Cremisi and Duprey, 1987: Paine *et al.*, 1992) of 5 and 10µg/ml. Cells were treated for 3 and 6 hours with drug, medium was removed and BrdU-free medium was added for 2 days. Immmunocytochemistry was then performed using Abs. to CK 8/18.
Result: Some +ve cells (5%) found in 6hr plates at 5 and 10µg/ml, rest -ve including 3hr plates.

*Exp.2:* used concs. of cycloheximide - 0, 5, 10, 20, 50 and 100µg/ml for periods of 1, 3, 6, 9.5, 22 and 36hrs. BrdU-free medium was then added for 48hrs, plates fixed and immunocytochemistry carried out with Abs to CK 8/18.
Result: Some +ve cells (5%) found in 6 and 9.5hr plates at concentrations of cycloheximide from 5-20µg/ml. All other plates were negative.

*Exp.3*: used low conc. of cyloheximide (50ng/ml) over a longer time period *i.e.* 24, 48 and 72 hours. Immunocytochemistry again carried out to CK 8/18.
Result: No +ve cells for CK 8/18.

*Exp.4:* similar to experiment 1 but used time periods of 5, 6, 7, 8, 9, 10, 11 hrs with cycloheximide at 5 and 10µg/ml, removed medium and added drug-free medium for 48 hours. Immunocytochemistry carried out with Abs to CK 8/18 as before.
Result: positive cells were found at all time points, again approx. 5% +ve

The results seem to indicate that cycloheximide may have some effect on the cells, especially at 5 and 10µg/ml for 6-10hrs. However, the number of positive CK 8 and 18 cells is quite low.

### 3.4.4.2 Actinomycin D

Actinomycin D has proved to be extremely toxic to DLKP cells at the concentrations used in the study, from 100ng/ml to 5000ng/ml. Upon removal of the drug, the cells showed poor recovery over the next 48 hours of the assay in drug-free medium.

Immunocytochemical analysis of the cells following treatment showed no induction of keratins 8 or 18 using similar time points to those used in the cycloheximide experiments.

## 3.5 DETERMINATION OF EPITHELIAL-SPECIFIC AND EPITHELIAL-ASSOCIATED MARKERS IN BRDU-TREATED CELLS

The effect of the differentiation-inducing agent BrdU was examined in an attempt to further characterise the new cellular phenotype as observed by Dr. Shirley McBride. BrdU has previously been shown to induce the expression of cytokeratins 8, 17 and 18 in DLKP cells. Studies were carried out on DLKP and other cell lines, DLKPA, H82, A549 and HL60, to determine if BrdU changed the expression of other epithelial-specific markers. The new cellular morphology was noted to be much larger, stretched and flattened suggesting a change in the adhesiveness of the cells. The $\beta_1$ integrin profile of the cells before and after BrdU treatment was also detetmined.

### 3.5.1 DLKP cells

Bromodeoxyuridine treatment of DLKP cells results in the induction of the cytokeratins, 8 and 18, as already described in Section 3.4. Morphologically the cells were altered on BrdU treatment. The cells appeared much more flattened and stretched with and apparent increase in surface area compared to untreated cells. Further immunocytochemical studies were carried out on the cells to characterise the new cellular phenotype. BrdU assays were carried out in 6-well assay plates as previously described in Section 2.13.

#### 3.5.1.1 Cytokeratin 19

This keratin is often expressed in simple epithelia in conjunction with keratins 8 and 18. Treatment of DLKP cells with 10μM BrdU for 7 days, the induction of keratin 19 was observed with about 10% of the cells showing positive staining. This level of induction was less than that usually observed for cytokeratins 8 and 18 (usually 30%). Fig. 3.5.1 shows positive cytokeratin 19 staining in BrdU-treated DLKP cells compared to negative staining in untreated control cells.

#### 3.5.1.2 Epithelial-specific antigen

ESA is a transmembrane protein that is mainly expressed at the baso-lateral domain of epithelial cell membranes. The Ab is use to discriminate cells of epithelial origin from cells of mesothelial origin. There is evidence to suggest that ESA may act as a cellular adhesion molecule.

Untreated DLKP cells are negative for ESA but on BrdU treatment for 7 days at a 10μM concentration, approximately 70-80% of the cells are faintly positive for ESA staining

with about 20% of these cells showing strong positive staining. Fig. 3.5.2 shows positive ESA reactivity in BrdU-treated DLKP cells.

### 3.5.1.3 Desmosomal protein

Desmosomes are thickened parts of the plasma membrane where cells are tightly attached to their neighbours. They are specifically associated with intermediate filaments and are specific to epithelial cells.
Cells exposed to 10μM BrdU for 7 days were analysed for desmosomal protein expression. All cells were negative for this protein before and after treatment with BrdU.

### 3.5.1.4 EGF-R

EGF-R plays a role in cellular proliferation and differentiation. The expression of EGF-R varies frequently in epithelial tumours.
Cell exposed to 10μM BrdU for 7 days were analysed for EGF-R reactivity. All cells were faintly positive before and after treatment with no apparent increase in intensity.

### 3.5.1.5 Beta-1 integrins

Integrins are very important receptors for extracellular matrix components. There has been an increasing interest in the role of these receptors in cancer, especially during invasion and metastasis. Reports from the literature show a trend for the down-regulation of $\beta_1$ integrins in poorly differentiated and aggressive epithelial carcinomas. Because of the observed morphological changes in DLKP cells after BrdU treatment, where the cells become more flattened and stretched suggesting changes in the adhesion properties of the cells, investigations were carried out using antibodies to a range of these integrins.

*$\alpha_1\beta_1$ integrin (VLA-1)*
Untreated cells were negative for VLA-1 expression. However on treatment of DLKP cells for 7 days with 10μM BrdU, induction of VLA-1 was observed with approximately 30% of the cells showing positive staining. Fig. 3.5.3 shows positive VLA-1 staining in BrdU-treated DLKP cells compared to negative staining in untreated cells.

### $\alpha_2\beta_1$ *integrin (VLA-2)*

Untreated cells show faint staining in approximately 20-30% of the cells. Treatment of the cells with BrdU at a 10μM concentration for 7 days, an upregulation of expression of VLA-2 was observed with approximately 80% of the cells positive. 20% of these cells showed very strong positive staining. Fig. 3.5.4 shows upregulation of VLA-2 in BrdU-treated DLKP cells compared to faint staining in untreated control cells.

### $\alpha_3\beta_1$ *integrin (VLA-3)*

Untreated DLKP cells show positive staining for VLA-3 with no apparent increase in intensity on BrdU treatment for 7 days.

### $\alpha_4\beta_1$ *integrin (VLA-4)*

Untreated DLKP cells were found to be negative for VLA-4 with no induction of expression observed on BrdU treatment for 7 days.

### $\alpha_5\beta_1$ *integrin (VLA-5)*

Untreated DLKP cells were found to be negative for VLA-5 with no induction of expression observed on BrdU treatment for 7 days.

### $\alpha_6\beta_1$ *integrin (VLA-6)*

Untreated DLKP cells were found to be negative for VLA-6 with no induction of expression observed on BrdU treatment for 7 days.

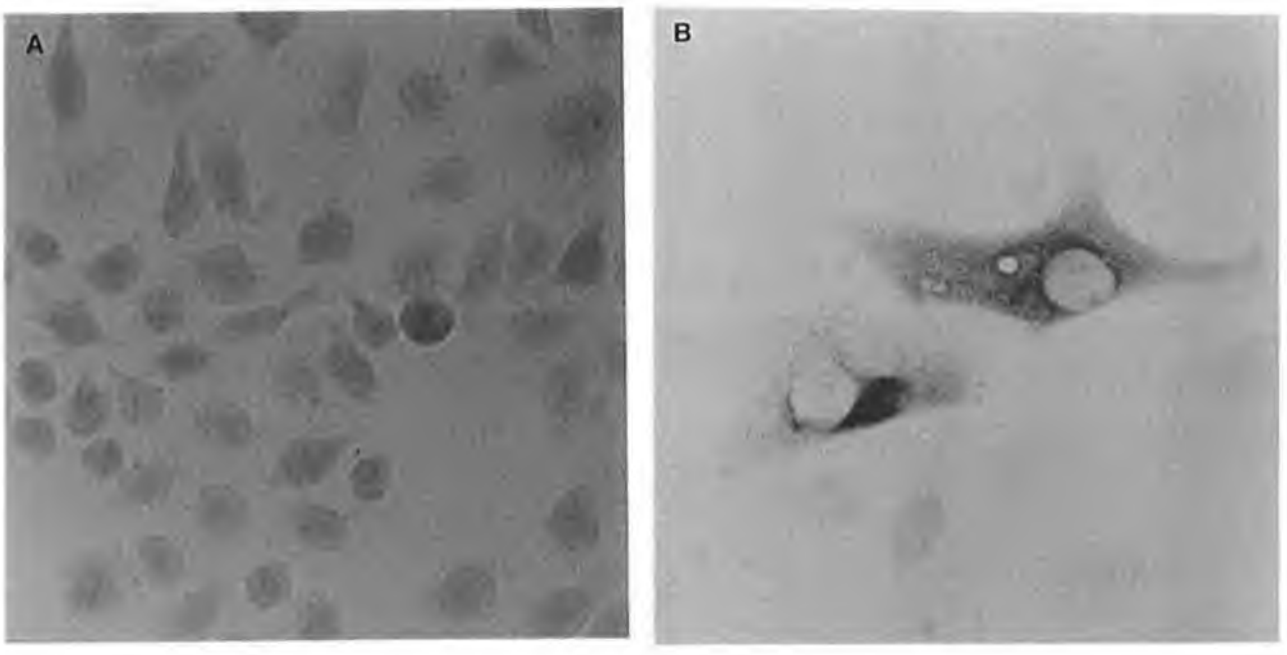


**Fig. 3.5.1** BrdU induced cytokeratin 19 staining in DLKP cells. (a) No immunoreactivity with anti-keratin 19 Ab was detected in untreated cells. (b) Positive reactivity in 10μM BrdU-treated cells with anti-cytokeratin 19 Ab.

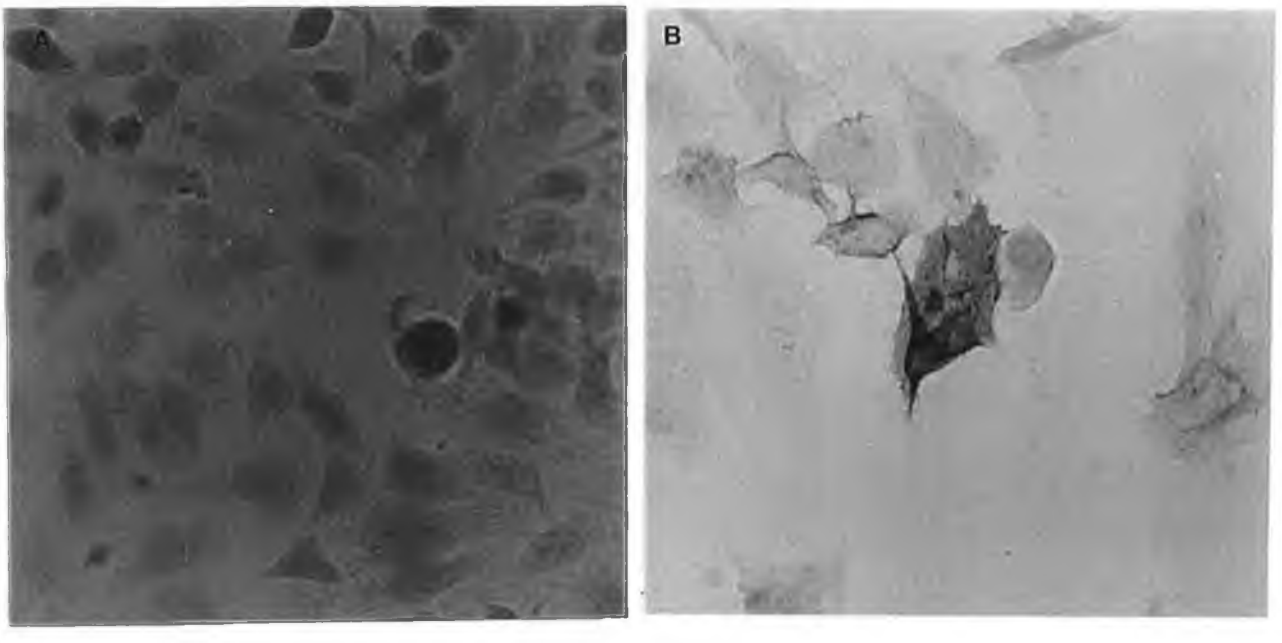


**Fig. 3.5.2** BrdU induced ESA staining in DLKP cells. (a) No immunoreactivity with anti-ESA Ab was detected in untreated cells. (b) Positive reactivity in 10μM BrdU-treated cells with anti-ESA Ab.

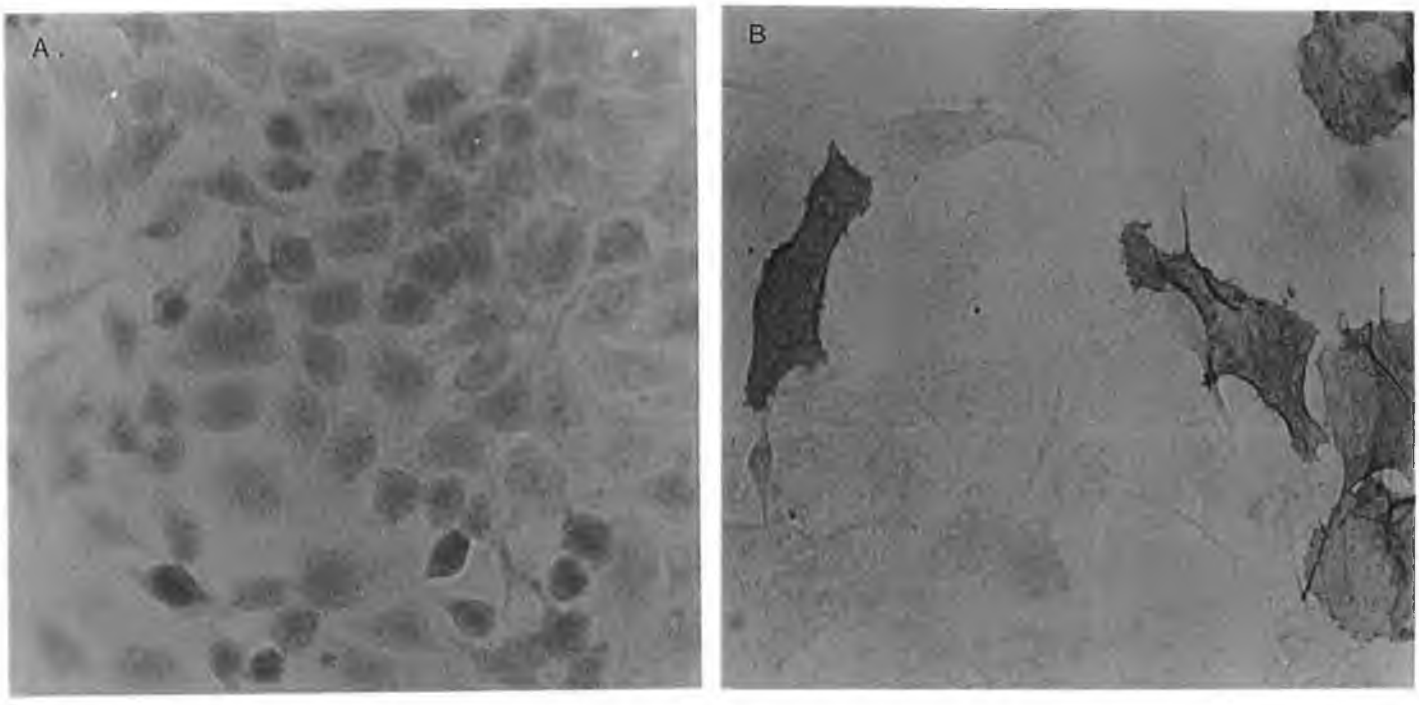


**Fig. 3.5.3** BrdU induced $\alpha_1$ integrin staining in DLKP cells. (a) No immunoreactivity with anti-$\alpha_1$ integrin Ab was detected in untreated cells. (b) Positive reactivity in 10μM BrdU-treated cells with anti-$\alpha_1$ integrin Ab.

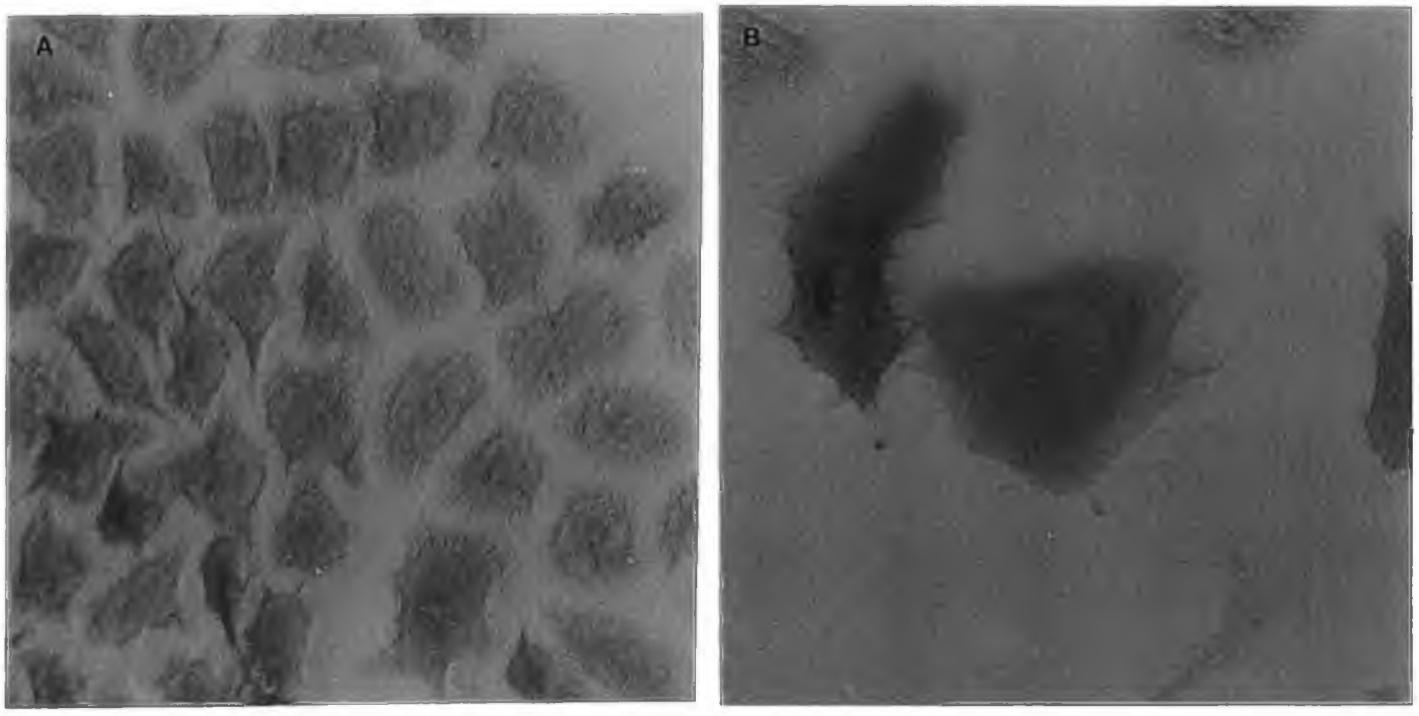


**Fig. 3.5.4** Upregulation of $\alpha_2$ integrin staining in DLKP cells. (a) Faint immunoreactivity with anti-$\alpha_2$ integrin Ab was detected in untreated cells. (b) Increased positive reactivity in 10μM BrdU-treated cells with anti-$\alpha_2$ integrin Ab.

### 3.5.2 Effect of BrdU on the expression of these antigens in other cell lines

#### 3.5.2.1 DLKPA

Cells were treated with BrdU for 7 days at a concentration of 10μM in all experiments carried out.

*Cytokeratin 19*
Untreated DLKPA cells were found to be negative for keratin 19. Induction of this keratin was observed after BrdU treatment for 7 days, with about 5% of the cells positively stained (Fig. 3.5.5(a)).

*Epithelial-specific antigen*
Untreated DLKPA cells, like DLKP, were found to be negative for the expression of ESA. However, after BrdU treatment for 7 days most of the cells (at least 80%) were found to show faint positive staining for ESA (Fig. 3.5.5(b)).

*$\alpha_1\beta_1$ integrin (VLA-1)*
Untreated DLKPA cells were found to be negative for VLA-1. BrdU treatment for 7 days resulted in the induction of this integrin in about 10% of the cells (Fig. 3.5.5(c))..

*Desmosomal protein*
Untreated- and BrdU-treated cells were found to be negative for the expression of desmosomal protein.

*EGF-R*
Untreated and treated DLKPA cells showed faint positive staining for EGF-R with no apparent increase in intensity, similar to BrdU treatment of DLKP cells.

This pattern of expression in DLKPA cells is the same for DLKP cells with similar induction of cytokeratin 18, ESA and $\alpha_1$ integrin and no change in the expression of EGF-R and desmosomal protein on treatment with BrdU.

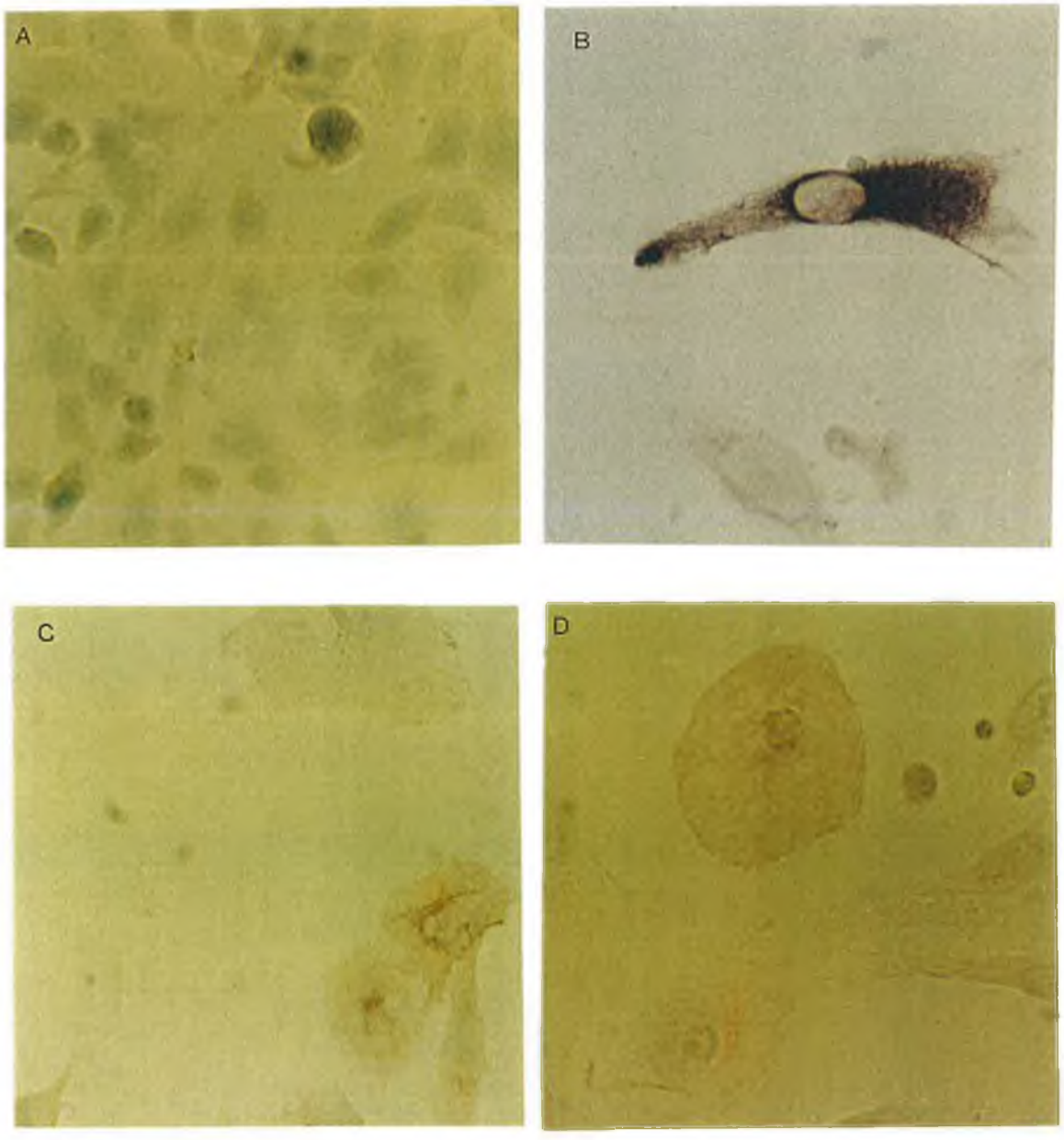


**Fig. 3.5.3** BrdU induced cytokeratin 19, ESA and $\alpha_1$ integrin staining in DLKPA cells. (a) No immunoreactivity with anti-$\alpha_1$ integrin Ab was detected in untreated cells. (b) Positive reactivity in 10μM BrdU-treated cells with anti-CK 19 Ab. (c) Positive reactivity in 10μM BrdU-treated cells with anti-ESA Ab. (d) Positive reactivity in 10μM BrdU-treated cells with anti-$\alpha_1$ integrin Ab.

### 3.5.2.2 H82

H82 is a human lung cell line and has been classified as variant SCLC. This cell line thus has no cytokeratin proteins expressed under normal growth conditions. The cells are similar to DLKP in that they express some NE markers. This cell line normally grows as aggregates in suspension but on treatment with BrdU, the cells begin to attach to the surface of the wells. Treatment with 10μM BrdU for 7 days results in attachment of over 90% of the cells to the surface of the wells. It has previously been shown that treatment with BrdU results in the induction of cytokeratin 8, 17 and 18 in H82 cells (McBride, 1995). There may also be changes in the expression of cell adhesion molecules in these cells in addition to the alterations in cytoskeletal proteins.

In all experiments, H82 were treated for 7 days with 10μM BrdU. After this time, attached cells were fixed in the 6-well plates while cytospins were made of the suspension cells as outlined in Section 2.14.2. Immunocytochemistry was then carried out on these cells.

*Cytokeratin 19*

Untreated H82 cells were found to be negative for keratin 19 expression. Treatment with BrdU resulted in keratin 19 expression in about 10% of treated, attached cells.

*ESA*

Untreated H82 cells were found to be negative for ESA expression. Treatment with BrdU showed no induction of ESA

*$\alpha_1\beta_1$ integrin (VLA-1)*

H82 normally are negative for VLA-1. However treatment with BrdU shows an induction of this integrin in about 5% of the cells.

*$\alpha_2\beta_1$ integrin (VLA-2)*

Untreated and BrdU-treated H82 showed negative staining for VLA-2.

Induction of $\alpha_1$ integrin and cytokeratin 19 was observed on treatment with BrdU similar to DLKP cells. However, no induction of ESA or upregulation of $\alpha_2$ integrin was observed.

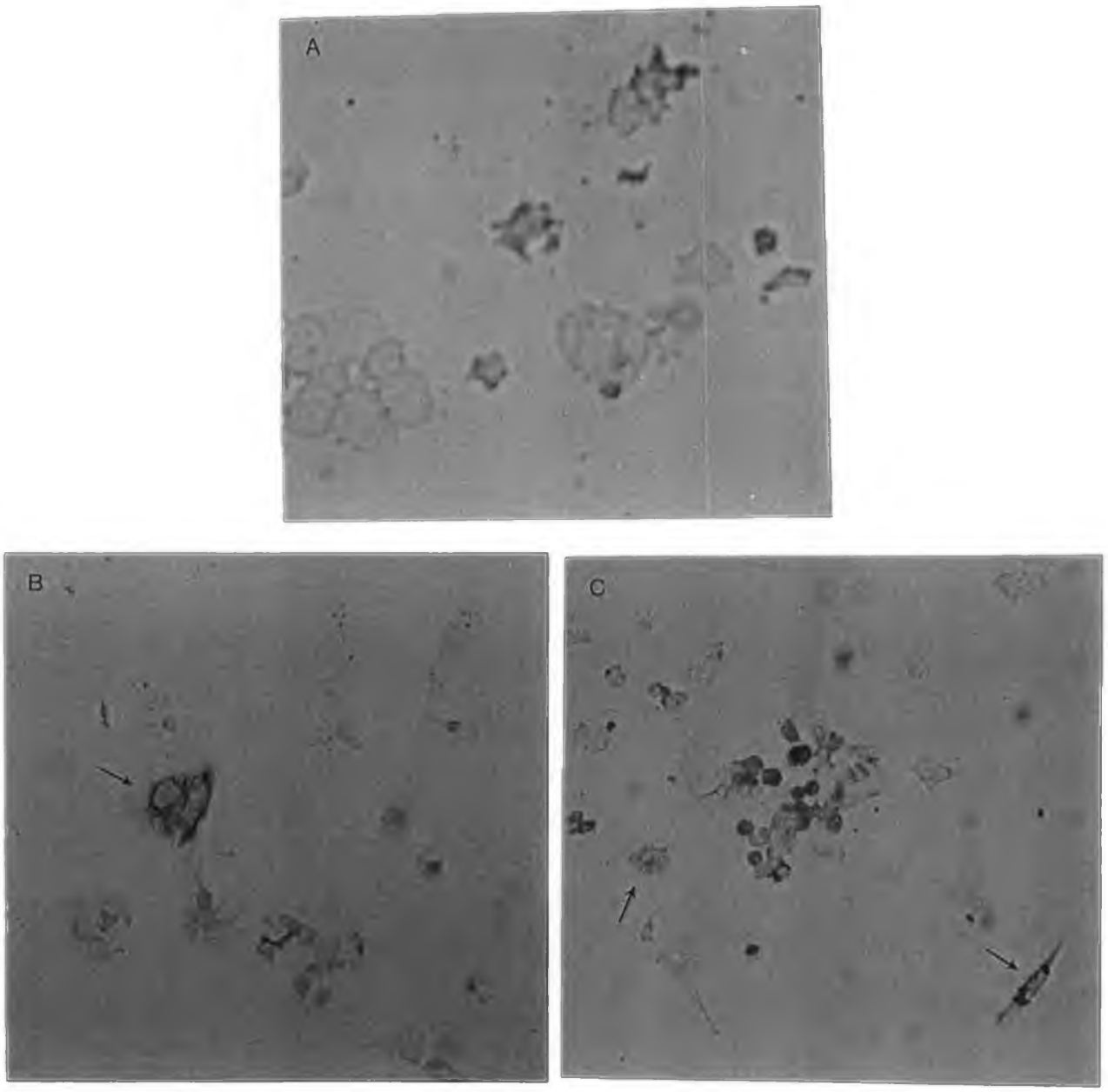


**Fig. 3.5.6** BrdU-induced cytokeratin 19 and $\alpha_1$ integrin staining in H82 cells. (a) No immunoreactivity with the anti-$\alpha_1$ integrin Ab was observed in untreated cells. (b) Positive reactivity in 10μM-treated cells with anti-CK 19 Ab. (c) Positive reactivity in 10μM-treated cells with anti-$\alpha_1$ integrin Ab.

### 3.5.2.3 A549

A549 cells have been classified as human lung adenocarcinoma cells. These cells express keratins 8 and 18 but not keratin 17 proteins. Treatment with BrdU results in the induction of expression of keratin 17 and an increased expression of keratins 8 and 18 (McBride, 1995).

*Cytokeratin 19*

Approximately 30% of the untreated A549 cells were positive for keratin 19. However, on treatment with BrdU, there is a strong upregulation of the expression of this protein with at least 80-90% of the cells positive for keratin 19 (Fig. 3.5.6(a)).

*Desmosomal protein*

Untreated- and BrdU-treated A549 cells were found to be positive for expression of desmosomal protein with no apparent increase in intensity.

*Epithelial-specific antigen*

Untreated A549 cells show approximately 20-30% of the cell with positive staining. On treatment with BrdU for 7 days, approximately all the cells show faint positive staining for ESA (Fig. 3.5.6(b).

*EGF-R*

Untreated and treated A549 cells were found to be positive for EGF-R with no apparent increase in intensity.

*$\alpha_1\beta_1$ integrin (VLA-1)*

Untreated and treated A549 cell were found to be faintly positive for the expression of VLA-1.

*$\alpha_2\beta_1$ integrin (VLA-2)*

Untreated and treated A549 cells were found to show strong staining for VLA-2 with no apparent increase in intensity.

*$\alpha_3\beta_1$ integrin (VLA-3)*

Untreated and treated cells were found to show strong staining for VLA-3 with no apparent increase in intensity.

*$\alpha_4\beta_1$ integrin (VLA-4)*

Untreated and treated A549 cells were found to be negative for expression of VLA-4.

*$\alpha_5\beta_1$ integrin (VLA-5)*

Untreated and treated A549 cells were found to be positive for expression of VLA-5 with no apparent change in staining intensity.

*$\alpha_6\beta_1$ integrin (VLA-6)*

Untreated and treated A549 cells were found to be negative for expression of VLA-6 with no apparent change in staining intensity.

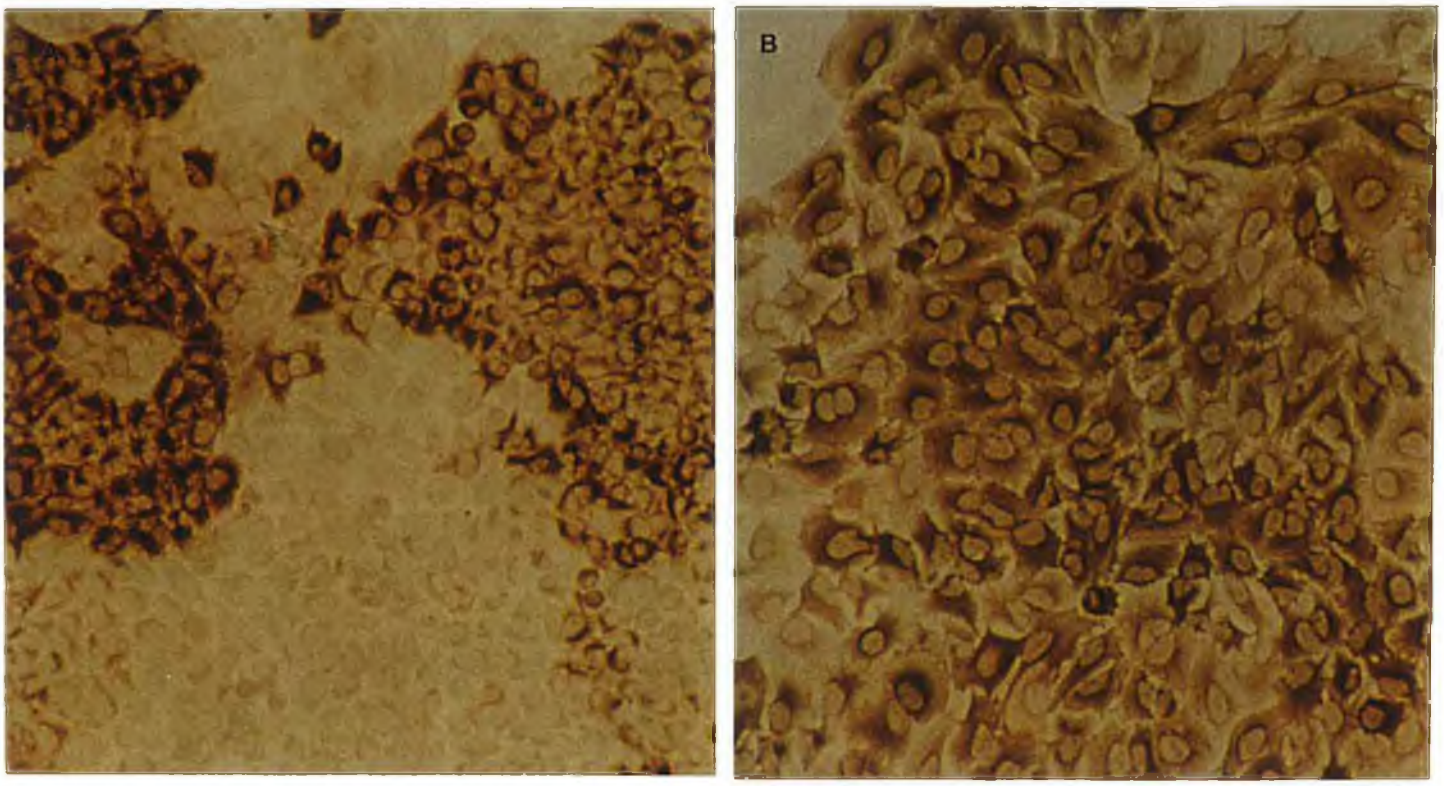


**Fig. 3.5.7(a)** Upregulation of cytokeratin 19 staining in A549 cells. (a) Scattered positives (30-40%) with anti-cytokeratin 19 Ab were detected in untreated cells. (b) Upregulation in 10μM BrdU-treated cells with anti-cytokeratin 19 Ab.

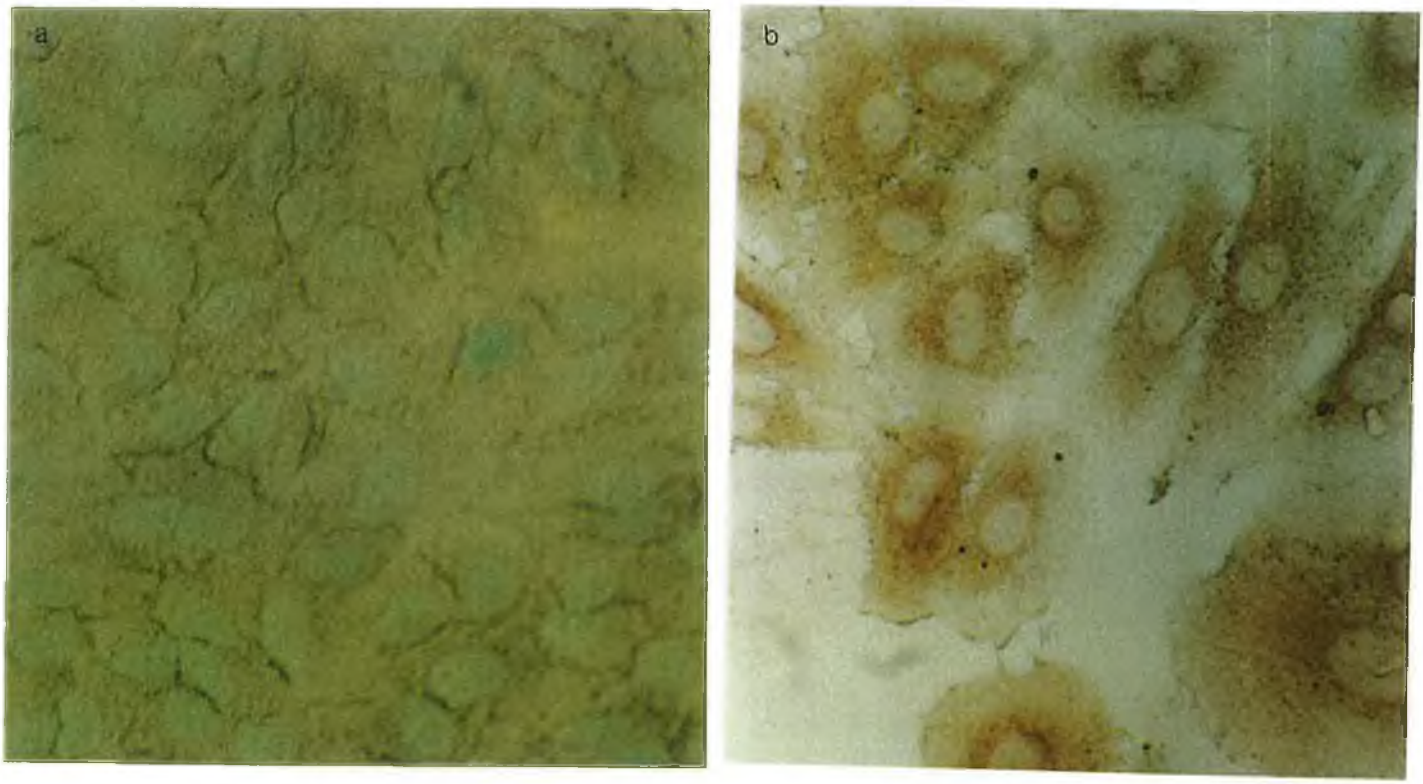


**Fig. 3.5.7(b)** Upregulation of ESA in BrdU-treated A549 cells. (a) Faint immunoreactivity with anti-ESA Ab was detected in untreated cells. (b) Upregulated positive reactivity in 10μM BrdU-treated cells with anti-ESA Ab.

### 3.5.2.4 HL60

HL60 is a human leukaemic cell line and is thus derived from a lineage distinct from epithelial and neural crest cell lines. These cells normally grow in suspension and therefore cytospins had to be made of these cells before immunocytochemical analysis could be carried out (as described in Section 2.14.2).

Untreated and treated HL60 cells were negative for the expression of cytokeratin 19, ESA, desmosomal protein, $\alpha_1\beta_1$ integrin, $\alpha_2\beta_1$ integrin, $\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrin and positive for the expression of $\alpha_4\beta_1$ integrin with no apparent increase in intensity.

Fig. 3.5.8 shows negative staining in untreated and BrdU-treated HL60 cells for anti-$\alpha_1$ integrin.

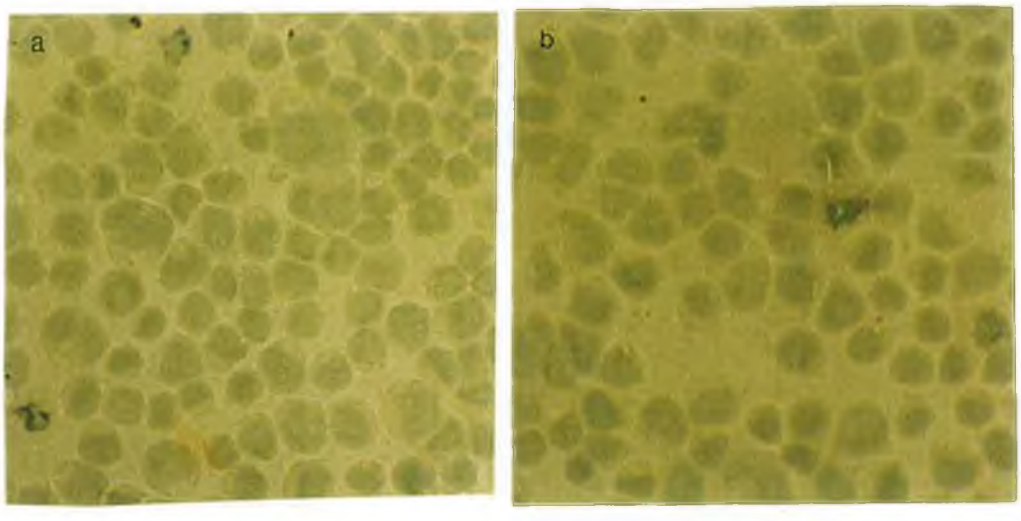


**Fig. 3.5.8** $\alpha_1$ integrin reactivity in untreated and BrdU-treated HL60 cells. (a) No immunoreactivity with anti-$\alpha_1$ integrin Ab was detected in untreated cells. (b) No immunoreactivity in 10μM BrdU-treated cells with anti-$\alpha_1$ integrin Ab.

### 3.5.3 Use of serum-free medium to investigate the differentiation effects of BrdU in a completely defined system

Investigations were carried out to determine if BrdU can induce expression of keratin proteins, ESA and integrins in a completely defined system. Serum is completely undefined and may have different factors which contribute towards the differentiation process and possibly mask some of the effect of BrdU.

Experiments were set up in 6-well plates with cells seeded at a concentration of $1x10^4$ per well and allowed to attach for 48 hours. The serum-free medium used was that extensively investigated in Section 3.2.1 and 3.2.2 for the growth of DLKP cells. Cells were treated with 10μM BrdU for 7 days with replenishment of the medium every 2 to 3 days. Plates were then fixed and immunocytochemistry was carried out using antibodies to cytokeratins 8, 18 and 19, ESA, $\alpha_1$ and $\alpha_2$ integrins as described in Section 2.14.

**Table 3.5.1** Effect of BrdU on antigen expression of DLKP cells in SFM

| Antigen looked at | Expression on BrdU treatment |
|---|---|
| CK 8 | induction |
| CK 18 | induction |
| CK 19 | induction |
| ESA | induction |
| $\alpha_1$ integrin | induction |
| $\alpha_2$ integrin | up-regulation |

This is exactly the same trend observed in experiments performed using serum supplemented to the medium at a concentration of 5% (v/v).

### 3.5.4 Analysis of induced and upregulated proteins by Western blotting and immunoprecipitation techniques.

BrdU-treatment of DLKP cells resulted in the induction of cytokeratin 19, ESA and $\alpha_2\beta_1$ integrin expression, observed by immunocytochemical techniques. Quantitation of these changes was carried out using Western blotting and the more sensitive immunoprecipitation technique.

#### 3.5.4.1 Western blotting

In all experiments, cell lysates were prepared as outlined in Section 2.16. Cell concentrations of $2x10^4$ cells per lane were run on 12% polyacrylamide gels for detection of cytokeratin 19 and ESA, which are both 40kDa proteins, under non-reducing conditions.

For detection of cytokeratin 19, DLKP cells were treated with BrdU for 7 and 14 days. A549, HL60 and MCF-7 were treated with BrdU for 7 days. Fig. 3.5.8 shows the blot obtained for a membrane probed with anti-cytokeratin 19 Ab. HL60, A549 and DLKP untreated and BrdU-treated cells were run on the gel. MCF-7 cells were used as a positive control in the experiment.

The upregulation observed by immunocytochemical staining in A549 BrdU-treated cells can clearly be noted from the blot. No protein was detected for treated and untreated DLKP or H82 cells, most likely due to the low level of induction of cytokeratin 19 expression. HL60 treated and untreated cells were also -ve for K19 expression.

Fig. 3.5.9 shows a blot for A549 cells treated with BrdU for 48 hours, 5 days and 7 days to show upregulation of the keratin 19 with increasing lengths of time in 10μM BrdU. Again, upregulation of the protein was observed on BrdU treatment in A549 cells over the time-course.

For the detection of ESA, DLKP cells were treated with BrdU for 7 and 14 days. A549 cells were treated for 7 days. A549 and DLKP untreated and treated cells were run on the gels. Fig. 3.5.10 shows a blot obtained for a filter probed with an anti-ESA Ab. Upregulation of ESA can be observed for DLKP-BrdU-treated cells, especially at 14 days treatment, compared to untreated cells.

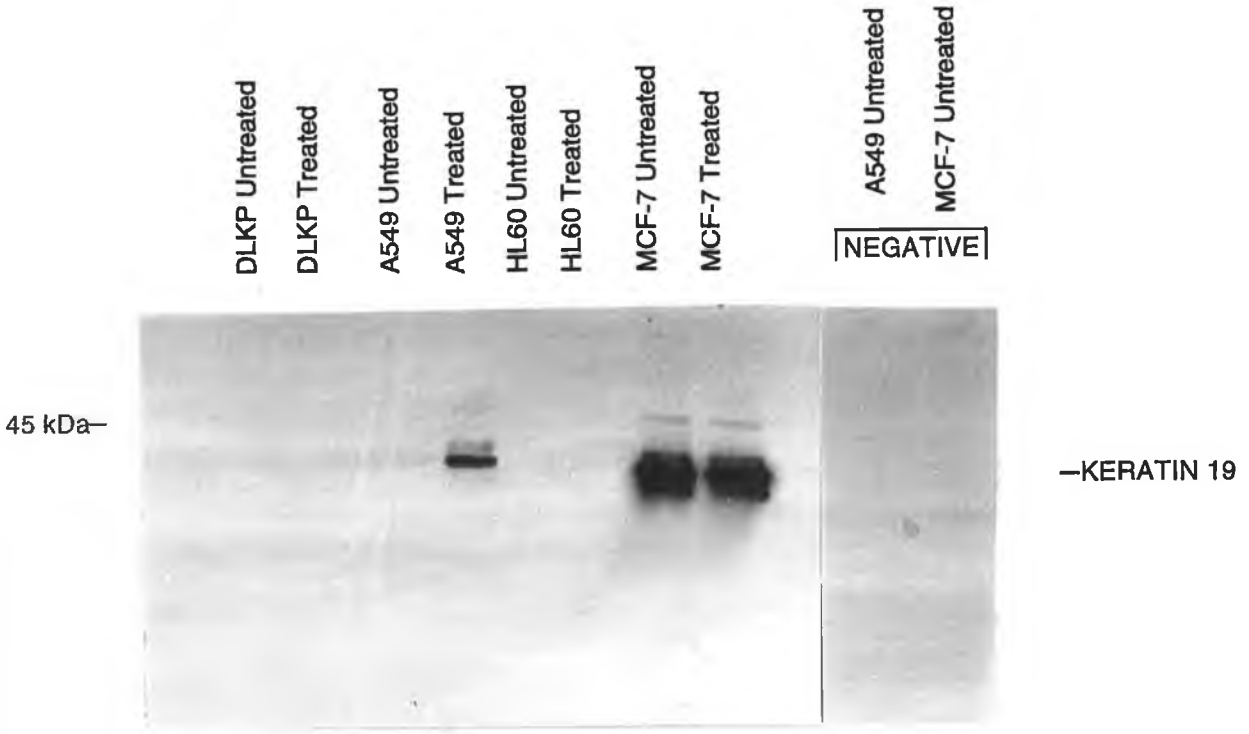


**FIG. 3.5.9** Western blot showing Keratin 19 expression before and after Bromodeoxyuridine treatment for seven days. MCF-7 was used as a positive control.

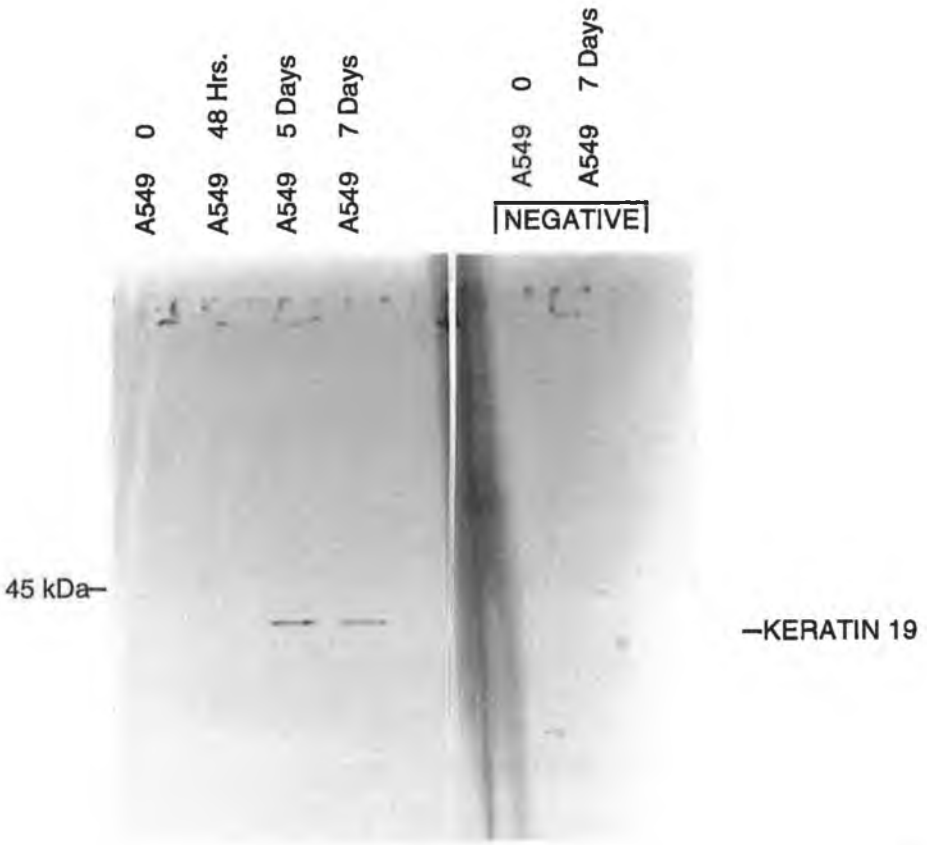


**FIG. 3.5.10** Western blot showing Keratin 19 expression in Bromodeoxyuridine treated A549 cells in a time course assay from 0 to 7 days.

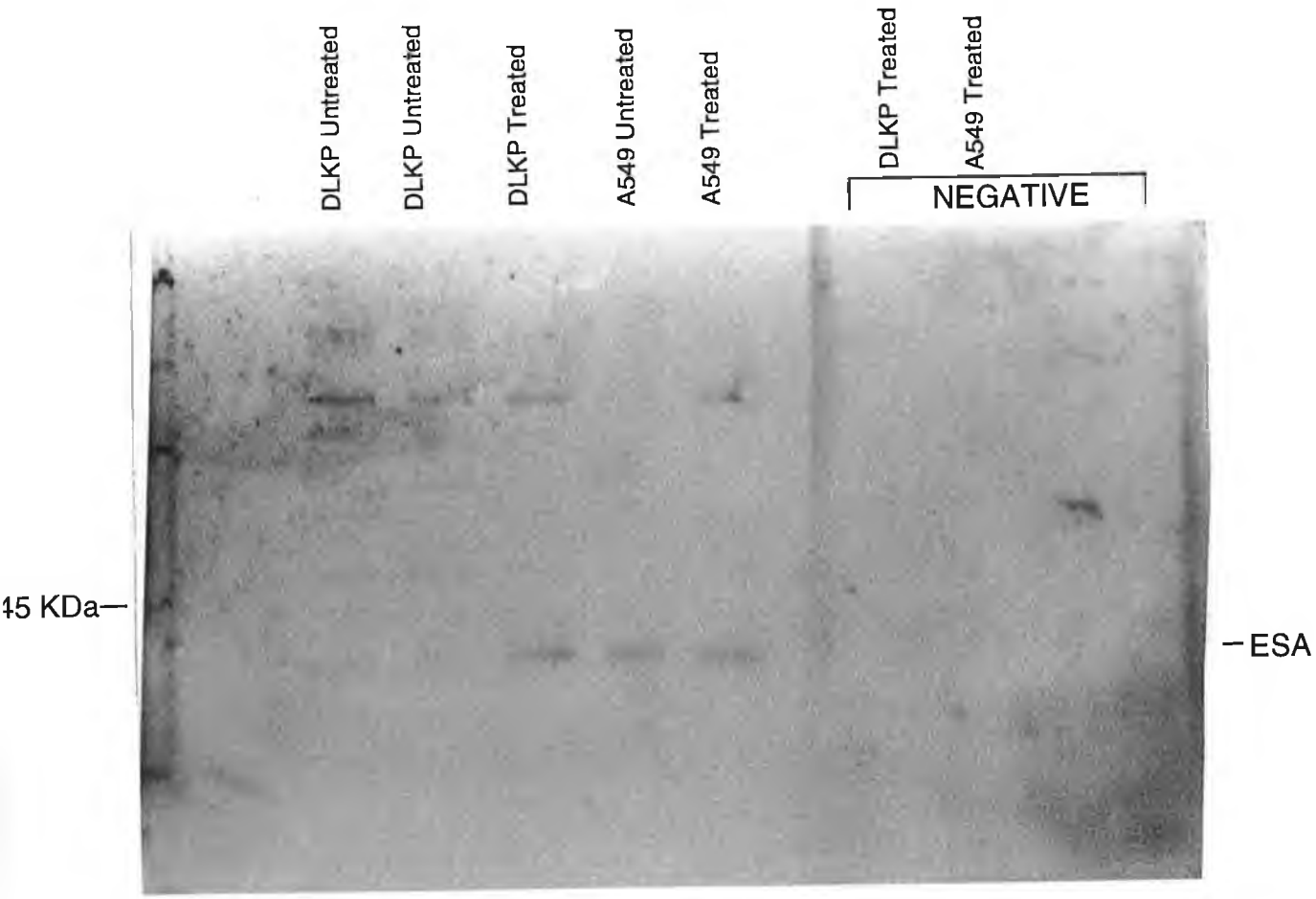


**FIG. 3.5.11** Western blot showing ESA expression before and after Bromodeoxyuridine treatment in DLKP and A549 cells.

### 3.5.4.2 Immunoprecipitation

This is a more sensitive technique than Western Blotting, which could possibly pick up the small changes in expression of proteins by BrdU treatment.

A Boehringer Mannheim kit was used in which cell lysates are labelled with biotin. The antibody (Ab) to be probed is then added. The Ab-protein complexes are subsequently precipitated using Protein A or Protein G beads, depending on the isotype of the Ab. The samples are then run on gels, blotted and probed using an anti-biotin Ab, with expression detected using ECL (see Section 2.17). Immunoprecipitation was carried out using Abs to CK19, $\alpha_1$ integrin and $\alpha_2$ integrin. CK19 samples were run on 12% gels while $\alpha_1$ and $\alpha_2$ integrin samples were run on 7.5% gels. A549 and DLKP cells were treated with BrdU for 7 days and then harvested for protein precipitation. In all experiments an irrelevant monoclonal antibody was used as the negative control.

Fig. 3.5.12 shows the blot obtained by immunoprecipitation of keratin 19 protein. Upregulation of CK19 expression was again clearly noted in A549 BrdU-treated cells. However, the technique was unable to pick up the changes in expression of K19 in DLKP cells.

Fig. 3.5.13 shows the blot obtained by immunoprecipitation of $\alpha_1$ integrin proteins in A549 and DLKP untreated and BrdU-treated cells. SKLU-1 was used as a positive control. Induction of $\alpha_1$ integrin in DLKP treated cells can be observed from the blot.

Fig. 3.5.14 shows the blot obtained by immunoprecipitation of $\alpha_2$ integrin proteins in DLKP and A549 untreated and BrdU-treated cells. Upregulation is clearly evident for DLKP cells on treatment with BrdU. A lot of smearing was observed in the negative control most likely due to non-specific binding of anti-biotin antibody in the cell lysates. However, a definite clear band is observed at approximately 160kDa in the samples precipitated with the $\alpha_2$ integrin antibody that is not observed in the negative control.

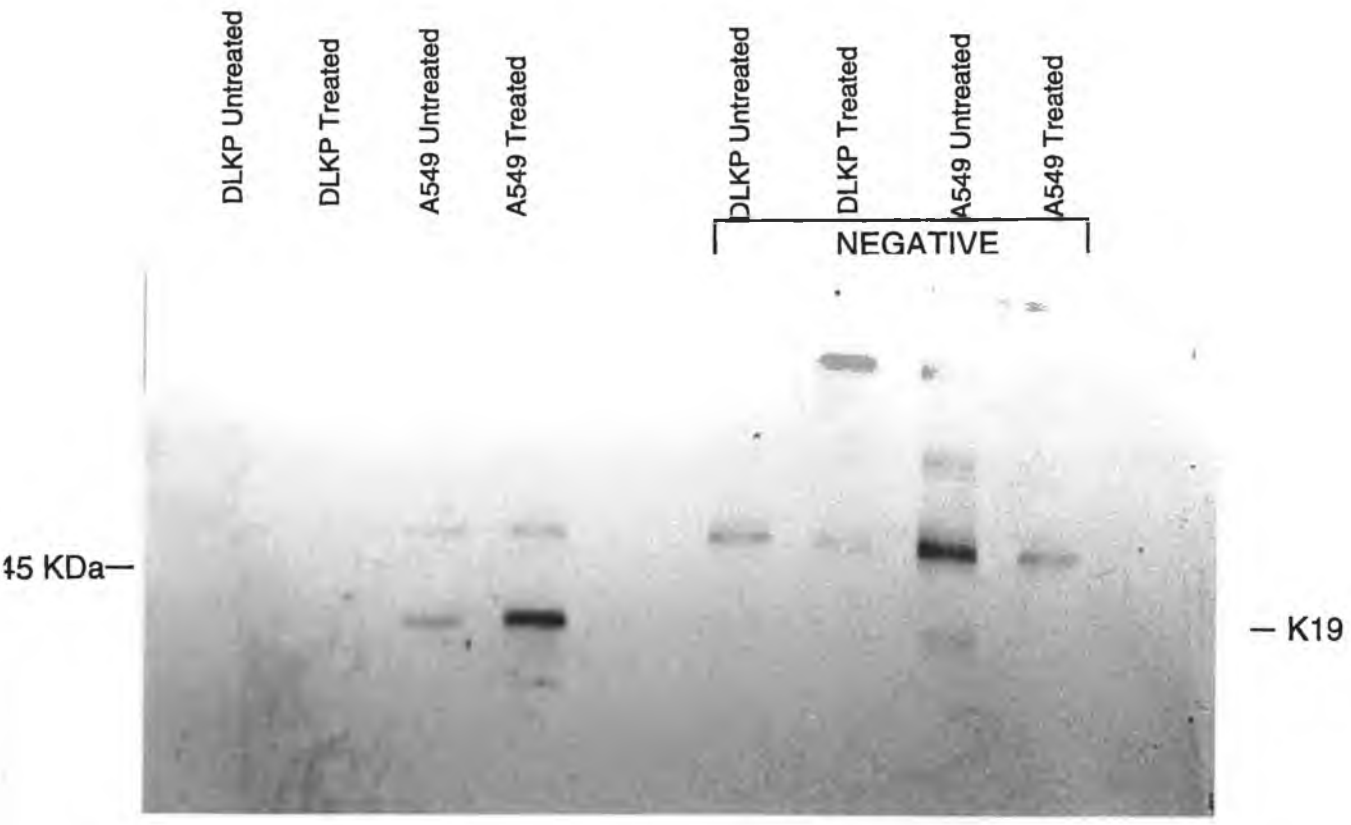


**FIG. 3.5.12** Immunoprecipitation of Keratin 19 Protein from A549 and DLKP untreated and Bromodeoxyuridine - treated cells.

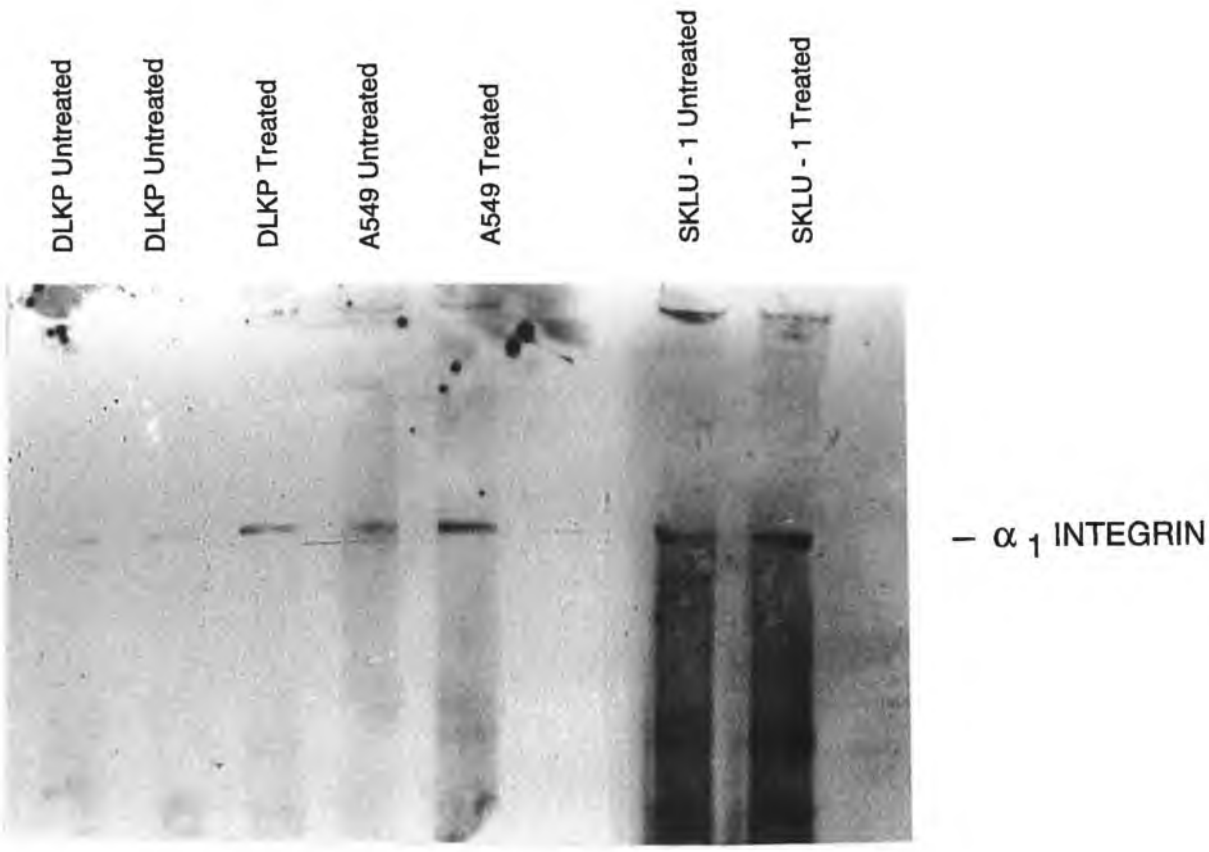


**FIG. 3.5.13** Immunoprecipitation of $\alpha_1$ Integrin protein from A549 and DLKP untreated and Bromodeoxyuridine - treated cells. SKLU - 1 were used as a positive control.

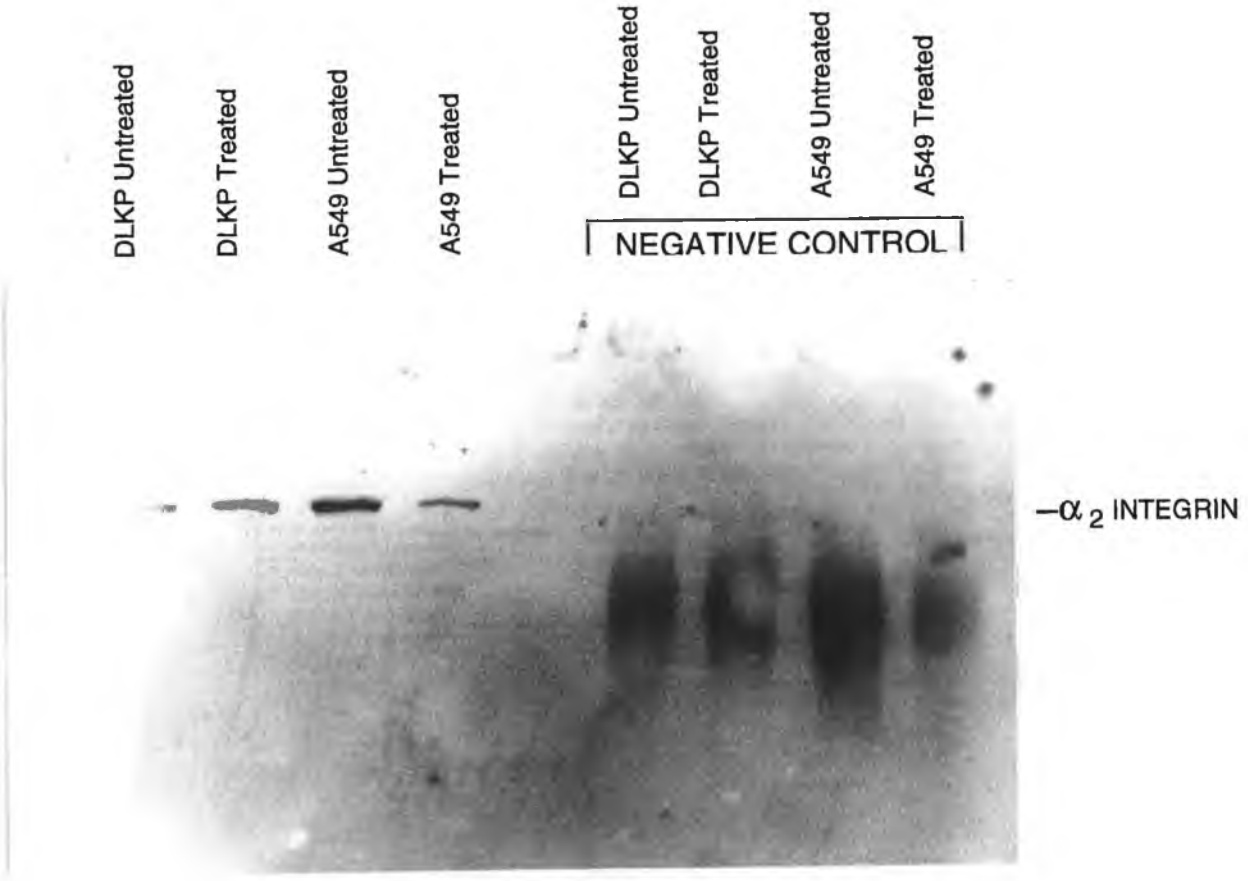


**FIG. 3.5.14** Immunoprecipitation of $\alpha_2$ Integrin protein from A549 and DLKP untreated and Bromodeoxyuridine - treated cells.

### 3.5.5 mRNA analysis of induced and upregulated cytokeratin 19 expression

Treatment of DLKP cells with BrdU causes the induction of CK19, ESA, $\alpha_1$ integrin and upregulation of $\alpha_2$ integrin protein expression. Dr. Shirley McBride determined that BrdU appears to act at a posttranscriptional level to reverse a block in keratin 8 and 18 expression in keratin-negative lung cancer cells, *e.g.* DLKP and H82, and to increase expression in keratin-positive lung cancer cells, A549. Experiments were carried out to try to determine if BrdU acts in a similar way in the control of cytokeratin 19 mRNA levels in these cells.

#### 3.5.5.1 RT-PCR of keratin 19 mRNA

RT-PCR was carried out for cytokeratin 19 on BrdU-treated and untreated cells. Sense and anti-sense K19 primers (Burchill *et al*, 1995) amplified a product of 214 base pairs. (See Section 2.21 for primer sequences). A549, HL60, H82 and DLKP cells were treated with BrdU for time points of 0, 15 minutes, 1 hour, 24 hours and 5 days. RT-PCR was carried out on RNA extracted from all these cell lines at the time points indicated above, as described in Section 2.20-2.23.

The mRNA levels for A549 remained unchanged over the 5 days treatment even though a dramatic upregulation of the protein was observed by immunocytochemistry, Western blotting and immunoprecipitation. K19 mRNA was found to be present in DLKP untreated cells, with no observed change in the levels of mRNA transcript over the 5 days of treatment even though induction of the protein was observed by immunocytochemistry. Similar results were obtained for H82. HL60 cells showed very weak mRNA bands even though the protein was never present before or after BrdU treatment.

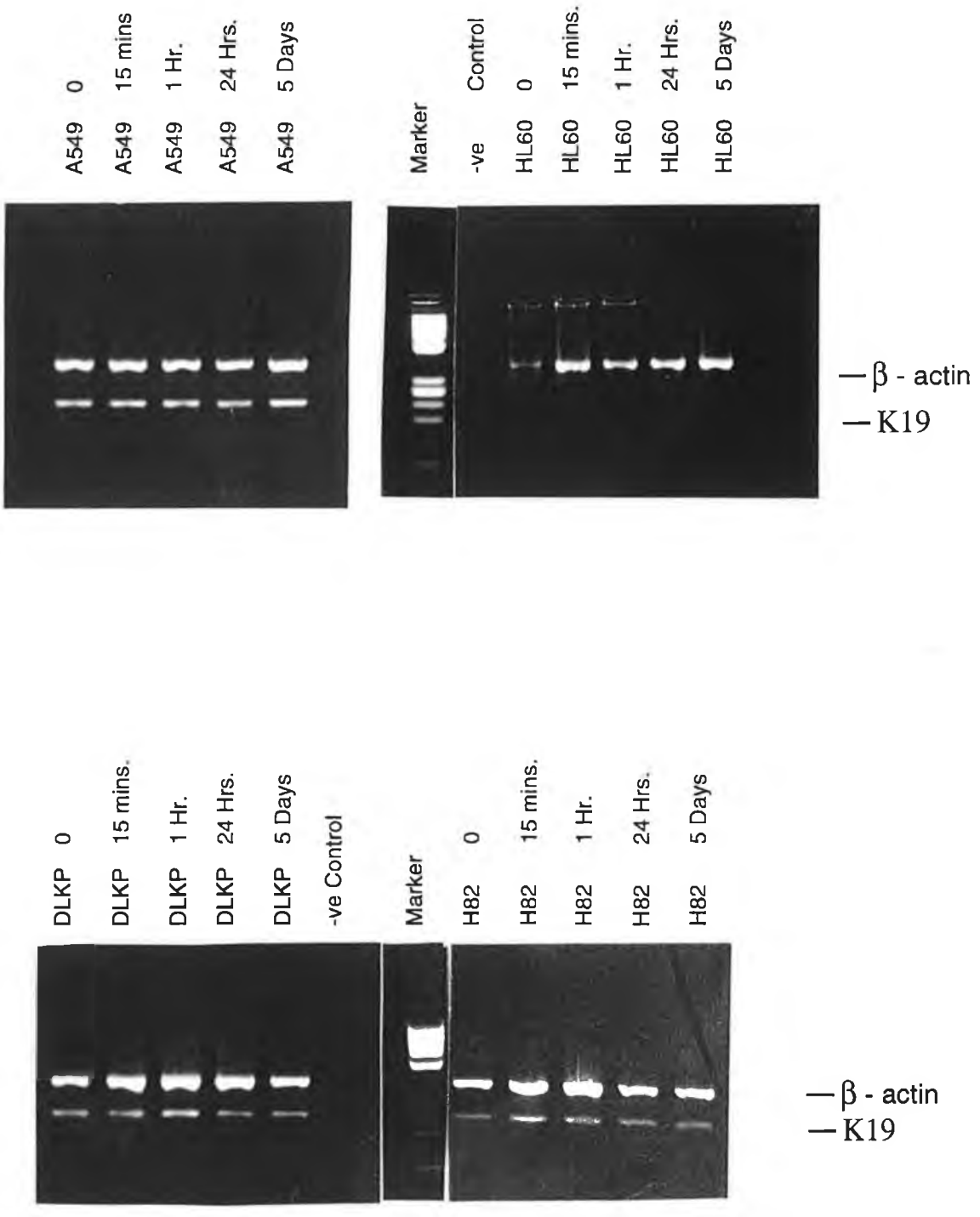


**FIG. 3.5.15** RT-PCR products resulting from amplification of keratin 19 RNA extracted from A549, HL60, DLKP and H82 treated with Bromodeoxyuridine over the time points indicated. The primers selected for keratin 19 produced a band of 214 base pairs.

### 3.5.6 Extracellular matrix adherence assays

*In vivo*, normal tissue cells interact with the ECM proteins through integrin receptors on their cell surface. These interactions are important for maintenance of tissue integrity and for mediating the expression of genes controlling proliferation and differentiation. Tumour cells *in vivo* also interact with ECM proteins but these interactions are frequently altered during carcinogenesis and tumour progression to an invasive and metastatic phenotype.

Because of the observed induction of the expression of $\alpha_1\beta_1$ integrin and the upregulation of the expression of $\alpha_2\beta_1$ integrin, a number of adherence assays were carried out using various extracellular matrix (ECM) components. Both integrins are collagen and laminin binding. Assays were thus carried out using laminin and Type IV collagen. Fibronectin was also used because DLKP has been found to be extremely responsive to this ECM component in serum-free media assays. Assays were also carried out to a mixture of these ECM components using basement membrane derived from EHS (Engelberth Holm-Swarm Mouse Sarcoma). This gel consists of collagens, non-collagenous glycoproteins and proteoglycans of the ECM.

Wells in 24-well plates were pre-coated with the various substrate proteins, after which uncoated sites were blocked with 0.1% BSA as described in Section 2.18. Cells were plated in basal Ham's F12 medium and attachment was observed after 15, 30 and 60 minutes. For all experiments, except those carried out using EHS, wells were stained with crystal violet which, after drying, was eluted with 33% glacial acetic acid (the CVDE assay as described in Section 2.10.2) and the absorbances of the resulting solutions were determined spectrophotometrically. Attachment was expressed as a percentage of the control wells which did not contain cells.

### 3.5.6.1 Response of BrdU-treated cells to Type IV collagen

Cells were treated with BrdU for seven days and then harvested before plating onto Type IV collagen, as described in Section 2.18.

BrdU-treated DLKP cells show increased adherence to type IV collagen over the time intervals 15, 30 and 60 minutes.

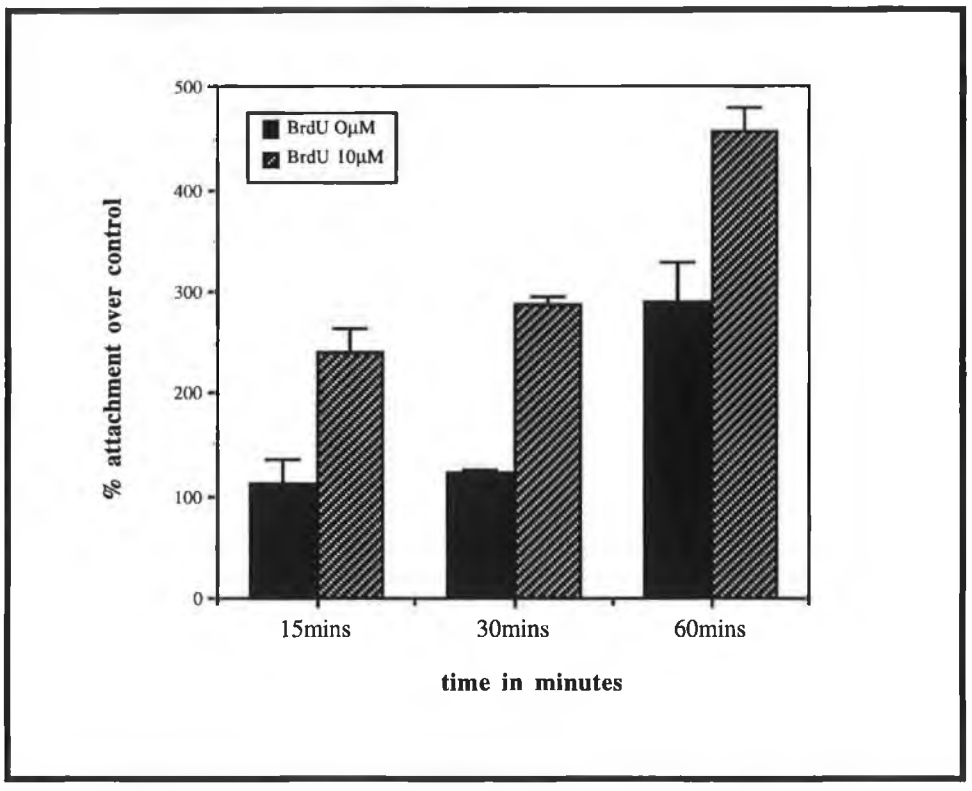


**Fig. 3.5.14** Response of untreated and BrdU-treated DLKP cells to type IV collagen

### 3.5.6.2 Response of BrdU-treated cells to laminin

DLKP cells were treated with BrdU for seven days as outlined in Section 2.18.

BrdU-treated DLKP cells show increased adherence to laminin over the time points 15, 30 and 60 minutes.

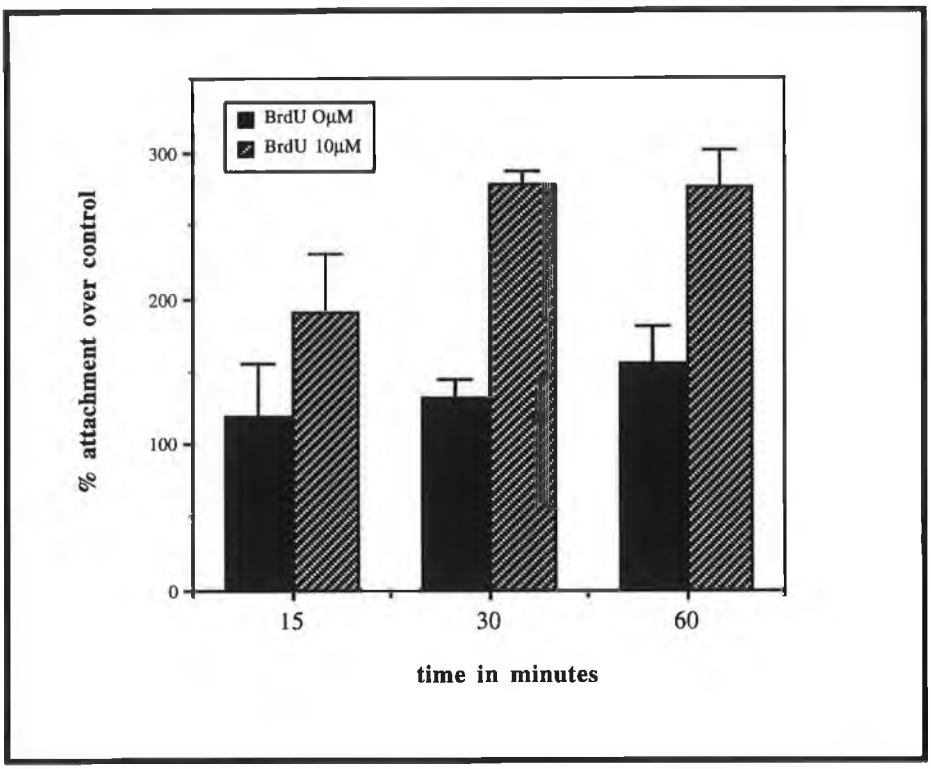


**Fig. 3.5.15** Response of untreated and BrdU-treated DLKP cells to laminin

### 3.5.6.3 Response of BrdU-treated cells to fibronectin

BrdU-treated DLKP cells show increased adherence to fibronectin over the time points 15, 30 and 60 minutes.

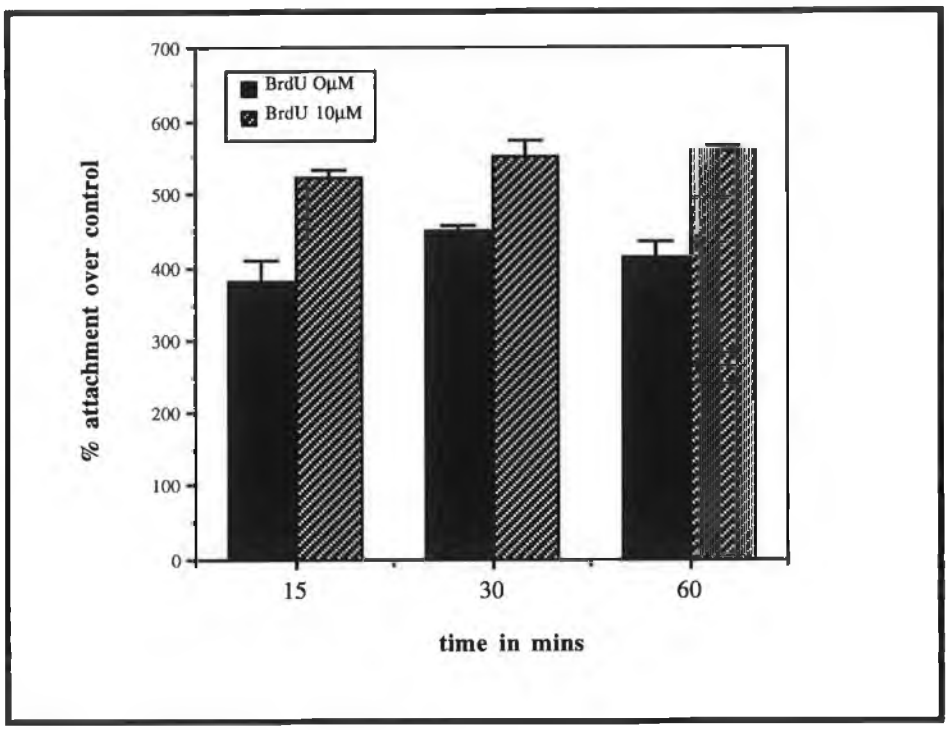


**Fig. 3.5.16** Response of untreated and BrdU-treated DLKP cells to fibronectin

### 3.5.6.4 Response of BrdU-treated cells to plastic

BrdU-treated DLKP cells show increased adherence to plastic over the time points 15, 30 and 60 minutes.

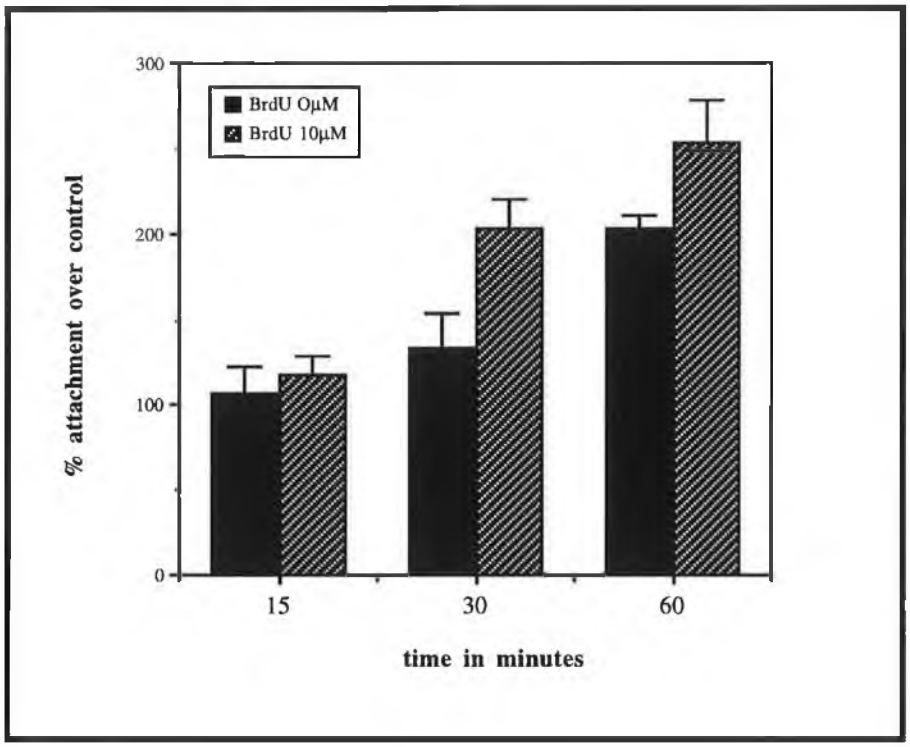


**Fig. 3.5.17** Response of untreated and BrdU-treated DLKP cells to plastic

### 3.5.6.5 Response of BrdU-treated cells to EHS

DLKP cells were treated for 7 days with BrdU and then harvested before plating onto wells pre-coated with EHS as outlined in Section 2.18.2. The end-point used in this assay was MTT because of difficulties encountered with non-specific staining of crystal violet on the EHS gel. The procedure for the MTT assay is described in Section 2.18.3. Results were expressed as a percentage of control wells which did not contain cells.

BrdU-treated DLKP cells show increased adherence to basement membrane (EHS) over the time points 15 and 30 minutes.

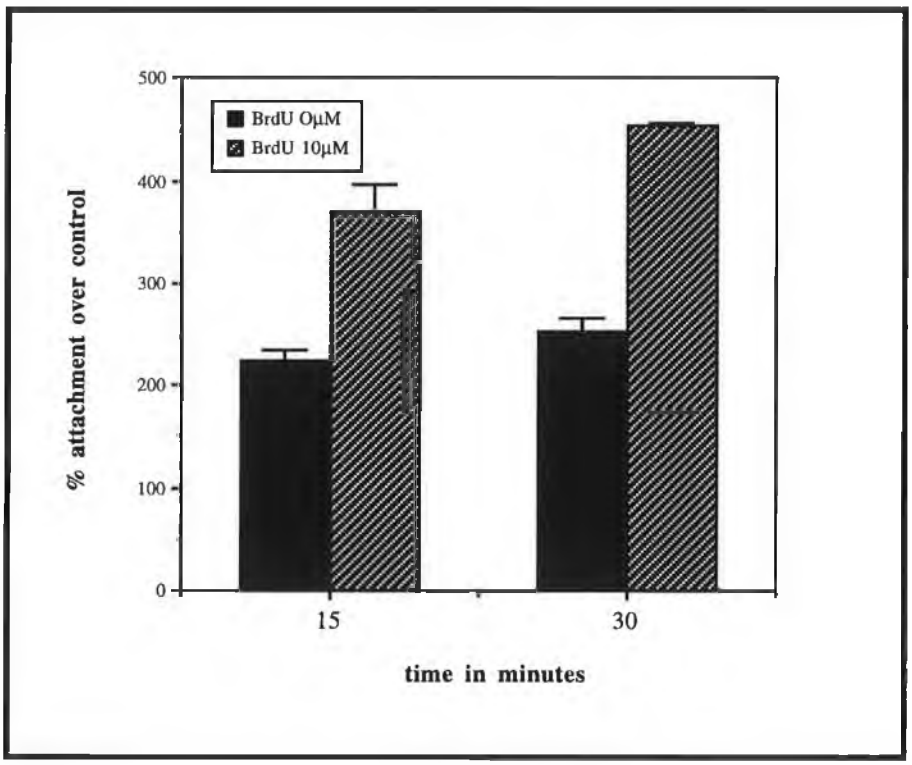


**Fig. 3.5.18** Response of untreated and BrdU-treated DLKP cells to EHS (basement membrane)

### 3.5.7 Adhesion blockade experiment

Adhesion blockade experiments were carried out in an attempt to reverse the changes in adhesive properties of the DLKP cells treated with 10μM BrdU as described in Section 2.19. An anti-$\alpha_1$ integrin antibody was added to untreated and 10μM BrdU-treated DLKP cells and the attachment properties of these cells to type IV collagen were compared to the same cells without antibody treatment. Attached cells were measured using the MTT assay as in Table 3.5.2.

**Table 3.5.2** Absorbance at 570nm of BrdU-treated and untreated DLKP attached to Type IV Collagen

| | *15 mins incubation* | | *30 mins incubation* | |
|---|---|---|---|---|
| **Absorbance at 570nm** | **0μM BrdU** | **10μM BrdU** | **0μM BrdU** | **10μM BrdU** |
| without Ab | 0.069 ± 0.001 | 0.141 ± 0.016 | 0.129 ± 0.004 | 0.238 ± 0.010 |
| With Ab | 0.038 ± 0.005 | 0.059 ± 0.009 | 0.053 ± 0.010 | 0.140 ± 0.021 |

Results expressed as average absorbance at 570nm ± standard deviation (n=3)

Addition of antibody resulted in a reversal of the attachment properties of the DLKP BrdU-treated cells to type IV collagen at both time intervals as shown in Fig. 3.5.18.

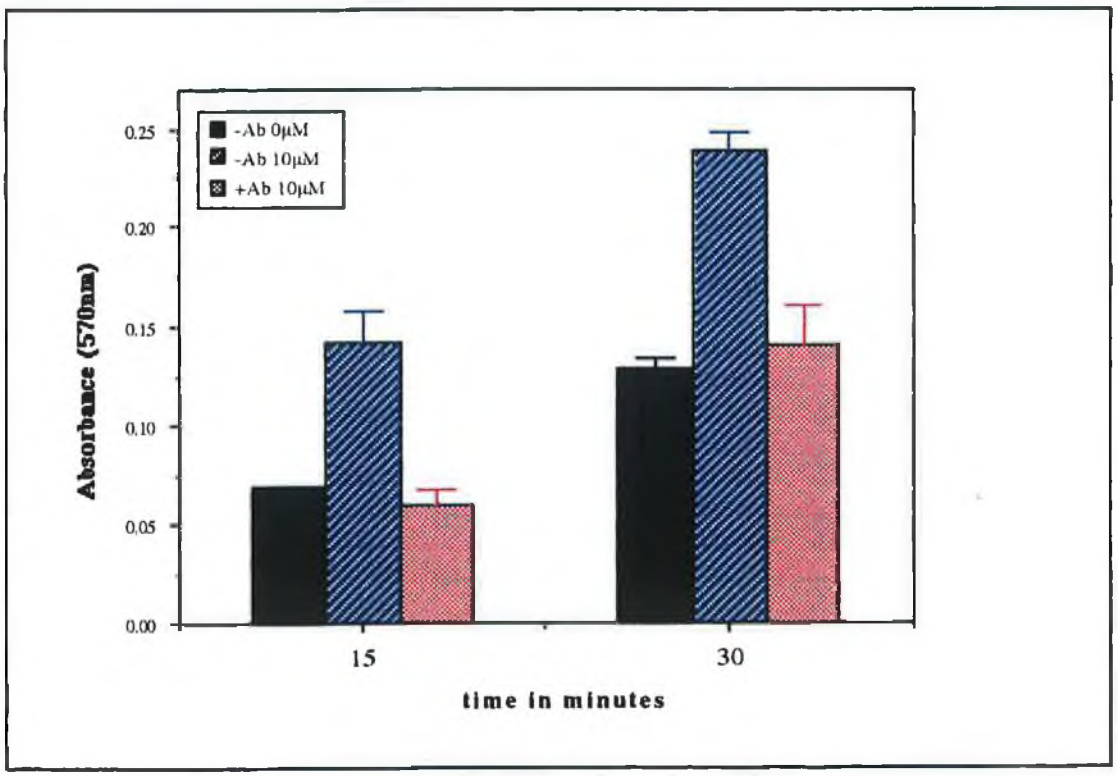


**Fig. 3.5.18** Reversal of attachment properties of DLKP BrdU-treated cells by addition of anti-$\alpha_1$ integrin Ab. Results are expressed as average absorbance at 570nm ± standard deviation (n=3), no addition of Ab at 0 and 10μM BrdU (- Ab), addition of Ab at 10μM BrdU (+ Ab 10μM).

# 4.0 DISCUSSION

## 4.1 GROWTH OF CHO-K1 CELLS IN SERUM-FREE MEDIUM

The Chinese Hamster Ovary (CHO) cell line has emerged as one of the most important and favoured for the commercial production of recombinant human proteins. These cells have been cultured for over 30 years and during that time a large body of basic knowledge on their biochemistry and their genetics has accumulated. Over 80 classes of mutants have been isolated from the cell line (Gottesman, 1985) and one of these, the DUK cell line which is dihydrofolate reductase (dhfr) negative, thereby allows the selection of transformants. The CHO cell line has been used for the isolation of mutants affecting intermediary metabolism; DNA, RNA, and protein synthesis; membrane functions and many complex forms of cell behaviour such as cell growth and endocytosis.

There have been many reports in the literature on the development of serum-free media for the growth of different CHO cell lines. Mendiaz *et al.* (1986) developed a defined medium for the growth of CHO-K1 in their studies on somatic cell genetics. Gasser *et al.* (1985) derived a medium for the growth of a wild type CHO cell line. Zang *et al.* (1995) reported on the production of two recombinant proteins, urokinase-type plasminogen activator (uPA) and humanised IgG kappa light chain, in serum-free medium. Coppen *et al.* (1995) reported on the production of human recombinant interferon-γ from the CHO-320 cell line which has been found to grow very well in the SFM developed by Hayter *et al.* (1991). Many researchers grow the recombinant cell line initially in serum-supplemented medium which they perfuse out of the system after a number of days when the required cell density is reached and then the medium is replaced by serum-free or protein-free medium. The cells then secrete the recombinant protein into the conditioned medium and subsequently purified. Ogata *et al.* (1993a,b) described the production of human soluble thrombomodulin in serum-free CHO-K1 culture by this method while Cole *et al.* (1993) reported on the production of recombinant human thyroid stimulating hormone by similar means.

### 4.1.1 CHO-K1 Suspension Culture

This study involved looking at the serum-free media (SFM) formulations investigated in Section 3.1 for the growth of the CHO-K1 cell line in SF suspension and microcarrier culture. The only mitogens present in the SFM were insulin and transferrin (see Table 2.7.1 for list of ingredients). It was clearly evident from the first experiments carried out that the Ham's F12 based SFM did not support the growth of the cells in suspension culture even though good growth was always achieved in monolayer culture. All the cells were found to be dead using trypan blue staining after 24 to 48 hours. Pluronic F68 (PF68) was then added at a concentation of 20µg/ml and after optimisation at a concentration of 50µg/ml, due to its known protective effect on the cells against adverse shear forces (Murhammer and Gooche, 1990; Handa-Corrigan *et al.*, 1989; Papoutsakis, 1991; Michaelis *et al.*, 1995). Pluronic F68 supplementation resulted in growth of the cell line in SF suspension culture to a certain extent. Long-term growth of CHO-K1 cells in suspension culture was also possible with PF68 supplementation where cells were subcultured up to five weeks.

Experiments were then carried out to compare changes in the plasma membrane fluidity (PMF) of these SF cells with serum-supplemented cells to see if this correlated to the poor growth of the cells in serum-free suspension culture. It is generally believed that the more fluid the membrane the more shear sensitive the cells are to external forces. PMF was evaluated by steady state fluorescence anistropy ($r_s$) of TMA-DPH. An increase in $r_s$ value indicates a decrease in PMF, hence the cells are more shear resistant. Ramirez and Mutharasan (1990) have shown that supplementation of SFM with 0.5% PF68 in the cultivation of hybridomas results in an increased $r_s$ value compared to control cultures. A similar effect was noted in the cultivation of CHO-K1 cells in serum-free medium (Fig. 3.1.8). Addition of PF68 resulted in an increase in $r_s$ value above unsupplemented SF culture over the 3 day growth period. There is a resultant decrease in PMF and a decrease in shear sensitivity in these cells thus allowing them to grow. These results and the findings of Ramirez and Mutharasan (1990) provide evidence that PF68 alters the plasma membrane of animals and thus its mechanism of action can at least be partly attributed to its property of decreasing the PMF. This agrees with the hypothesis of Murhammer and Gooche (1990) that the protective effect of PF68 resides in its ability to intercalate in the plasma membrane. Thus possibly, the lack of growth of CHO-K1 cells in SFM without PF68 supplementation could be attributed to an increase in shear sensitivity or a decrease in PMF of the cells.

Good growth of CHO-K1 cells was always achieved in serum-supplemented suspension culture. Ramirez and Mutharasan (1992) suggested a possible role for the

shear protective effect of serum. It is suggested that its protective action may be at least in part, to a condensing effect on the plasma membrane fluidity (PMF) by the transfer of cholesterol or analogous compounds from the culture medium to the plasma membrane. The $r_s$ values for CHO-K1 cells growing in serum-supplemented culture was found to be higher compared to cells growing for 3 weeks or 6 months in serum-free monolayer culture (see Table 3.1.1 and Fig. 3.1.8). Ramirez and Mutharasan (1992) found that increasing serum concentrations from 0 to 9% resulted in an increase in $r_s$ value for a mouse hybridoma cell line, HB32, during agitated culture. They also found that addition of LDL, HDL and cholesterol to these hybridoma cultures in SF or complete SSM also decreased their shear sensitivity ($r_s$ increases). Serum-free cells appear to have a more fluid membrane due to the absence of cholesterol in the medium. Serum is not present, so it cannot protect against hydrodynamic forces. No transfer of cholesterol or other lipids to the cellular membane can occur so the cells die rapidly due to their increased sensitivity to adverse shear forces. Addition of PF68 allows the cells to grow, thus the cells have been made more resistant to shear forces somehow.

Other surfactants could be tried especially polyethylene glycol (PEG) which has been found to promote the growth of a number adherent and non-adherent cell lines in SFM (Shintani *et al.*, 1988). PEG and another surfactant, PVA (polyvinyl alcohol), are non-ionic weak surfactants and possibly act by lowering the surface tension of the medium and improving the transport of metabolites into the cells.

### 4.1.2 Microcarrier culture of CHO-K1 cells in serum-free medium

The use of microcarriers to aid growth of CHO-K1 cell growth in serum in SFM was investigated. CHO-K1 cells can grow very well either attached to a substrate or in suspension culture. For the large-scale cultivation of cells that will adhere to a surface, one of the most promising and widely used techniques is to grow the cells on the surface of microcarriers suspended in media. This method offers a large surface area for adherent cells to grow on, allows a simple media/ cell separation and perfusion, provides a readily assessed and controlled homogenous culture environment, and can also support the growth to over $10^7$ cells/ml for some cell lines (Croughan *et al.*, 1987). However, animal cells on microcarriers are especially susceptible to destruction by excessive agitation. This susceptibility is probably due to the lack of a strong protective cell wall, the relatively large size of animal cells and from lack of individual cell mobility. These anchored cells cannot freely rotate or translate and accordingly cannot reduce the net forces and torques experienced when they are exposed to excessive hydrodymanic forces. Experimental evidence (Croughan *et al.*, 1988; Cherry *et al.*, 1989) show that cell damage results from the interaction between particles (microcarriers) and eddies of a size similar to particle size. These eddy sizes are known as Kolmogorov eddy sizes and are responsible for micromixing at a very fine scale. Croughan *et al.* (1988) showed that cells on microcarriers are damaged even at very low bead concentrations when the Kolmogorov eddy size becomes approximately equal to, or less than, the bead size. Bead to bead interactions also appear to play a role in cell damage in microcarrier bioreactors under intense agitation conditions. The frequency and severity of interactions are important determinants of cell injury; thus agitation intensity and microcarrier concentration are important (Cherry *et al.*, 1988, 1989).

In these experiments, cell growth was found to be much less in the microcarrier serum-free (SF) culture compared to SF suspension culture plus Pluronic F68 (PF68) supplementation. The use of microcarriers afforded a certain degree of protection on the cells against adverse shear forces but it was much less than the protective effect of PF68. Further experiments could be carried out using microcarriers plus PF68 supplementation. Due to the greater susceptibility of SF cells to shear forces compared to serum-supplemented cells, it is important to optimise concentrations of microcarriers in serum-free culture and also to investigate the agitation rate employed, in order to reduce the cell damage to the microcarrier culture.

### 4.1.3 Low Calcium concentration suspension culture of CHO-K1

Within 24 hours of SF suspension growth, the CHO-K1 cell line has been observed to clump extremely tightly compared to cells growing in SS culture. In SF stirred suspension culture, there are many reports that CHO cells tend to form aggregates with the size distribution ranging up to several hundred cells per aggregate (Coppen *et al.*,1995; Renner *et al.*,1993; Lehmann *et al.*,1991; Perry *et al.*,1978). Aggregation has also been reported for other cell lines; *e.g.* human recombinant kidney epithelial cells (Peshwa *et al.*, 1993); BHK cells (Moirera *et al.*, 1995; Perrin *et al.*, 1995); and Vero cells (Litwin 1992).

Several mechanisms may underly cellular aggregation. Cross linking of cells by DNA from lysed cells was reported by Renner *et al.* (1993) in the growth of the CHO-SSF3 cell line; secretion of lectins by cells was reported by Lehmann *et al.* (1991); or there may be several types of intercellular junctions involved, such as desmosomes and adherens-type junctions reported by Coppen *et al.* (1995). Usually within the centres of the larger aggregates a lot of necrotic material exists which is probably derived from cells dying of nutrient or oxygen deprivation and a build-up of waste materials.

Coppen *et al.* (1995) found that aggregation of the cell line CHO-320 producing human recombinant interferon-γ is caused by the formation of intercellular junctions. Membrane thickening and the presence of dense proteinaceous layers on the cytoplasmic faces of the opposed membranes was observed. They detected the presence of $Ca^{++}$ dependent cadherins and also found increased expression of vinculin and spectrin, which are well characterised cytoskeletal proteins (Geiger *et al.*, 1989) at the regions of cell-cell contact. In addition, Coppen *et al.* observed that their cell line showed no accumulations of DNA within the aggregates by transmission electron microscopy (TEM) or DAPI staining. Aggregation was found to be an active process associated with exponential growth and not with the death phase as would be expected if DNA molecules were released from lysed cells. They added DNase I to their medium either before or after aggregation had begun and no effect on the aggregation of the cells was observed.

However, this appears to contradict what Renner *et al.* (1993) had found with CHO.SSF3 cells, *i.e.* that DNase I supplementation at a concentration of 100μg/ml resulted in the break up of the aggregates to a single cell suspension within 12 hours of addition. Addition of DNase I to aggregated CHO-SSF3 SF culture also resulted in a 47% increase in specific growth rate over an 80 hour period and a 100% increase in the maximal biomass yield. Also the presence of released DNA within the clusters was

revealed by DAPI staining and TEM. It is reasonable to assume in their culture that shear stress caused by stirring and agitation of cultures is responsible for damaging cells, initiating DNA release and creating new nucleation centres for cluster formation.

The study in this thesis was concerned with reducing the level of aggregation within SF CHO-K1 suspension culture by decreasing the amount of calcium ions in the medium and to see if this would increase cellular growth. Little decrease in aggregation was noticed from microscopic observations between the high calcium media (Ham's F12 SFM, 226μM $Ca^{++}$ and Ham's F12/DMEM (1:1) SFM, 790μM $Ca^{++}$) and low calcium (Ham's F12/MEM.S SFM, 113μM $Ca^{++}$) while using a SFM without calcium present (MEM.S SFM) resulted in loss of cellular growth properties completely. Growth of the cell line in H/M SFM was quite comparable to the H SFM as seen in section 3.1.1.5. Growth of cells in H SFM and H/D SFM was also found to be quite comparable. Very little difference was seen in the microscopic observations of natural aggregation between the reduced calcium and the high calcium media. However, Peshwa *et al.* (1993) showed decreased aggregation of human recombinant epithelial cell line at a lower concentration of calcium chloride in the medium (100μM compared to 500μM).

In this study, as with CHO-SSF3 (Renner *et al.*, 1993) the presence of FCS in the culture medium caused a looser aggregation of CHO-K1 cells as compared to medium without serum supplementation from microscopic observations. As serum has direct effects on the cell surface and its structural elements, fewer cells are ultimately aggregated. Therefore, it appears that the medium type, whether containing high or low calcium concentrations, has little effect on the extent of aggregation in serum-free suspension culture of CHO-K1 cells. Severe aggregation was noted in the low calcium medium equivalent to the high calcium media suggesting that the aggregation was caused by alternative mechanisms rather than the $Ca^{++}$-dependent junctions between the cells. The most likely explanation is that the shear forces caused by the agitation results in many of the cells dying, releasing their nucleic acids which act like a glue between cells, causing the cells to aggregate and eventually leading to death of cells within the clumps, as reported by Renner *et al.* (1993) for CHO-SSF3 cells.

It is reasonable to assume that in all probability, DNA accumulation between cells is not a universal factor in CHO cell aggregation and that aggregation could be associated with $Ca^{++}$ levels in the medium and formation of intercellular junctions, and also with the release of lectins from the cells. It is important to elucidate the mechanism(s) of aggregation in CHO-K1 cells so that they can be controlled. Comparisons could then be made between the growth of the cell line in SF single cell suspension culture, SF aggregated culture and SF microcarrier culture.

It may be important to inhibit aggregate formation in terms of evaluating CHO productivity in batch culture. It is important to evaluate whether elimination or reduction of aggregation in carefully controlled chemostat culture improves the specific production rate. However, on the other hand, formation of aggregates can be used to effect cell retention in bioreactors, thereby increasing cell concentration and yield of product from the reactor (Peshwa *et al.*, 1993; Moirera *et al.*, 1994). Thus all the different methods of cultivating animal cells, especially the commercially important CHO cell lines, as single cells or as aggregates in suspension culture or attached to microcarriers, have to be addressed in terms of specific growth rate, specific production rate, ease of cell separation from media containing required product and, of course, expense.

## 4.2 LONG-TERM GROWTH OF CHO-K1 IN SERUM-FREE MEDIUM

Many processes which involve a production phase in serum-free medium still require the presence of serum at the cell inoculation stage. This carries some risk of viral or protein contamination of the final product. Long-term growth of a cell line in serum-free media is a good test for a true, feasible SF medium which may be important for optimal productivity. Keenan (1994) carried out investigations into the serum-free growth of the CHO-K1 cell line using the SFM developed by Mendiaz *et al.* (1986) as a starting base. It was found that this SFM was unable to sustain growth of the cell line over a long period of time though good growth was usually achieved for the first few passage in this SFM. Keenan (1994) found that this SFM was unable to sustain growth longer than at most 9 passages or 36 days. One of the main objectives of the study was to increase the length of time this cell line could be sub-cultured under SF conditions and to determine what factor or factors are involved with the death or poor growth of the cells after long-term subcultivation. The longest sub-culture that was achieved in this study using H SFM was 8 passages (32 days culture) but usually the average cultivation length lasted on average between 4 to 6 passages.

### 4.2.1 Extension of serum-free medium sub-cultivation for CHO-K1

The first series of experiments were carried out using a medium derived from a product of the NCTCC, SF Briclone, to try and extend passaging of the cell line. SF Briclone was used because of its ability to enhance the clonal growth of hybridomas. The active ingredient appears to be IL-6. It was used in all assays at a 5% v/v concentration and when added to the H SFM it was found to generally increase cell numbers after 4 days cultivation and also extended the passaging of the cells by approximately two passages (8 days in culture). SF Briclone was also found to have a proliferative effect on NRK cells (Dr. Joanne Keenan, PhD 1994). The Briclone was then fractionated into a retentate containing all components > 3000Da and a filtrate with all components < 3000Da. The 1x filtrate showed the greatest stimulation of growth over the other fractions in a 7 day growth assay. However, subcultivations using the SFM plus 5% filtrate resulted in no improvement in the long-term passaging of the cell line. The 1x and 10x retentate also showed increased cell numbers over the unfractionated SF Briclone supplementation. The 10x retentate generally resulted in lower cell numbers than the 1x retentate. However, in the long-term subculture, the opposite effect was observed. Perhaps as the cells are subcultured, residual serum proteins are gradually being diluted out of the system and the cells' requirements for media supplements becomes greater. The 10x retentate may be better able to provide these factors thus accounting for the observed increased growth over supplementation with other SF

Briclone fractions. The variability in results suggests that a number of factors are involved in the growth promoting effects (less than and greater than 3000Da). Also the condition of the cells and possibly even damage due to trypsinisation procedures may also play a part in these variable results. It was thus decided to discontinue with these experiments due to this variability and the undefined nature of SF Briclone. Generally, however, the SF Briclone showed increase cell numbers during passaging of the cells compared to unsupplemented SFM and the cultivation time was generally extended by about a week.

As a consequence, it was then decided to try and extend the long-term sub-cultivation of the CHO-K1 cell line in SFM using defined components but without using expensive cytokines or hormones. The components tested were BME vitamin solution, Pluronic F68, Fibronectin, LDL, DNase I and conditioned medium collected from the CHO-K1 cell line itself.

BME vitamins is a solution of B vitamins and its effect on the cells at the recommended concentration was assessed. No increase in passaging of the cells was observed in the two experiments described which suggests that a B vitamin is not being diluted out over long-term sub-cultivation.

Conditioned medium from the CHO-K1 cells was collected and used at a 5% concentration. However, no increase in passaging of the cells or improvements in cell numbers were observed using this CM. This does not necessarily imply that no autocrine activity occurs between CHO-K1 SF cells. Possibly the secreted autocrine factors may have been extremely labile and the activity may have been lost before it was assayed. The overall concentration of the factor may been too low, or the autocrine factor may depend on some component in the SSM for a commitment to cell growth and division.

Pluronic F68 was tested because of its apparent growth promoting activities. Bertheussen (1993) used Pluronic F68 at a 20µg/ml concentration in the SFM developed for the growth of L929, HeLa S3 and a hybridoma cell line, 1E6. A concentration of 50µg/ml of PF68 was subsequently found to optimally stimulate growth of CHO-K1 cells in monolayer culture (see Fig. 3.1.4). It is often used as an additive for serum-supplemented suspension culture to prevent denaturation of serum proteins during agitation (Swim and Parker, 1960) and aids in protecting the cells from adverse shear forces as already discussed in 4.1.1. In SFM, PF68 may have growth stimulatory properties by acting as a solubilising agent to increase the solubility of sparingly soluble compounds (Collett and Tobin, 1977). It is also reported to form

complexes with other phenols and other small molecules (Marcus *et al.*, 1956) which may reflect a weak albumin-like property as a detoxifying toxic species in the medium. The use of PF68 in the H SFM extended sub-cultivation by one passage. The PF68 appears to enhance the growth of the cells but it does not appear to be enough to extend passaging of the cells significantly.

Fibronectin at a concentration of 10µg/ml gave the most promising results so far with an increase of 2 to 3 passages in the long-term sub-cultivation of the cell line. In late passages, with fibronectin as compared to the control flasks where a lot of cellular aggregation is usually noted to occur if the cells have not already died, the cells were observed to be nicely spread out and growing well. The requirement for an attachment and a spreading factor such as fibronectin is usual for anchorage-dependent cells though some cell lines appear to be well capable of attaching and spreading in their absence. Hewlett (1991) found that A431 epithelial cells, Vero and MDCK cells are quite capable of attachment, spreading and growth in a protein-free medium. However, their principal growth parameters are down modulated in the simple SFM. Some anchorage-dependent cell lines appear to have a facultative requirement for attachment factors. Hewlett (1991) found that the CHO-P cell line when placed in SFM no longer attached to the plastic substrate but grew in suspension as an aggregated culture. When the medium was supplemented with serum or fibronectin, anchorage-dependent growth in the SFM occured. This appears to be the case with the CHO-K1 cell line except that aggregation often occurs after a number of passages in SFM (see Fig. 3.1.12). Mendiaz *et al.* (1986) and Dr. Joanne Keenan in her Ph.D thesis showed no requirement for fibronectin in early passage SF growth. However, it appears that control of the ECM is critical for long-term growth of the cell line. The long-term presence of fibronectin may play a role in enhancing the synthesis of ECM proteins so that cellular attachment processes can be initiated before the cell is irreversibly damaged. Cell attachment appears to be a primary regulator of cell growth, proliferation, gene expression and secretion for non-transformed cells (Daniels and Solurch, 1991). In these experiments, cultivation was really only increased by 8-12 days. Perhaps a lot of the cells had been irreversibly damaged before fibronectin addition or perhaps other factors are being diluted out. Other attachment factors which could be tried include laminin, collagen or elastin over long-term growth.

Lipoproteins, especially HDL and LDL, are often added to SF formulations. They are macromolecular complexes of lipids and apolipoproteins. Nakama and Yamada (1993) found that the most effective factor on the SF growth of the human hepatoma cell line, HepG2, was LDL supplemented at a concentration of 100µg/ml. Chen *et al.* (1986) showed optimal proliferation of both human EC (vascular endothelial) and SMC

(smooth muscle cells) with either HDL or LDL at concentrations ranging from 20 to 60μM. Thus LDL was investigated to see if a lipoprotein source would increase the long-term proliferation of the CHO-K1 cell line. However, no increase in passage number was obtained.

Often at high passages in SF monolayer culture of CHO-K1 cells, aggregation was noted to occur. Renner *et al.* (1993) used DNase I at a concentration of 100μg/ml in the SF suspension growth of the CHO-SSF3 cell line to avoid aggregation. DNase I was added to the SFM at a similar concentration to try to prevent formation of such aggregates. Perhaps the cells at high passages in SF culture have suffered a lot of trypsinisation damage due to serial passaging of the cells with the subsequent release of nucleic acids from lysed cells. Nucleic acids can serve as a kind of glue causing aggregation of the cells. Thus cells within the centre of these aggregates would soon die due to lack of $O_2$ and nutrient availability with subsequent release of more nucleic acids and proteases by the cells. As SFM contains no protease inhibitors such as trypsin inhibitor, the action of these extra proteases can damage surviving cells eventually causing their own destruction. In the experiment carried out in Section 3.1.3.2 using DNase I supplementation, the cells were found to spread out very nicely but no increase in passaging of the cells was obtained. This could possibly have been due to the DNase I concentration being slightly inhibitory in monolayer culture or the cells being far too damaged after the serial passaging to recover at this stage. Soybean trypsin inhibitor could be added to try and reduce damage to the cells.

It would be interesting to test a combination of the effective factors used, especially with fibronectin, Pluronic F68 and also with DNase I. These factors could be added at passage 4 as in the experiments described in Section 3.1.3.2 and compared to long-term growth with supplements added at passage 1 in SFM. Other components could be tested such as growth factors and hormones but this would lead to increases in expense and protein content of the SF formulation. However such experiments would be worth trying in order to find out what factors are involved with the poor growth of the cell line in long-term serum-free culture.

### 4.2.2 Low calcium medium and long-term serum-free growth

A low $Ca^{++}$ medium was used to try to reduce aggregation in CHO-K1 SF suspension culture, as already discussed in 4.1.3. Firstly, it was decided to see if this low $Ca^{++}$ medium actually supported the growth of the cell line in monolayer culture before it was applied to suspension culture. The results shown in Section 3.1.3.3 indicate that this SFM is capable of supporting the growth of the cell line. Most importantly, it appears to be capable of supporting long-term growth of the cell line. A sub-cultivation lasting 52 passages (11 months in culture) has been successfully obtained with this medium and cellular aggregation has not been a problem. This medium is thus extremely successful in the continuous subcultivation of CHO-K1 cells because the other media tested with different supplements did not allow growth beyond 7 or 8 passages (one month in culture). In the low $Ca^{++}$ medium, the cells appear to go through a crisis stage and then show adaptation to the SF conditions with cell counts often exceeding those obtained for passage 1 or 2 cells. The cells in long-term culture appear smaller than early passage cells and are not as elongated or as fibroblastic-like, hence more space is available for cells to grow leading to higher cell counts.

The $Ca^{++}$ ion has been proposed as a key component in cell proliferation control mechanisms. $Ca^{++}$ depravation causes normal cells to stop growing (Balk *et al.* 1973). There seems to be a change in regulatory functions of calcium during neoplastic transformation (Boynton and Whitfield, 1976; McKeehan and Ham, 1978; Hazelton *et al.* 1979; Swierenga *et al.* 1983; Ribeiro and Armelin, 1984; Klann and Marchok, 1984) where transformed or tumourigenic cell lines show a requirement for reduced $Ca^{++}$ concentrations, compared to normal cell lines. Before undergoing spontaneous transformation, 3T3 cells can be reversibly growth arrested with $Ca^{++}$ starvation. As the cells become spontaneously transformed, they have been found to grow well in low calcium medium (Ribeiro and Armelin, 1984).

CHO-K1 cells were found to be incapable of proliferating in $Ca^{++}$-free medium. However, on addition of $Ca^{++}$ to the medium, at the concentration found in basal HF12 or H/M SFM, proliferation of these cells was possible, showing a requirement for $Ca^{++}$. CHO-K1 cells are capable of proliferating continuously in a reduced calcium medium (113μM concentration), while higher concentration media (226μM and 790μM concentration) cause the cells to aggregate after a number of passages and eventually die off. The H/D SFM (790μM $Ca^{++}$) usually caused the death of CHO cells after 2-3 passages while H SFM (226μM $Ca^{++}$ ) caused death after 4-6 passages, on average. CHO-K1 cells are a transformed cell line, thus their requirement for calcium would be lower than that for normal or weakly tumourigenic cell lines. The transformed state of

CHO-K1 and its tendency to aggregate in monolayer (and suspension) culture demonstrate that for successful long-term growth of these cells, the calcium concentration in the medium should be taken into account. This dependency on calcium concentrations for long-term growth has been proven by subculture experiments where calcium was added to the low calcium medium (113 μM H/D), to a concentration equivalent to H SFM, and caused the cells to aggregate and die off after 3 passages in one experiment and 8 passages in a second separate experiment. Perhaps the cells show good growth initially in the high $Ca^{++}$ media (H and H/D SFM) due to the presence of trace serum proteins on the cells. Long-term growth in serum-supplemented medium may be due to binding of exogenous $Ca^{++}$ by serum proteins such as albumin.

The best approach to defining the system involved adding $Ca^{++}$ to a calcium-free media. Adding an excess of the specific $Ca^{++}$ chelator, EGTA to growth media lowers the $Ca^{++}$ concentrations to a level that can damage cell membranes (Durham and Walton, 1982). Raising the $Ca^{++}$ concentration can cause precipitation of calcium phosphate which, by virtue of its particulate nature, can stimulate the proliferation of some cell lines (Barnes and Colowich, 1977).

CHO-K1 cells subcultured in high calcium media (H/D SFM and H SFM) show increased aggregation at higher passages in SFM compared to lower amounts of aggregation in the lower $Ca^{++}$ medium (H/M SFM). Mattey *et al.* (1990) showed that desmosomal adhesion between MDCK cells in culture may be controlled by manipulation of the extracellular $Ca^{++}$ concentration. As these are adhesive intercellular junctions, increasing their numbers would be expected to increase cellular adhesiveness. Peshwa *et al.* (1993) reported on the effect of $Ca^{++}$ ion concentration on the cultivation of the recombinant human epithelial 293 cell line. This cell line grows as aggregates in suspension culture. They found that increasing concentrations of $Ca^{++}$ from 100 to 500μM resulted in an increase in aggregate formation. They also found that the viability in lower concentrations appeared to be better than in high $Ca^{++}$ concentrations, possibly due to the diffusional limitations in the high $Ca^{++}$ aggregates because of the larger size and denser packing of cells. Possibly the effect of $Ca^{++}$ on the aggregation of 293 is caused by increasing numbers of desmosomal junctions between cells. Coppen *et al.* (1995) found that the SF aggregation of their cell line, CHO-320, is possibly caused by intercellular junctions. They confirmed the presence of $Ca^{++}$ dependent adhesion molecules, cadherins, associated with cell-cell adherent junctions, which ties in with the observations of Peshwa *et al.* (1993). Perhaps the low $Ca^{++}$ concentration of the H/M SFM, which allowed the indefinite growth of CHO-K1 cells compared to H SFM, can partly be attributed to the low concentration of the $Ca^{++}$ ion.

The low $Ca^{++}$ medium possibly allows long-term growth due to less intercellular junction formation between cells and thus less cellular aggregation. It would be interesting to look at the expression of $Ca^{++}$-dependent adhesion molecules such as cadherins and desmosomes in the low and high $Ca^{++}$ serum-free media.

A recent report has shown that transfection of VE-cadherin (vascular endothelial cadherin) into CHO and L929 cells confers inhibition of growth (Caveda *et al.* 1996). In other types of tissue, cadherins can act as tumour suppressors, where reduced cadherin expression or activity is associated with tumour cell invasive potential and loss of differentiated characteristics (Takeichi, 1993; Frixen *et al.* 1991; Vleminckx *et al.* 1991; Birchmeier and Behrens, 1994). Perhaps growing the CHO-K1 cells long-term in SFM causes overproduction of cadherin proteins, which may cause inhibition of growth of the cells. Possibly VE-cadherin can transfer growth inhibitory signals through association with catenins, intermediate filaments and other cytoplasmic molecules (Caveda *et al.* 1996). It appears that cadherins and catenins can directly contribute to inhibition of cell growth, induced by cell density.

Thus the concentration of calcium in cell culture medium has important implications for growth of normal and transformed cell types. In primary culture, the media conventionally used contain high quantities of calcium, usually above a 1mM concentration. If a cell line is required, it makes sense to use low calcium media. It has been observed in the literature that there is a good correlation between loss of $Ca^{++}$ control and progression towards tumourigenicity, as has already been discussed. The role of calcium in the differentiation of many tissues has also to be taken into consideration during primary culture. High concentrations of $Ca^{++}$ ions induce squamous differentiation in epidermal keratinocytes (Lechner *et al.* 1984; Merchant, 1990) and has been suggested in other epithelial systems (Bologne *et al.* 1986). Maybe this is occurring in CHO cells growing long-term in SFM (high Ca) where $Ca^{++}$ is inducing differentiation in the cell line, forming intercellular junctions (cadherins) and intermediate filaments, such as vinculin and spectrin, as observed by Coppen *et al.* (1995) in CHO-320 cell line.

Thus, an excellent model now exists to cultivate CHO-K1 cells long-term in SFM which may have important consequences for commercial production of recombinant proteins in a completely defined system. Continuous subcultivation of the CHO-K1 cells is now possible.

## 4.3 DLKP CELLS IN SERUM-FREE MEDIUM

A model for the growth of a human lung epithelial cancer cell line was then investigated in SFM with a view to understanding characteristics of tumour cell growth, adhesion properties and differentiation in a completely defined system.

DLKP is a human lung cancer cell line derived in our laboratory by Dr. Geraldine Grant from a lymph node biopsy of a primary lung tumour. The tumour has been histologically diagnosed as a poorly differentiated squamous carcinoma. Cytological (Gilvarry *et al.*, 1990) and cytogenetic (Law *et al.*, 1992) analyses have been reported elsewhere. The DLKP cell line has been extensively characterised and appears to be a heterogeneous population of cells which appear to interconvert between themselves. Three distinct cell populations have been cloned out of the parental population (McBride *et al.*, 1997).

Establishing cell lines from lung carcinomas on a routine basis provides an important tool to study the disease. Development of serum-free media for the growth of such cell lines provides a defined system for investigating the cells. Serum-free media permit greater flexibility for manipulating growth conditions by altering levels of hormones, growth factors or attachment substrates. The effects of differentiating agents and chemotherapeutic drugs can also be evaluated in a defined environment without the masking effects of serum. The possibility of multi-dependent mechanisms of growth regulation can be more easily investigated under SF culture conditions. Siegfried *et al.* (1994) reported on the secretion of gastrin releasing peptide (GRP) by a non-small lung carcinoma cell line, A549, adapted to SF growth conditions. This peptide is an important growth modulating factor in lung epithelium development and an autocrine loop involving GRP and its receptor have been demonstrated in many small cell tumour cell lines. Thus the use of SF media has been essential for studying the significance of autocrine growth mechanisms.

There are many reports in the literature on the development of serum-free media for lung carcinoma cell lines (Brower *et al.*, 1986; Collodi *et al.*, 1991; Gazdar, 1994). Over the last 15 years, many SCLC and NSCLC cell lines have been established by utilising serum-free, hormone and growth factor supplemented defined media, HITES and ACL-4 (Oie *et al.*, 1996).

### 4.3.1 Growth of DLKP cells in serum-free medium

The serum-free medium developed by Mendiaz *et al.* (1986) and further investigated by Dr. Joanne Keenan for the growth of CHO-K1 cells was used as a starting base for the growth of the DLKP cell line under SF conditions. The main reason for picking this medium was due to its simplified composition and the presence of only one mitogenic compound, insulin. The cell line was found to grow quite well in the SF conditions as indicated in Section 3.2. However, growth was not nearly as good as that obtained in serum-supplemented culture but was better than basal F12 only. Care has to be taken when culturing these cells in SFM. The cells need to be fed or passaged every 4 to 5 days, as after this time many of these cells begin to detach from the flask surface. Culture in SFM also appears to influence the morphology of the parental cell line. The majority of the cells appear to proliferate in the SFM without actually forming colonies compared to the SS culture (see Fig. 3.3.6(a,c)). The cells usually remained quite rounded and were loosely attached. After 4 to 5 days many of these cells would detach from the flask surface. These cells have been found to be viable because they grow very well when they are placed in serum-supplemented culture after pelleting out and resuspending the detached cells in SSM.

The ability of DLKP to proliferate successfully in basal medium (up to 3 passages) and continuously in defined serum-free medium suggests that these cells are producing autocrine growth factors capable of stimulating their own growth. DLKP can possibly be characterised as variant SCLC or NSCLC-NE (McBride, PhD 1995) and these are characterised by more rapid growth, an undifferentiated morphology and *c-myc* proto-oncogene amplification and may be less dependent on external peptide stimulation (Carney *et al.*, 1985). Doyle *et al.* (1990) identified a number of proteins that appear to distinguish the CM of classic SCLC with its distinct neuroendocrine phenotype, from the more anaplastic variant-SCLC. They have adapted a number of SCLC lines to hormone and serum-free medium. These tumour cells are able to produce all of the essential autocrine and paracrine factors required for their own growth.

Long-term growth of the cell line has been found to be successful. The cells have been successfully subcultured for 2 and a half years in the H SFM. In all long-term sub-cultivations carried out, the cell counts were found to increase from about passage 8 to 10 onwards. DLKP cells appear to adapt to the conditions in which they are forced to grow where long-term SF cells show increased growth rates to parental cells in serum-free and basal media. The 2 year SF cells also appear to grow more slowly in serum-supplemented medium compared to their parental counterparts suggesting that serum

may be slightly cytotoxic to these cells, a case of protein overload! The cells at the higher passages in SF culture also appear to become more tightly attached and spread. The cellular morphology of these high passage SF cells is quite unlike any of the clones which have been derived from the parental population. The SFM appears to influence the cell morphology in long-term culture. There appears to be a change in the expression of some adhesion proteins as indicated by the better attachment and spreading of the cells cultured long-term in serum-free medium. Perhaps the SFM has resulted in pushing the cell line along some differentiation pathway with a resultant expression of various adhesion and differentiation antigens. Immunocytochemical analysis of cells growing for 3 months and two years in SFM showed no change in expression of keratin filaments, EGF-R, desmosomal protein, neurofilaments, NCAM, serotonin, and α-transglutaminase compared to passage 1 cells in SFM. However, the beta-1 integrin profile of the parental cells was found to be slightly different in the 3 month and 2 year SF cells. There appeared to be a slight increase in expression of $\alpha_2$ and $\alpha_3$ integrins in the long-term SF cells. Expression of $\alpha_1$, $\alpha_5$ and $\alpha_6$ integrins remained negative. DLKP express low amounts of $\alpha_2$ and $\alpha_3$ integrins and perhaps the SF conditions cause them to express more of these integrin proteins as they are already inherently expressed, while the induction of expression of the other $\beta_1$ integrins was not possible. Ruck *et al.* (1994) have reported on the growth of a bladder carcinoma cell line for at least 2 years in SF conditions. They investigated the antigenic phenotype of the cells growing with or without serum using antibodies to a variety of cell surface antigens such as ICAM, EGF-R, IGF-1 and other adhesion molecules and integrins. All of the antigens were found to be expressed in similar amounts irrespective of whether the cells were grown in SF or SS media for two years.

When these long-term SF cells were placed back into serum-supplemented medium from about passage 10 onwards, the cell population appeared to be almost totally homogeneous compared to the parental population. The cell morphology appeared to resemble the DLKP-SQ squamous-like clone. The emergence of a squamous cell phenotype in cell lines has been reported previously. Terasaki *et al.* (1987) have reported on the emergence of a squamous cell phenotype in a small cell lung cancer cell line by using delipidised serum-supplemented medium which lacks retinoic acid. The changes were reversible on addition of RA to the medium. It is possible that the absence of this agent from the SFM for DLKP cells caused the cells to differentiate towards a squamous phenotype.

### 4.3.2 Cryopreservation of DLKP cells in SF conditions

Serum has been shown to be effective in enhancing the survival of frozen cells (Dougherty, 1962) and it is thus often added before freezing, even for cell lines that are maintained in SF conditions. If serum is used in such systems, many washings and long-term recultivation under SF conditions are required for complete removal of serum-derived factors. Even with these precautions, use of serum at the freezing stage does, to some extent, compromise the serum-free staus of the cells. Thus to maintain a true SF system for both DLKP and DLKPA cells, investigations into the SF freezing of the cell lines were carried out.

Cryoprotectants are broadly divided on the basis of whether or not they permeate cells. Cells are usually permeable to DMSO and glycerol where the cells are protected against freezing damage caused by intracellular ice crystals and osmotic effects. Cells are usually impermeable to PVP (polyvinylpyrrolidone) and serum proteins (Ashwood-Smith, 1987). Methylcellulose, PVP and other synthetic polymers have been used as for a number of years as protective agents in low serum media. Yoshida and Takeuchi (1991) used Methylcellulose to act as a non-permeating cryoprotectant because of its high molecular weight (140,000MW). A combination of a permeating and a non-permeating compound seem to have the best effect on survival of the cells in freezing medium. They found that a combination of 0.1% MC or 3% PVP with 10% DMSO resulted in a viability on thawing of between 72-76% for primary mouse astrocytes. They found that the percentage of cell attachment was higher when the cells were frozen with 0.1% MC and 10% DMSO. Ohno *et al.* (1988) used this combination for the successful freezing of mouse LP.3 cells with an 81% and 74% viability after a cryopreservation period of 4 and 6 months respectively. Merten *et al.* (1995) have used the same freezing medium for the successful freezing of a number of important industrial cell lines, such as BHK and Vero. The percentage viabilities on thawing were much higher than previous reports, greater than 94% for Vero and almost 100% for BHK.

Both the DLKP and DLKPA cell lines have been successfully frozen in a SF freezing medium composed of 10% DMSO and 0.1% Methylcellulose. Greater than 75% viability has generally been obtained for DLKP and greater than 78% for DLKPA which are results comparable to other researchers. Further work to try and improve viability on thawing could be carried out using PVP or glycerol.

### 4.3.3 Improvements in the SFM for the growth of early passage DLKP

The importance of basal medium optimisation for SF growth of cell lines cannot be understated. The basal medium provides the cells with many soluble, low molecular weight nutrients such as ions, carbohydrates, vitamins, amino acids and nucleosides. Barnes and Sato (1979) found that using a 50:50 mix of F12 and DME media is essential for the growth and survival of the MCF-7 cell line under SF conditions. No growth is observed when DME is used as the basal medium and a suboptimal growth rate is obtained with F12. Some cell lines may have defective metabolic or transport systems and thus require additional supplementation. Hybridoma cell lines secreting high levels of protein shoud be ideally grown in SFM containing a basal medium that is rich in amino acids. The work carried out in Section 3.2.6.1 shows that Ham's F12 basal medium is more effective than F12/ DME (1:1) in supporting the growth of the DLKP cells in SFM.

Insulin is the most common hormone supplement of culture media and is considered to be essential for the growth of nearly all cell lines in culture including hybridoma cell lines (Glassey *et al.*, 1988). It is included in most culture media at concentrations ranging from 5-10µg/ml which is much higher than physiological concentrations due to the short half of the molecule in convential media formulations. For most cell lines in SFM, the use of supraphysiological concentrations has been related to the mitogenic activity of insulin being mediated through the IGF-1 receptor. Mendiaz *et al.* (1986) have shown that insulin stimulates the growth of CHO-K1 cell line in SFM over a concentration range from 1ng to 10µg/ml. Murakami and Masui (1980) have shown that insulin at a concentration of 0.5µg/ml is required for the optimal growth of a human colon carcinoma cell line in SFM. For DLKP cells, a concentration of insulin of 2.5µg/ml was found to give optimal growth stimulation which falls well within the range observed by most researchers.

Transferrin is an iron-transport molecule and is a protein constituent of the β-globulin fraction of blood serum. It appears to be an obligatory requirement of most SF media formulations as most cell lines investigated under serum-free conditions show a positive response to it. Typical concentrations in SFM range from 1 to 30µg/ml. Transferrin is thought to exert its effect by interacting with cell surface receptors and facilitating the transport of iron into the cells across the plasma membrane. It also appears to chelate deleterious trace elements (Glassy *et al.*, 1988). The results of the transferrin assay carried out in Section 3.2.6.3 show that type of transferrin appears surprisingly to be slightly inhibitory over the range of concentrations tried. Hewlett (1991) has shown

that MDCK cell line show no requirement for transferrin when seeded at relatively high densities. Mendiaz *et al.* (1986) found that transferrin was not an essential requirement for the cells as $FeSO_4$ substituted equally well. Keenan and Clynes (1996) have shown that transferrin can be replaced by simple iron compounds for MDCK cells grown and subcultured in serum-free medium. Mather and Sato (1979) found that the observed growth stimulatory effects on the growth of a mouse melanoma cell line, MR2, in SFM were dependent on the absence of $FeSO_4$ in the medium. In the absence of added iron, there was found to be a fifteen-fold increase in the cell growth at a transferrin concentration of 1µg/ml. However, at the same concentration in the presence of $FeSO_4$, only a two-fold increase in cell numbers was observed. Further work needs to be carried out by looking at the effects of transferrin on growth with and without $FeSO_4$ being present in the SFM ($FeSO_4$ is present in the H SFM at a concentration of 5µM).

Successful growth of many anchorage-dependent cells in SFM usually requires the addition of attachment factors normally present in serum. Thus a number of attachment factors, laminin, fibronectin and Type IV Collagen were investigated to determine if DLKP showed improved growth with supplemented attachment factors. Although all cells are capable of producing attachment factors, the incorporation of attachment factors into a SFM greatly increases the deposition of the extracellular membrane.

Fibronectin is a multimeric glycoprotein associated with a host of biological functions including cell growth, adhesion, migration and wound repair (Hynes, 1990) and is located in the extracellular matrix of most tissues. Many cell lines have a requirement for attachment factors in order to spread and initiate cellular growth. For example, fibronectin has been found to be involved in the growth control of a rat ovarian cell line, RF-1, at a concentration of 8µg/ml (Orly and Sato, 1979). Fibronectin at a concentration of between 5-10µg/ml has been found to have a very significant stimulatory effect on the growth of the DLKP cell line. In the assays carried out in Section 3.2.6.4, almost a two-fold increase in cell numbers was achieved over the controls without fibronectin supplementation. The cells were also noted to attach very well to the flask surfaces with a more flattened, epithelial-like morphology than in unsupplemented SFM. Dr. Shirley McBride has shown that the DLKP cells and the three clones derived from this population attach very rapidly to wells coated with fibronectin. It appears that fibronectin plays an important role in regulating the growth control of the DLKP cell population.

Laminin at a concentration of 5µg/ml was also found to be stimulatory to the growth of DLKP cells in serum-free medium. The cells also appear to form short "neurite-like" processes compared to a more squamous, epithelial-like flattened morphology when the

cells are cultivated in fibronectin supplemented medium. Culture of a retinoblastoma cell line, 1-79, in SFM containing 10μg/ml laminin showed similar attachment and formation of neurite-like processes (Kyritsis *et al.*, 1986). Higher cell numbers were generally achieved for DLKP cells growing in laminin-supplemented medium rather than fibronectin supplemented medium. Similar trends were observed in 1003 embryonal carcinoma cells (Darmon, 1982) and rat epithelial cells (Junker and Heine, 1987). Laminin has also been shown to induce anchorage-dependent growth and slight neuronal differentiation in SCLC cell lines (Giaccone *et al.*, 1992). It would be interesting to investigate possible changes or induction of differentiation in DLKP cells after treatment with these ECM components, especially with the observed neurite-like processes in laminin treated cells. When laminin was added to a retinoblastoma cell line in SFM, the cells began to attach to the surface and started to from neurite-like processes. After 3-4 days, treatment of the cells with dibutryl-cyclic AMP (Db-cAMP) or sodium butryate resulted in extensive differentiation of the cells. Db-cAMP provoked the formation of long ramifying neurite-like processes, whereas butyrate induced the appearance of epithelial-like cells with a flat morphology (Kyritsis *et al.*, 1986). Laminin appears to have an initiating role in RB cell attachment and differentiation. Studies similar to this could be investigated in DLKP cell line which appears to have some degree of neuronal differentiation since it has been shown to express neurofilaments (McBride *et al.*, 1997).

Addition of Type IV Collagen to the SFM has been found not to improve the growth of DLKP cells in SFM and no apparent change in morphology was observed. This ties in with the obsevations made by Dr. Shirley McBride during experiments investigating attachment properties of the clones. Poor attachment to Type IV Collagen was always observed during time course assays.

Thus, DLKP grow significantly better in SFM when supplemented with the extracellular matrix proteins, fibronectin or laminin. The induction and maintenance of cell growth, differentiation and normal tissue architecture is a multistep process that requires interaction between the cell and the ECM (Rodriguez-Boulen and Nelson, 1989). Basement membrane components have also been shown to stimulate the growth of lung tumor cells *in vivo* (Topley *et al.*, 1993).

### 4.3.4 Sensitivity of DLKP to chemotherapeutic drugs in SFM

The sensitivities of the DLKP cell line, both long-term and short-term in SF conditions, to a number of chemotherapeutic drugs was determined. VP16 (etoposide) inhibits topoisomerase II activity leading to increased DNA damage. Adriamycin is an intercalating agent which also inhibits topoisomerase II activity and blocks DNA synthesis and RNA transcription while Vincristine is a tubulin inhibitor which disrupts spindle formation during cell division. These three drugs are among the most effective single agent responses of anti-cancer drugs used (Cook *et al.*, 1993). The phenomenon of multiple drug resistance (MDR) is often one of the main reasons for the high fatality rates associated with the disease. The human multidrug resistance I gene (mdr-1 gene) encodes P-glycoprotein, which appears to catalyse the outward transport of anticancer agents in cancer cells (Endicott *et al.*, 1989; Gottesmann, 1993). This appears to be one of the mechanisms involved in the phenomenon of MDR.

The SFM developed for DLKP cells was used to perform toxicity assays in a totally defined system. Serum proteins can inhibit the *in vitro* cytotoxicity of some anti-neoplastic drugs while other components of serum, such as growth factors, hormones and prostaglandins may affect the toxicity of the drug being tested. Tsai *et al.* (1996) screened 24 human cancer cell lines with nine anticancer agents in both serum-free and serum-supplemented medium and found that SSM had a negative effect on the cytotoxic action of some of the drugs including methotrexate, 5-fluorouracil and mitomycin C. The cellular protective effect on methotrexate and 5-fluorouracil are mediated *via* hypoxanthine and thymidine contained in undialysed serum (Yoshida *et al.*, 1978; Martin *et al.*, 1980; Sobrero *et al.*, 1986). Cisplatin and vincristine have been found to bind avidly to serum proteins (Donigan and Owellen, 1973; Wilson, 1986). Many growth factors and hormones exert their effects at such low concentrations that even small changes in concentration from one serum batch to another could result in a lot of result variation. Serum-free medium allows such assays to be carried out without risk of such variability and thus allows the concentration of such factors to be varied when actually required.

For VP16, it was observed that the $IC_{50}$ values were higher for the cells P.22 in SF compared to P.1 SF or SS culture, a 1.8-fold increase was noted. This difference was much more significant for cells exposed to Adriamycin where the P.22 cells showed a 3-fold increase in $IC_{50}$ value over normal culture serum-supplemented cells (P.1 SS). The P.1 SF cells also showed a 0.5-fold increase in $IC_{50}$ value compared to the same cells assayed in SSM. This general trend was not observed for cells assayed in

Vincristine where the cells were found to be extremely sensitive to the drug in SFM. Perhaps the reason for this increase in $IC_{50}$ value for two of the drugs tested in SFM is due to the fact that the cells grow more slowly in SFM. Many of the effects of MDR drugs occur during DNA replication, thus a cell line with a high turnover rate may be effected to a greater extent than one which divided at a slower rate. Due to the faster growth rate of SS cells, the $IC_{50}$ value is lower for these cells. Tsai *et al.* (1996) found that cell lines tend to be more sensitive to different drugs when they are tested in the particular medium type in which they preferentially proliferate.

Perhaps removal of serum from the system has caused the increased expression of the mdr-1 gene. There have been reports of increased mdr-1 expression caused by serum-starvation which would mimic the conditions observed in this SFM where the only mitogenic factor present is insulin. Expression of mdr-1 gene appears to be activated by various environmental stresses. For example, in human neuroblastoma and colon cell lines, differentiating agents such as retinoic acid and sodium butyrate increase expression of P-glycoprotein (Bates *et al.*, 1989). Heat shock and arsenite have also been found to increase its expression in human renal cancer cell lines (Chin *et al.*, 1990). Tanimura *et al.* (1992) have shown that the human mdr-1 promoter can be activated by serum deprivation in two human cancer cell lines, KB and SW-13. This group used an assay system using chloroamphenicol acetlytransferase (CAT) gene driven by the human mdr-1 gene promoter for their study. The presence of serum appears to be inhibitory to the activation of the mdr-1 promoter because the addition of serum to the cells cultured in SFM resulted in decreased CAT expression. Muller *et al.* (1995) have shown that the P-glycoprotein half-life of 4 different cell lines was normally between 14 to 17 hours. However, removal of serum from the medium results in the half-life increasing by 4- to 6-fold depending on the cell line. This observed serum effect suggests the involvement of growth factors in the control of P-glycoprotein stability and underlines the importance of culture conditions in determining the actual toxic effects of drugs. They also found that the effect of P-glycoprotein half-life is reversible when serum is added back. For the long-term SF cultured cells, especially for Adriamycin, a reduction in $IC_{50}$ values was noted between the SF and SS assays which could be indicative of a return to the original half-life of the P-glycoprotein normally observed in serum-supplemented culture. Although overall there is not a significant change in $IC_{50}$ values between SF and SS assays, a definite 3-fold increase in $IC_{50}$ value was observed for Adriamycin between P.22 SF cells and parental cells normally assayed in SSM.

RT-PCR analysis could be carried out on these long-term or short-term SF cells to see if there has been an increase in the expression of the mdr-1 gene. The protein is expressed normally at extremely low levels in DLKP serum-supplemented cells and is often undetectable even by RT-PCR analysis (O' Driscoll, 1994).

## 4.4 GROWTH OF DLKP CLONES IN SERUM-FREE MEDIUM

The growth capacities of the DLKP clones were found to vary under serum-free conditions. DLKP-I was found to be the only clone among the three that was capable of proliferating in the SFM investigated for DLKP. DLKP-I appeared to be the most independent of exogenous growth and attachment signals. This suggests that DLKP-I is producing the autocrine factors which DLKP-SQ and DLKP-M are lacking and enables these cells and the parental population to grow in defined, serum-free conditions. This may explain why the growth rate of DLKP-I in defined SFM often exceeds that of DLKP, as in the mixed heterogeneous parental population, this clone accounts for only 30% of the total population.

However, addition of a single factor, fibronectin, was found to be capable of stimulating growth of DLKP-SQ and -M. Subcultivation of these clones was also possible with fibronectin supplementation. Thus, attachment factors may be what DLKP-I produces allowing them to proliferate in SFM without supplementation of an attachment factor. One interesting observation was that the morphology of DLKP-I was similar to DLKP-M in this supplemented SFM. It has been suggested that DLKP-I may be an intermediate phenotype between those of -SQ and -M or possibly a stem cell-like population which can give rise to either of the two other phenotypes, depending on culture conditions. On prolonged culture in serum-supplemented medium, DLKP-SQ and -M can interconvert with -I but DLKP-SQ and -M do not appear to interconvert directly (McBride *et al.*, 1997). Thus, cultivating DLKP-I in SFM supplemented with fibronectin may push them towards the DLKP-M phenotype.

A useful model now exists to further investigate interconversion between these subclones of the heterogeneous DLKP population. DLKP and its clonal variants may prove to be a useful model for the study of tumourigenic progression. The adhesion properties of metastasising cells are extremely complex (Birchmeier and Behrens, 1994) and their interactions with ECM proteins must allow for substrate adhesion and motility. DLKP-M were found to have unusual attachment properties to fibronectin where the cells were found to adhere rapidly to the protein and then detach again; thus, they may represent a metastatic-like phenotype in the parental DLKP population (McBride *et al.,* 1997). The apparent ability of DLKP-I to interconvert to DLKP-M with fibronectin supplementation should allow this possible metastatic potential to be further investigated. The interconversion process is observed in serum supplemented conditions but the undefined nature of serum prevents this process being controlled, hence the ideal use of a totally defined system to continue these investigations.

## 4.5 GROWTH OF DLKPA CELLS IN SERUM-FREE MEDIUM

Multiple drug resistant variants of the DLKP cell line have been established in our laboratory (Clynes *et al.*, 1992) as models for MDR in non-small cell carcinoma cell lines. DLKPA is one such variant which was selected by subjecting the cells to increasing concentrations of the drug adriamycin and is several more times resistant than DLKP to a range of toxic compounds. The cell line shows a 300-fold increase in resistance to adriamycin compared to DLKP.

The growth of the cell line was investigated under SF conditions, both in short term and long-term culture, with a view to developing a system that allows the effects of different chemotherapeutic drugs alone or in combination with other factors such as growth factors, hormones or prostaglandins to be tested without the risk of serum interference and variability in results. Cryopreservation of the cell line under serum-free conditions was also possible using a combination of 10% DMSO and 0.1% Methylcellulose in SFM.

The SFM used for both DLKP and CHO-K1 SF culture, H SFM, was found to support the growth of the cell line both in short-term culture, over a period of 10 days and in long-term culture. It appears that indefinite culturing of the cell line is possible in SF conditions. The morphology of the DLKPA cell line in SFM is similar to that in serum-supplemented culture. Unlike DLKP SF cells, the DLKPA cells grow very tightly attached to the flask surface and are also much more spread out. The cells also grow to confluency and in colonies unlike DLKP SF culture. Many researchers have noted alterations in cell morpholgy during selection of drug-resistant cell lines (Biedler, 1981,1975). These changes in morphology appear to be more pronounced and obvious in a controlled SF environment. There have been reports in the literature of a relationship between P-glycoprotein expression and cellular differentiation. Treatment with differentiating agents such as RA have shown to result in increased expression of P-glycoprotein (Mickley *et al.*, 1989; Bates *et al.*, 1989). Biedler *et al.* (1991) have shown altered intermediate filament protein expression in multi-drug resistant neuroblastoma cells compared with their sensitive counterparts. Expression of cellular adhesion molecules due to the very tight attachment of the DLKPA cells in SFM should be investigated to demonstrate changes in cellular phenotype. Possibly changes in cellular adhesion molecules may be related to the differentiated state of this MDR cell line.

## 4.6 INVESTIGATIONS INTO KERATIN 8 AND 18 EXPRESSION IN DLKP AND DLKPA

Novel induction of keratin 8 and 18 expression with BrdU treatment of keratin-negative DLKP cells has been observed (McBride and Clynes, manuscript submitted). Experiments were carried out to determine if a similar induction of keratin expression by BrdU could be demonstrated in DLKPA cells. Studies were also carried out to determine if keratin expression was a result of cytotoxic stress by BrdU rather than a differentiating effect, using an extremely toxic anti-cancer drug, Adriamycin. Long-term K8 and K18 expression was also investigated in DLKP cells. Protein synthesis inhibitor experiments were also carried out to try to determine if there might be a protein causing de-stabilisation or degradation of keratin filaments in keratin-negative DLKP cells. There appears to be strong evidence that K8 and K18 expression is regulated at a post-transcriptional level.

### 4.6.1 Effect of BrdU on keratin 8 and 18 expression in DLKPA

DLKPA is a 300-fold adriamycin resistant variant of the human lung carcinoma cell line, DLKP. The cell line is also more resistant to a range of anti-cancer drugs including VP16, vincristine and cisplatin (Clynes *et al.*, 1992). There have been varying reports in the literature regarding relationships between multiple drug resistance and keratin filament expression. Intermediate filaments, along with microtubular proteins, play a key role in the maintenance of cell shape and in the spatial organisation of the cellular organelles. They are also thought to be involved in the modulation of membrane transport (Mills and Mandel, 1994). Thus, changes in the intermediate filament content and structure could potentially alter the cellular cytoskeleton and affect a multitude of cellular events, including drug transport. L cells (mouse fibroblasts) transfected with cytokeratins 8 and 18 exhibited a multiple drug resistance phenotype when exposed to a number of drugs including adriamycin, methotrexate and vincristine (Bauman *et al.*, 1994). An increased level of cytokeratin 8 expression was observed in resistant variants of the human ovarian cancer cell line, OAW42, (Moran *et al.*, 1997). On the other hand, Parekh and Simpkins (1995) have shown that a cisplatin-resistant human ovarian cell line contained markedly lower levels of cytokeratin 18 when compared to the sensitive parental cells. This group has also shown that the cisplatin-resistant variants are hypersensitive to the cytotoxic effects of taxol, vincristine and vinblastine. Decreased levels of cytokeratin 14 was also reported in a cisplatin-resistant variant of a human squamous cell carcinoma cell line (Katabami *et al.*, 1993).

The multiple drug resistant cell line, DLKPA, was found to be negative for the expression of keratin filaments using a pan-cytokeratin antibody and antibodies to keratins 8 and 18. Treatment with BrdU induced expression of keratin 8 and 18 proteins with an apparent change in morphology, similar to that observed in BrdU-treated DLKP cells. Thus keratin filament protein expression does not appear to be associated with multiple drug resistance in DLKPA cells. However, it would be interesting to carry out some toxicity or drug transport assays on BrdU-treated cells to see if there are any changes in resistance to the various anti-cancer agents.

### 4.6.2 Toxic effect of BrdU

Bromodeoxyuridine at the concentrations used in the study was found to be extremely toxic to the cells. To rule out the possibility that cytokeratin expression was a result of cytotoxic stress caused by BrdU, experiments were carried out using concentrations of the highly toxic chemotherapeutic drug, adriamycin, that would result in a similar level of toxicity to the cells. No induction of cytokeratins 8 or 18 was observed in DLKP, DLKPA, H82 and HL60 cells, and no upregulation of expression was observed in A549 cells. The induction/ upregulation of keratin expression is due to/ caused by the differentiating, and not the cytotoxic, effects of BrdU.

### 4.6.3 Long-term keratin 8 and 18 expression in DLKP cells

The effect of BrdU on induction of keratin 8 and 18 expression in DLKP appears to be reversible. Removal of BrdU from the medium results in the cells retaining K8 and K18 positivity for at least two weeks. As the cells were being continually passaged, the number of keratin positive cells gradually decreased. Twenty-eight days after removal of BrdU from the medium, all the cells were found to be negative for K8 and K18 expression. The experiments which have been done do not distinguish between two possibilities; the cells induced to express cytokeratins are terminally differentiated and are diluted out during subculture; or expression is gradually lost if BrdU is not continuously present. A combination of these two events could also be occurring. Further experiments could be performed to determine if these keratin positive cells are capable of replicating. Double-labelling immunofluorescence studies could be carried out using antibodies to keratins 8 or 18 and proliferation markers such as Ki67 or PCNA. The keratin positive cells could also be sorted using flow cytometry and these cells could then be investigated for ability to replicate. If BrdU has induced a degree of differentiation, these cells may also have slower growth rates compared to the parental untreated cells.

### 4.6.4 Protein synthesis inhibitor studies on DLKP cells

BrdU has been found to induce keratin 8 and 18 expression in DLKP cells at a post-transcriptional level (McBride and Clynes, manuscript submitted). This suggests that BrdU may exert its effect directly or indirectly on protein translation, or stabilisation of translation products already present. Keratin 8 and 18 proteins are already present in A549 cells before treatment and are upregulated following BrdU treatment. It thus seems plausible that a stabilisation of keratin translation products is occurring rather than an induction of translation. Perhaps in DLKP and H82, a rapid degradation of keratin proteins in untreated cells results in their being undetectable by immunocytochemical methods.

One of the hypotheses put forward was that a protein may be causing rapid degradation of these keratin proteins. Cycloheximide and Actinomycin D protein synthesis inhibitor studies were carried out to try and determine if there might be some evidence for a protein causing de-stabilisation or rapid degradation of translation products for keratins 8 and 18. The principal behind the experiment was that cycloheximide and actinomycin D would prevent synthesis of the protein thus allowing stabilisation and resultant expression of the keratin filaments. Cycloheximide treatment for 6 to 10 hours at concentrations ranging from 5 to 10μg/ml had some effect on the cells with a definite induction of K8 and K18 expression, observed by immunocytochemistry. Levels of expression were less than that observed for BrdU-induced K8 and K18 expression. This is probably due to cycloheximide compromising the synthesis of the keratin proteins themselves. However, the results were found to be promising and do lend some weight to the proposal that BrdU may be stabilising keratin translation products and thus acts at a post-transcriptional level to regulate control of K8 and K18 expression in epithelial cell lines. Cycloheximide has been shown to induce keratin expression in other cell lines. Cremesi and Duprey (1987) demonstrated that treatment of an undifferentiated embryonal carcinoma cell line led to the transcriptional induction of the endo A gene, which is the mouse homologue of cytokeratin 8.

Similar studies carried out using Actinomycin D showed no induction of keratin 8 and 18 expression. This may be due to these two protein synthesis inhibitors having different mechanisms of action on the cells.

## 4.7 STUDIES TO INVESTIGATE THE DIFFERENTIATING EFFECT OF BRDU IN HUMAN LUNG CANCER CELL LINES

When DLKP are grown in SFM, there appears to be a change in the morphology of the cells especially when the medium is supplemented with the extracellular matrix proteins, fibronectin and laminin. Treatment of DLKP with BrdU has been shown to induce the expression of epithelial specific proteins with a dramatic change in morphology. The cells become more flattened and spread. The effect of the differentiation-inducing agent BrdU was examined in an attempt to further characterise the new cellular phenotype with a particular emphasis on the expression of adhesion receptors such as integrins. The response of these cells to various ECM proteins was also examined to investigate possible changes in the adhesive properties of the cells.

### 4.7.1 Determination of epithelial-specific and epithelial-associated markers in BrdU-treated cells

The new cellular phenotype was noted to be much larger, stretched and flattened, suggesting a change in adhesion properties of the cells. There appears to be a trend for the down-regulation of $\beta_1$ integrins in poorly-differentiated epithelial tumours of the lung (Smythe *et al.*, 1995; Roussel *et al.*, 1994), colon (Pignatelli *et al.*, 1990) and breast (Zutter *et al.* 1995). A decrease in integrin expression may favour tumour invasion and unregulated growth (Ziober *et al.*, 1996). Collagen-laminin binding integrin expression has often been seen to correlate with the degree of pathological de-differentiation of the primary tumour, with the more poorly-differentiated tumours exhibiting lower levels of expression (Bonkhoff *et al.*, 1993; Korhonen *et al.*, 1992). DLKP has been classified as a poorly-differentiated carcinoma of the lung and appears to be highly malignant and aggressive, which may be reflected in its successful proliferation in serum-free medium (See section 3.2). Thus, the $\beta_1$ integrin profile of these cells before and after BrdU treatment was examined.

I found that treatment of DLKP with BrdU changes the expression of a number of the $\beta_1$ integrins. Induction of $\alpha_1$ integrin expression has been shown by immunocytochemistry and immunoprecipitation. There have been few reports in the literature on the association between $\alpha_1$ integrin and the invasive/metastatic behaviour of tumour cells. However, decreased levels of $\alpha_1$ are found in human metastatic melanoma (Schadendorf *et al.*, 1993) and axillary metastases of the breast (Gui *et al.*, 1994). Loss of $\alpha_1$ integrin has been observed in lung adenocarcinomas, compared with strong expression in normal lung pneumocytes (Roussel *et al.*, 1994).

Upregulation of $\alpha_2$ integrin was also observed by immunocytochemistry and immunoprecipitation. More information on $\alpha_2$ integrin exists in the literature, though it is somewhat contradictory (See Section 1.4). Overall, it appears that invasive breast cancer has the strongest correlation with lack of $\alpha_2$ integrin expression and the differentiation state of the tumour. High levels of $\alpha_2$ are found in normal breast ducts and ductules. However, these levels fall in moderately differentiated, and are markedly reduced or completely absent in, poorly-differentiated ductal carcinoma and breast disease (Zutter *et al.*, 1995; Arihiro *et al.* 1993). Reduction in $\alpha_2$ integrin expression has been observed in poorly-differentiated non-small cell carcinoma of the lung (Smythe *et al.*, 1995), but it appears that down-regulation of $\alpha_2$ may be associated with the differentiation status, but not metastasis, of the NSCLC. This may tie in with observations made by Dr. Shirley McBride (PhD 1995) where faint positive staining was noted in DLKP and two of the clones (DLKP-I, DLKP-SQ), but the clone which appears to possibly have metastatic properties, DLKP-M, has a stronger $\alpha_2$ integrin expression. Normal bronchial and alveolar epithelial cells express high levels of this integrin (Damjanovich *et al.,* 1992; Mette *et al.*, 1993). Thus DLKP is an excellent model to further investigate the metastatic potential of those cells before and after treatment with bromodeoxyuridine.

No change in the expression of $\alpha_3$ integrin was observed by immunocytochemistry in BrdU-treated DLKP cells. In most normal epithelia, $\alpha_3$ integrin is restricted to the basal and suprabasal cells and is generally absent in more differentiated cells and may be involved in cell-cell adhesion events (Symington *et al.*, 1993; Carter *et al.*, 1994). It appears to be altered in some tumours with the presence of $\alpha_3$ integrin correlated with tumour progression and high expression in metastatic lesions (Bartolazzi *et al.*, 1993). Strong expression of $\alpha_3$ integrin was observed in DLKP cells which may correlate with the low degree of differentiation of this tumour cell line. DLKP was also established from a primary lymph node metastasis which may explain the high expression of this integrin in the cell line. No apparent change in expression of this integrin was observed by immunocytochemical techniques.

The expression of $\alpha_4\beta_1$ integrin was also investigated in DLKP cells. This integrin is not usually expressed in any significant amounts in normal or malignant lung tissue (Damjanovich *et al.*, 1992; Mette *et al.*, 1993). No induction of $\alpha_4$ integrin expression was observed in BrdU-treated DLKP cells.

BrdU has been found to upregulate expression of $\alpha_3$, $\alpha_5$, and $\alpha_6$ integrin expression in a human melanoma cell line (Thomas *et al.*, 1993). However, no reports have been found in the literature on the induction and upregulation of integrin proteins in response

to differentiation agents in human epithelial cells lines. The mechanism of action of BrdU in differentiation is unknown but the induction and upregulation of integrin expression may afford an excellent opportunity to investigate this mechanism. The literature on regulation of integrin expression is extremely sparse and DLKP provides an excellent model to further investigate integrins and their role in differentiation and cancer progression.

The finding that keratin 8 and 18 expression in DLKP was induced by BrdU treatment suggested the value of looking at the expression of keratin 19. Keratin 19 is often co-expressed with keratins 8 and 18 in simple epithelial tissues. Induction of keratin 19 was observed in BrdU-treated DLKP cells, though the level of induction was less than that observed for keratin 8 and 18 (5% compared with 20%).

The expression of epithelial specific antigen (ESA), which is also known as epithelial glycoprotein (EGP), was also investigated in DLKP cells treated with BrdU. DLKP and its clones are normally negative for expression of ESA (McBride *et al.* 1997). However induction of this protein has been observed by immunocytochemistry and Western blots, by treatment with BrdU. EGP protein expression is exclusive to epithelial cells (Momberg *et al.* 1987; Moldenhauer *et al.* 1987) and is usually expressed at the basolateral surface of the majority of simple cuboidal or columnar, pseudostratified columnar and transitional epithelia (Spurr *et al.* 1986; Edwards *et al.* 1986; Burmol *et al.* 1988). The function of this protein is unknown, though the gene has been cloned by a number of different investigators (Szala *et al.* 1990; Strnad *et al.* 1989; Simon *et al.* 1990). EGP was suggested to play a role in fundamental cell-cell or cell-matrix interactions of epithelial cells because it was found to have sequence homology to nidogen, a component of the extracellular matrix (Simon *et al.* 1990). Litvinov *et al.* (1994) have shown that EGP expression can supress invasive colony growth of L cells, a fibroblast cell line, in EHS-matrigel. They suggest that in EGP-negative epithelial tissues, the *de novo* expression of the molecule in tumour cells may be critical at early stages of tumourigenesis. They suggest a role similar to E-cadherin, the main type of intercellular adhesion of epithelia, which is often found down-regulated in poorly-differentiated carcinomas and later stage carcinomas. Re-introduction of EGP into EGP-negative cell lines may be capable of diminishing the strength of normal-tumour cell adhesions and thus reduce the invasiveness of a tumour cell line into normal surrounding tissue.

The effect of other differentiating agents on the expression of ESA has been investigated in epithelial cell types. DMSO has been shown to reduce ESA expression in human colonic epithelial cells, while sodium butyrate had no effect in these cells

(Omary *et al.* 1992). The marked change in the expression of ESA after BrdU treatment should provide a useful model to study potential functions for ESA, especially if its function as a cellular adhesion molecule, which may have a role in tumour differentiation and progression, is proven.

The effect of BrdU on the expression of cytokeratin 19, ESA and some of the beta-1 integrins was also investigated in other lung epithelial cell lines, DLKPA, H82 and A549, and in a non-epithelial cell line, HL60.

Treatment of DLKPA showed a similar induction of cytokeratin 19, ESA and $\alpha_1$ integrin expression with no change in expression of EGF-R and desmosomal protein. These results are similar to those found for the parental drug-sensitive cell line, DLKP.

Treatment of A549, a human lung adenocarcinoma, with BrdU had a very dramatic effect on cytokeratin 19 expression. Before BrdU treatment, 30% of the cells were usually positive for keratin 19 expression but after treatment for 5 to 7 days, at least 80-90% of the cells were found to be positive by immunocytochemistry. This upregulation was clearly demonstrated by Western blots and immunoprecipitation of keratin 19 proteins. Upregulation of keratins 8 and 18 was also observed in DLKP after BrdU treatment (McBride and Clynes, manuscript submitted). However, this was not as dramatic an upregulation as that observed for keratin 19. Expression of EGF-R and desmosomal protein was found to be similar before and after BrdU treatment. Upregulation in expression of ESA was also observed on BrdU treatment. However, no apparent change in expression of $\alpha_1$ and $\alpha_2$ integrin was observed in this cell line which is different to DLKP. A549 are quite a well-differentiated cell type and already inherently express $\alpha_1$ integrin and high levels of $\alpha_2$ integrin.

H82, a variant-SCLC cell line, was also investigated for the expression of these proteins after BrdU treatment. DLKP has been found to express some antigens typical of V-SCLC classification. This cell line has been shown to differentiate towards a more tightly aggregated, classic SCLC morphology in the presence of retinoic acid (Doyle *et al.,* 1989). Treatment of H82 with BrdU results in induction of K8, K17 and K18 (McBride and Clynes, manuscript submitted). The cells which usually grow as aggregates in suspension begin to attach to the flask surface and spread becoming more epitheloid in appearance on BrdU treatment. Induction of keratin 19, and of $\alpha_1$ and $\alpha_2$ integrins was observed in a small number of these attached cells (<5%). The expression of ESA was found to remain negative after BrdU treatment which differs from that observed in DLKP BrdU-treated cells.

HL60, a leukaemic cell line which grows as single cells in suspension, did not express keratin 19, ESA, $\alpha_1$, $\alpha_2$ or $\alpha_3$ integrins before or after BrdU treatment.

It thus appears that BrdU is capable of inducing differentiation along lineage-specific pathways. It is incapable of inducing expression of epithelial-specific markers such as keratin 19 and ESA in a non-epithelial cell type such as HL60. It is also incapable of inducing beta-1 integrin expression in these cells as these integrins are most commonly expressed in epithelial cells which require these receptors in order for them to be anchored to the basement membrane through integrin ligand-ECM interactions.

### 4.7.2 Effect of BrdU in serum-free medium

Induction of keratin 8 and 18, ESA, $\alpha_1\beta_1$ integrin and upregulation of $\alpha_2\beta_1$ integrin was also observed when serum-free medium was used. This shows that BrdU is working on its own in the system without interference from factors found in serum which may contribute towards the differentiation process. The use of serum-free medium in such a system is ideal for the use of physiologically relevant differentiating agents such as retinoic acid and interferons.

### 4.7.3 Messenger RNA studies

RT-PCR analysis was carried out to determine if the induction and upregulation of keratin 19 by BrdU was a result of alterations in the transcription of the gene. The mRNA levels in A549 were remained unchanged over the 5 day treatment even though protein levels were significantly upregulated, as demonstrated by immunocytochemistry, Western blotting and immunoprecipitation of keratin 19 protein. Keratin 19 mRNA was also detected in DLKP and H82 prior to treatment with BrdU. No increase in keratin 19 mRNA transcripts was apparent after BrdU treatment for 5 days even though induction of keratin 19 protein was clearly evident by immunocytochemistry. Keratin 19 mRNA was also detected in HL60 cells at much lower concentrations before and after treatment even though expression of K19 protein was never detected by immunocytochemistry or Western blots. Several reports describe the detection of keratin mRNA transcripts in cells which do not express the corresponding protein. Burchill *et al.* (1995) detected K8, K19 and K20 mRNA in normal blood cells. Thus, the presence of K19 mRNA is not too surprising in HL60 cells which are in fact a leukaemic cell line.

Much remains to be discovered about the mechanisms which control expression of the various keratin proteins. Most of the studies have shown regulation at the transcriptional level (Roop *et al.*, 1988; Blouin *et al.*, 1992). Retinoic acid has been demonstrated to regulate keratin 19 expression at the post-transcriptional level in keratinocytes (Crowe, 1993). Translational (Tyner and Fuchs, 1986) and post-translational (Bisgaard *et al.*, 1994) control of keratin expression have also been reported. However, no reports have appeared in the literature on the mechanism of regulation of keratin expression by BrdU. Pampinella *et al.* (1996) observed K8 and K18 expression in rabbit serosal cells following BrdU treatment while untreated cells expressed only keratin 18. However, mRNA levels were not measured. K8 and K18 mRNA appears to be regulated in DLKP and H82 cells at the post-transcriptional/ translational level showing similar levels of expression of keratin 8 and 18 mRNA transcripts in untreated and treated cells by RT-PCR and Northern blots (McBride and Clynes, manuscript submitted). No upregulation of K8 and K18 mRNA was observed in A549 while HL60 was also found to express low amounts of K8 and K18 mRNA transcripts. It appears that keratin 19 expression is regulated at the post-transcriptional/ translational level in a similar manner to keratins 8 and 18 in these epithelial cell lines.

It was not possible to carry out RT-PCR studies on the integrins due to the lack of availabilty in the literature of complete gene sequences for the $\alpha_1$ and $\alpha_2$ integrins. Only cDNA sequences for these genes have been reported (Briesewitz *et al.*, 1993; Takada

and Hemler, 1989). Thus, it was not possible to design primers because of the danger of primer sequences spanning introns and amplifying genomic DNA rather than the actual gene of interest. Northern blotting was carried out using a rat $\alpha_1$ integrin cDNA probe (Ignatius *et al.*, 1990) but I was unable to get the probe to detect human RNA from the various cell lines tested. Further studies will be concerned with making human cDNA probes for both these integrins to try to elucidate how BrdU is working to induce and upregulate the expression of such proteins.

### 4.7.4 Change in adhesion properties of BrdU-treated cells

BrdU treatment of DLKP cells resulted in induction of $\alpha_1\beta_1$ integrin and upregulation of $\alpha_2\beta_1$ integrin. Both these receptors are collagen and laminin binding. BrdU-treated cells also show a change in morphology, with the cells becoming more stretched and flattened, possibly indicative of a change in adhesion properties of the cells.

Functional attachment assays were thus carried out in response to these changes in receptor expression and cellular morphology. The DLKP treated cells were found to show increased adherence to all three ECM proteins looked at, fibronectin, laminin and Type IV collagen, over the 15, 30 and 60 minute time intervals investigated. Increased adherence of treated cells to EHS was also observed. EHS is composed of collagens, non-collagenous glycoproteins (such as laminin and fibronectin) and proteoglycans of the basement membrane, and would be present in the basement membrane *in vivo*. Treated cells also showed increased adherence to plastic, though the rate of attachment to plastic was much slower than the rates observed for laminin or fibronectin.

Poorly-differentiated and invasive cell types often showed down-regulation of epithelial cell-cell attachments, thus altering normal cell-matrix interactions which may enable metastasising cells to migrate and invade the basement membrane. BrdU-treated cells show greater adherence to collagen and laminin which may be associated with the up-regulation of the collagen and laminin binding $\alpha_1$ and $\alpha_2$ integrin. These cells also appear to attach better to the basement membrane (EHS). Possibly, the DLKP cell line has lost some of its metastatic capabilities with the change in expression of such proteins, as well as the ESA protein. The cells may then be less inclined to move around the body as a metastatic cell type, and remain anchored to the site in which the primary tumour originally occurred. The induction of such proteins may make DLKP a less migratory cell type.

Changes in the adhesiveness of cells has been reported previously in response to BrdU and retinoic acid treatment. Thomas *et al.* (1993) found increased adherance of melanoma cells to Type IV collagen after BrdU treatment. Ross *et al.* (1994) found that retinoic acid treatment of F9 embryonal cells results in increased expression of the $\beta_1$ subunit of integrin receptors. A consistent, small increase in attachment of F9 carcinoma cells to plastic as well as gelatin, laminin and fibronectin after RA treatment of these cells was observed.

Invasion assays using Matrigel and the determination of expression of matrix-degrading enzymes by these BrdU-treated cells could show changes in the possible metastatic

# 5.0 CONCLUSION AND FUTURE WORK

## 5.0 CONCLUSION AND FUTURE WORK

A serum-free medium has been developed which supports the continuous, long-term cultivation of CHO-K1 cells. It appears that the concentration of calcium in the medium is critical to this long-term growth in serum-free medium but not in serum-supplemented medium. Cultivation of the cells in high calcium media results in cessation of growth after 4-6 passages on average, while the use of a low calcium SFM allows the cells to proliferate continuously. This may have important considerations for primary culture and for other cell lines that fail to proliferate in SFM. Traditional media use high calcium concentrations, usually above a 1mM concentration. High concentrations may induce differentiation in these cells and suppress proliferation thus preventing their long-term culture. There is also a good correlation between loss of $Ca^{++}$ control and progression towards tumourigenicity where transformed cell lines often show a reduced requirement for calcium compared to normal cells. This suggests that it is of considerable importance to consider the calcium content of the medium before proceeding with the culture of specific cell types. Employment of such a medium investigated for CHO-K1 cells would be of considerable importance. The development of a SFM that allows continuous proliferation of CHO-K1 cells may also have important consequences for the commercial production of recombinant proteins in a completely defined system.

CHO-K1 cells can be cultivated in suspension culture in SFM when Pluronic F68 or microcarriers are added to the spinner flasks. Cell death usually occurs within 24 hours when the SFM is not supplemented with Pluronic F68. This appears to be due to the effects of shear forces in the spinners caused by changes in fluidity of the membrane (PMF). A change in PMF shown by fluorescence anistropy measurements correlates with the poor growth of the cells where a decreased PMF value was associated with an increase in shear sensitivity of the cells. The SFM could be scaled up to assess the growth of the cells in larger scale suspension culture. Other shear protective agents and recombinant SFM supplements (especially insulin or transferrin) could also be investigated in these systems.

Successful cryopreservation of CHO-K1, DLKP and DLKPA was achieved in SFM using methylcellulose or polyvinylpyrrolidone to replace serum in the freezing medium. This is of particular interest because the use of serum at the freezing stage compromises the serum-free status of the cells, especially if they are being cultured long-term in SFM.

The DLKP and DLKPA cells have been found to grow continuously in SFM and appear to adapt to the conditions in which they are forced to grow after a period of time in SFM. This is of benefit when performing chemotherapeutic drug toxicity assays where often serum proteins may mask the effect of the drug, and small changes in serum composition may affect the cytotoxicity of the drug. Differentiation studies could also be carried out in SFM when investigating the effects of physiological differentiating agents such as retinoic acid, interferons, *etc.* Serum is too ill-defined to carry out such studies with a good degree of accuracy.

Serum-free medium has also been developed for the clonal variants of the DLKP parental cell line. It appears that the intermediate-like phenotype, DLKP-I, may be producing its own attachment factors including fibronectin, which the other two clones are incapable of producing. This clone can grow in SFM without fibronectin supplementation whereas the other two clones cannot. The development of SFM for the three clonal populations of DLKP is a potentially useful model to study interconversion among a heterogenous tumour cell population. This process could then be controlled or manipulated more easily in a defined, SFM because of the ill-defined nature of serum-supplemented medium.

Treatment of DLKP with the differentiation-inducing agent, BrdU, resulted in changes in the expression of a number of integrins on the cell membranes. Induction of $\alpha_1$ integrin and upregulation of $\alpha_2$ integrin was observed in treated cells. This appears to be an epithelial-specific effect, in this case, as no change in integrin expression was observed in HL60 leukaemic cells. These changes in integrin expression also resulted in a functional change in the cells. The cells became more adherent to a number of ECM proteins, laminin, collagen and fibronectin, and also to basement membrane (EHS). This change in adhesive properties of the cells was capable of being reversed by the addition of an anti-$\alpha_1$ integrin blocking antibody.

The induction and upregulation of integrin receptors by a simple, chemical differention-inducing agent in lung epithelial cells has not been reported previously, though a report has been found in the literature demonstrating a change in integrin expression in melanoma cells by treatment with BrdU (Thomas *et al.*, 1993). Further studies with DLKP cells may aid in the elucidation of control of integrin expression in lung epithelial cells. The literature is quite scant on information about the specific role of the $\alpha_1$ integrin in normal cells. The DLKP cell model which may represent a poorly-differentiated stem cell-like lung population appears to be a particularly clean system to

investigate such mechanisms of regulation and normal function. Studies should also be carried out on the effect of BrdU on the expression of the $\beta_1$ subunit of these integrins.

Many of the $\beta_1$ integrins are down-regulated in poorly-differentiated epithelial cancers and this may have implications for increased invasive and metastatic potentials of the cells. Many of these integrins are down-regulated in DLKP cells where the only $\beta_1$ integrin that is expressed in significant amounts is the $\alpha_3$ integrin. It would be interesting to see if BrdU treatment of DLKP induced a change in the invasive potential of these cells by performing *in vitro* Matrigel invasive assays.

Treatment of DLKP with BrdU also induced the expression of other epithelial specific markers including epithelial specific antigen and keratin 19. RT-PCR analysis of keratin 19 mRNA before and after BrdU treatment suggests that BrdU is working at the post-transcriptional/ translational level to overcome the block preventing proper translation of keratin mRNA transcripts. Epithelial specific antigen is thought to be a cellular adhesion molecule. However, very little is known about its normal function and its role in tumour progression. An excellent opportunity thus exists with the DLKP differentiation model to further study the regulation and function of this protein.

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