THE ROLE OF FIBROBLASTS IN THE DIFFERENTIATION OF HUMAN NON-SMALL CELL LUNG CARCINOMA

by

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LIST OF ABBREVIATIONS

cAMP	cyclic adenosinemonophosphate
BSA	bovine serum albumin
CPC	cetylpyridinium chloride
DAB	diaminobenzidine
DMEM	Dulbecco's modified Eagles medium
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DPPC	disaturated phosphatidylcholine
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme linked immunoadsorbant assay
FCS	foetal calf serum
FDF	fibroblast derived factor
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
FPF	fibroblast pneumocyte factor
FPLC	fast protein liquid chromatography
GAGS	glycosaminoglycans
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxylethyl]piperazine-N'-[2-ethanesulphonic acid]

HPLC	high pressure liquid chromatography
IEF	isoelectric focusing
IFN	interferon
IGF-I	insulin-like growth factor one
i.p.	intra-peritoneal
i.v.	intra-venous
MES	2-[N-morpholino]ethanesulphonic acid
MOG	Medical Oncology Glasgow
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NMF	N-methylformamide
NSCLC	non small cell lung carcinoma
PA	plasminogen activator
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
SCLC	small cell lung carcinoma
SDS	sodium dodecyl sulphate
TAT	tyrosine aminotransferase
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylene diamine
TGF	transforming growth factor
TNF	tumour necrosis factor
Tris	Tris (hydroxymethyl) aminomethane
Tween-20	polyoxyethylene sorbitan monolaurate

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SUMMARY

Failure to differentiate may represent an intrinsic genetic defect or an aberration in the regulation of gene expression. An element of this deficiency may arise from an alteration in heterologous cell interactions resulting from modifications in cell contact, extracellular matrix, or soluble transmissible agents.

A549 cells, derived from an alveolar type II pneumocyte tumour were selected to study regulation and re-expression of the differentiated phenotype because:

1. They possess specific markers for differentiation
2. Previous work had indicated stromal dependence for the differentiation of the normal pneumocyte
3. The system is regulated by a well characterised inducer, hydrocortisone
4. The cells grow well in culture and have previously been well characterised by others

As others had shown previously that the differentiation of normal type II pneumocytes is regulated by glucocorticoid via its interactions with stromal fibroblasts, the role of human foetal lung fibroblasts (MOG-LF113) on the *in vitro* and *in vivo* response of A549 was investigated.

A549 cells showed an increase in pulmonary surfactant synthesis with an enhanced response to glucocorticoid when cultured in a filter well as opposed to on a conventional plastic substrate. The addition of foetal lung fibroblasts either in coculture in the filter well, or separated by the filter well membrane, with A549 on the filter and MOG-LF113 growing on the dish below, further enhanced pulmonary surfactant synthesis, with greatest stimulation observed under transfilter conditions in the presence of glucocorticoid.

Glucocorticoid treated MOG-LF113 conditioned medium was also capable of enhancing pulmonary surfactant synthesis, although to a lesser extent than the transfilter experiments. Electron microscopy studies showed cells treated with conditioned medium had a greater incidence of multilamellar bodies, the intracellular storage sites for surfactant. This medium had no effect on the growth of either A549 or MOG-LF113 in vitro, although it was found to be mitogenic to Swiss 3T3 fibroblasts.

It was possible to purify partially a factor, (or a mixture of factors), designated MOG-FDF/IV, from the conditioned medium, and treatment of A549 with MOG-FDF/IV stimulated pulmonary surfactant synthesis some twelve-fold.

MOG-FDF/IV had no effect on A549 cell growth in monolayer, but reduced clonogenicity in suspension. Plasminogen activator activity was reduced in treated cultures, and there was an increase in total cellular and secreted glycosaminoglycans.

Intra-peritoneal administration of MOG-FDF/IV to nude mice bearing A549 xenografts markedly reduced the growth of the tumour. This was accompanied by an alteration in the tumour histology, with extensive tissue reorganisation and the formation of glandular-like structures.

Preliminary observations with xenografts of three other tumours suggest that the carcinostatic effect of MOG-FDF/IV may be specific, at least to lung.

Finally, since an alteration in phenotypic expression may change the sensitivity to cytotoxic drugs, the effect of induction of differentiation on sensitivity to Adriamycin was tested. Pretreatment of A549 with MOG-FDF/IV had no effect on the chemosensitivity of the cells. This was true even when A549 cells were cultured under conditions conducive to maximum pulmonary surfactant synthesis.

PREFACE

THESIS LAYOUT

This thesis is divided into self-contained chapters, each with its own introduction, results section and discussion. Chapter 1 outlines the main topic of the thesis. The main methodology used is described in Chapter 2. Chapter 3 characterises the principle cell lines used in this study, as well as their response to steroid. The consequences of fibroblast-A549 interactions are described in Chapter 4, while Chapter 5 describes the partial purification of fibroblast conditioned medium. Chapter 6 looks at other phenotypic markers in A549, and there is a brief section on chemosensitivity and differentiation. Chapter 7 looks at studies *in vivo*, while in Chapter 8, a general discussion, the implications of the results and suggestions for future studies are discussed.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 BIOLOGICAL BASIS OF CANCER

Normally, there is a well controlled balance between cell division, cell differentiation and senescence, but this delicate balance is disrupted in neoplastic tissues. The onset of malignancy is due, at least in part, to some kind of block in the differentiation pathway, since many malignant cells show signs of abnormal or incomplete differentiation. The development of a less well differentiated cell in a given population is sometimes referred to as "dedifferentiation". In many ways, this term is a misnomer, since it implies that a cell actually progresses backwards along the differentiation pathway, as a result of carcinogenic insult. This has not been clearly established and is conceptually more difficult to explain. A more likely scenario is that an anaplastic malignant cell arises from the progeny of a tissue stem cell, (one which is still capable of self-renewal and which has not yet become fully differentiated), which for some reason has been blocked or diverted in the process of becoming a fully differentiated cell. By removing this block, perhaps by the intervention of pharmacological or biological agents, it may be possible to allow the cells to proceed towards terminal differentiation.

1.2 DIFFERENTIATION

But what exactly is differentiation? Literally, it has 3 meanings; to develop variation in, to specialise or to acquire a distinct character. Scientifically, differentiation can be described as a series of recognisable steps leading to structurally and functionally mature cells which generally lack the capacity for further proliferation.

In the adult, there are 2 main pathways of differentiation (Freshney, 1985). In self renewing tissues such as the skin or the haematopoietic system, a small population of totipotent or pluripotent undifferentiated stem cells give rise to committed precursor cells which progress towards terminal differentiation. These cells lose the capacity to divide as they reach the terminal stages, ultimately giving rise to fully differentiated mature cells which do not normally divide. Alternatively, a differentiated cell such as a fibroblast may dedifferentiate (but still retain lineage fidelity), in response to a local reduction in cell density or growth factor intervention, thereby losing some of it is differentiated properties and re-enter the cell cycle where it divides to give new progeny. When the correct cell density is achieved, cell proliferation ceases and differentiation is reinduced.

1.3 FAILURES IN NEOPLASIA

As already stated above, the crucial difference between neoplastic cells and normal cells is an apparent lack of normal differentiation due to a blockade in the normal pathway towards differentiation. What then, are the principle causes for such a failure in response mechanisms ? The differences between normal and transformed cells can usually be described in terms of the loss or repression of specific endogenous genes and the the incorrect expression of others. This results in an absence of differentiated cell products and the acquisition of properties commonly associated with tumour growth and spread.

There are probably four principle reasons for the failure of neoplastic cells to proceed towards terminal differentiation. (1) genomic alterations, (2) overexpression or amplification of normally "silent" oncogenes, (3) loss or modification of a receptor or associated signal transduction, and (4) alteration in the supporting matrix or stroma. Each of these points will be discussed in greater detail below.

1.3.1 Genomic alterations

Many agents have the potential to cause neoplastic change, and their probable mode of action is to cause DNA damage, resulting in chromosome aberrations, deletions or mutations. Carcinogenic

chemicals and irradiation have long been known to be mutagenic under certain conditions, and one long-standing theory is that cancer is caused by one or more genetic mutations. These can take two forms. Firstly, by incorrect interactions within the DNA. This can be caused by either a misrepair of damaged DNA, or base mispairing by direct covalent bonding of the carcinogen with with purine or pyrimidine bases in the DNA molecule. A second type of DNA damage is a frameshift mutation, whereby the addition and/or deletion of base pairs is such that the "reading frame" of the triplet base code is pushed out of synchrony.

Growth-suppressor genes have also been implicated in neoplastic transformation. Inheritance of mutated or deleted suppressor genes may confer susceptibility to cancer. Under normal circumstances these genes can suppress cell transformation, but if they undergo deletion or become functionally altered, a tumour can arise (Klein and Klein, 1985). The most thoroughly analysed example of such a gene is the Rb gene, the gene responsible for retinoblastoma, where both alleles are lost in retinoblastoma tumour cells (Weinberg, 1988).

1.3.2 Oncogenes

Other genomic alterations such as oncogene activation have also been implicated in the development of neoplasia. Many different types of human and animal tumours have been shown to contain

overexpressed or amplified oncogenes, and it has been suggested that these may be responsible for neoplastic transformation. Oncogenes are not unique to transformed cells, indeed they are present in a quiescent form in most normal cells where they are known as proto-oncogenes (Hunter, 1984). A study by Yokata et al (1986), using both normal and neoplastic human tissues, showed that oncogene activation appeared to correlate with tumour development. In the case of the H-ras oncogene, activation is associated with a single base mutation resulting in an altered and chronically active G-protein product (Reddy et al, 1982; Tabin et al, 1982).

Amplification of the cellular myc, myb or H-ras oncogenes were found in more than one third of human solid tumours studied. Apparent allelic deletions in H-ras and myb were correlated with tumour progression and metastasis, whilst amplification of myc occurred in advanced tumours or in aggresive primary tumours. Of more than seventy normal tissues studied, none expressed alterations in cellular oncogenes (Yokata et al, 1986).

Therefore cellular oncogenes appear to be critical elements in neoplastic transformation in mammalian cells, such that in a transformed cell, oncogenes encode proteins which function abnormally, inappropriately or at the wrong concentration. This results in a circumvention of the normal cell control mechanisms, which regulate cell division and differentiation.

1.3.3 Receptors and growth factors

Regulatory changes in either growth factor production or in receptor expression are also likely contributors for the onset of neoplasia. If there was an alteration in the cell surface receptor, it is not difficult to imagine the consequences of this, particularly if the receptor was for a growth regulatory factor. Over-expression of a PDGF-like peptide has been asociated with expression of the *sis* oncogene (Doolittle *et al*, 1983; Waterfield *et al*, 1983), and the *erb*-B oncogene codes for a truncated EGF-receptor which is constantly active (Downward *et al*, 1984). Neoplastic cells also tend to have a reduced capacity for growth regulatory factors which are essential for normal homeostatic control, often showing autocrine control of growth, and this can give them a growth supremacy over their normal counterparts (Moses and Robinson, 1982).

1.3.4 Alteration of the stroma

It is well known that tumour cells secrete mitogenic peptides, of which one of the actions is to induce a transformation-like reaction in fibroblasts (Todaro and De Larco, 1978). This may alter the capacity of the tumour-associated stroma to act as an epithelial inducer. Therefore by recombining tumour cells with foetal stroma from the correct site and the correct phenotypic status, the tumour cells may then receive the correct inductive

signal from the stroma, thus regaining their ability to respond. The significance and role of cell-cell and cell-stromal interactions is well established in the normal foetal development programme, and this concept will be discussed in greater detail below.

1.4 REVERSIBILITY OF NEOPLASTIC CHANGE

Malignant transformation gives rise to an altered pattern of differentiation, resulting in a clone of cells with unlimited proliferative capacities. However, a malignant tumour is not usually composed exclusively of undifferentiated stem cells, but can retain abortive representations of the mature stem cells or structures ordinarily found in non-neoplastic tissues (Reiss et al, 1986). These morphological characteristics suggest that under the correct conditions, at least some tumour cells still retain the ability to give rise to differentiated progeny in the tissue of origin.

The idea that a tumour is but a caricature of normal tissue has been illustrated well in studies by Pierce and Wallace (1971). Their model system was a squamous cell carcinoma, which contains squamous epithelial "pearls", the presence of which are regarded to be excellent replicas of squamous differentiation. When animals bearing such tumours were injected with ^{3}H-thymidine, there was no label in the pearls two hours after injection. However, the label

was found in many undifferentiated cells. By 96 hours, labelled cells began to appear in the pearls, supporting the conclusion that previously undifferentiated cells had migrated into the pearls. On autoradiographic examination under EM, it transpired that they had undergone differentiation into squamous epithelial cells. When the pearls were dissected out and transplanted into recipient mice, they never produced tumours, and under the conditions of the experiment, equivalent amounts of undifferentiated tissue produced tumours in at least one third of all cases (Pierce and Wallace, 1971).

This has been substantiated by Illmensee and Mintz (1975; 1976), who dramatically demonstrated that single murine embryonic carcinoma cells injected into early stage mouse embryos (ie blastocysts), gave rise to mature tissues which formed an integral part of normal chimeric mice which arose from the embryos.

A more recent study by Pierce et al (1987) followed the fate of ^{3}H-thymidine labelled embryonic carcinoma cells which were injected into mouse blastocysts. Autoradiography revealed that the cancer-derived cells had differentiated in accordance with their localisation.

Therefore, under the correct inducing conditions, it appears that neoplastic cells still retain the capacity to form differentiated cells.

1.5 DIFFERENTIATION AND ORGANOGENESIS

Cellular differentiation is a necessary component of organogenesis in developing organ systems. Heterologous cell interactions between cells of epithelial and mesenchymal origin play a central role in coordinating the differentiation of a large number of different organ systems, and it is well established that normal epithelial differentiation requires mesenchymal support during organogenesis (Auerbach and Grobstein, 1958; Cooper, 1965; Wessells, 1970). There are four main parameters which regulate differentiation:

1. matrix interactions
2. cell-cell interactions
3. polarity and cell shape
4. soluble factors (Freshney, 1985).

1.5.1 Matrix interactions

The differentiated state is maintained by the extracellular matrix (ECM), a dense supporting scaffold which separates tissue compartments, mediates cell attachment and influences tissue architecture (Kleinman et al, 1981). The ECM can be regarded as a macromolecular filter, which plays a role in differentiation and mitogenesis (Kleinman et al, 1981; Liotta, 1986). Malignant change can be perceived as a defect in differentiation, whereby the cells in some way escape from the normal matrix control. The ECM itself tends to become altered in neoplasia (Hynes, 1976) and this may

influence tumour differentiation, proliferation and invasion (Liotta et al, 1983; Liotta, 1986). The loss of appropriate cell-cell interactions, whether due to deletion of certain glycopeptides on the cell surface (Hynes, 1976; Sherbert et al, 1982), alteration in the sialation of cell surface proteins (Van Beck et al, 1973), or to the proteolytic activity of the tumour (Unkeless et al, 1974), or induced in the stroma by the tumour (Gross et al, 1983), may result in the tumour cell being deprived of the correct inductive signals from the stroma.

To maintain the differentiated state, there is a continuous feedback of information between interacting cells and the extracellular matrix (ECM), such that both matrix and transmembrane macromolecules (collagen, laminin, integrins, cadherins, proteoglycans) and soluble factors (growth and differentiating factors e.g. TGF-beta) can mediate these epithelial-mesenchymal interactions (Schor and Schor, 1987 - see also Figure 1).

Many investigators have shown that growth and differentiation of normal cells can be enhanced by culturing the cells on ECM coated material e.g. laminin is important for epithelial cell growth and differentiation (Kleinman et al, 1981), while the presence of fibronectin is necessary for the adhesion and spreading of fibroblasts (Thom et al, 1979). The ECM component, fibronectin, has been shown to be permissive for EGF initiated DNA synthesis in rat hepatocytes (Sawada et al, 1986), who suggested that ECM components could modify hepatocyte DNA synthesis by means of post-

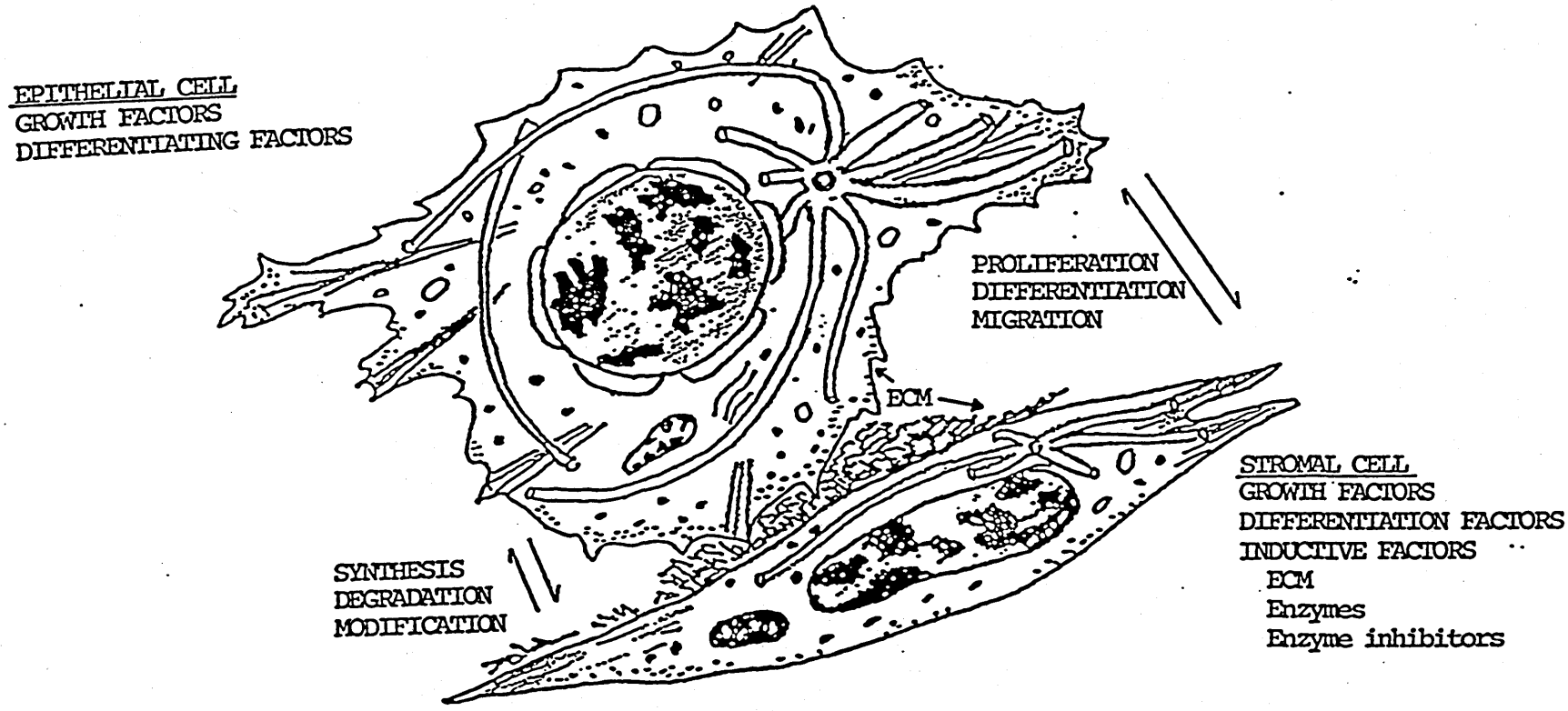


FIGURE 1

Schematic representation of stromal-epithelial interactions.

EGF-receptor mechanisms. Freshly isolated rat type II pneumocytes showed increased cell proliferation and differentiation when cultured on an ECM which was rich in fibronectin. This stimulated production of their own ECM, compared to the equivalent cells cultured on plastic (Rannels et al, 1987).

Studies by Bissell (1988) using mammary epithelial cells growing on ECM material have shown that the ECM can regulate protein synthesis and milk secretion. Both these observations are consistent with a model of "dynamic reciprocity", proposed by Bissell et al in 1982, whereby the ECM can influence gene expression via transmembrane proteins attached to the actin myofilaments, which have connections with the cell nucleus. Alterations in gene transcription can, in turn, influence the composition of the matrix. This effect has been verified and extended by several other groups. Blum and Wicha (1987) showed that the expression of the genes responsible for milk proteins required an intact cytoskeleton, since long-term treatment (24 hours) with cytochalasin B, a cytoskeletal inhibitor, inhibited the synthesis of milk proteins, while short term treatment (1 hour), enhanced the secretion of alpha-casein and transferrin.

Li et al (1987) emphasised the role of the ECM in receiving and integrating structural and functional signals, since cultures of mammary epithelium growing on reconstituted Engelbreth-Holm-Swarm (EHS) tumour matrix (which is mainly laminin), permitted more than 90% of the cells to produce beta-casein, as well as stimulating duct formation. Components of the ECM, glycosaminoglycans and

proteoglycans, have been shown to be important regulators in the synthesis and stabilty of tissue specific mRNA's and in the expression of gap junction proteins in cultures of normal hepatocytes (Fujita et al, 1987).

However, it is not clear to what extent transformed cells require or respond to the ECM, since they frequently show reduced adhesion to substrates in vitro and are more likely to have anchorage independent growth (Macpherson and Montaigner, 1964). There are several possibilities why transformed cells tend to lack the requirement for the ECM. It may be that they lack the appropriate cell surface receptor to bind to the ECM, or the production of proteolytic enzymes by tumour cells may simply digest away the ECM. Also, a neoplastic cell produces its own autocrine growth factors, which may override signals from the ECM, causing a reduced dependence on the ECM. Neoplastic cells, therefore, tend to be less dependent on the ECM for growth. Nevertheless, a recent study by Daneker et al (1989), using a panel of colon carcinoma cell lines, has demonstrated that the ability of these cells to interact with the ECM depended on the degree of differentiation of the cells, with poorly differentiated cells generally adhering and spreading significantly better than moderately well differentiated lines, further supporting the role of the ECM in structuring development.

1.5.2 Cell-cell communication

Another prerequisite for cellular differentiation is cell-cell communication. Cells can communicate in 3 ways. Firstly, they can share the products of metabolism by means of gap junctions. Gap junctions mediate communication between cells by allowing inorganic ions and other water soluble molecules to pass directly from the cytoplasm of one cell type to the other, thereby coupling the cells both metabolically and electrically. This usually occurs between like cells, allowing an even distribution of second messengers e.g. cAMP, although it can occur, albeit less frequently, between disparate cells e.g. across basement membranes (Finbow and Pitts, 1981; Pitts et al, 1988).

Secondly, and especially during organogenesis, there may be a contact interaction between opposed cell surfaces. This may involve interactions between cell surface molecules or the ECM, and may be influenced by hormones, particularily hydrocortisone (Simon-Assmann et al, 1988).

The third form of cellular communication may involve transfer of a diffusible factor between interacting cells in a paracrine fashion. This may be dependent on the ECM, since in the stromal induction of haematopoietic cells, the matrix has been shown to bind growth factors (Roberts et al, 1988).

A further regulatory aspect of cell differentiation to be considered is cell-cell interactions between heterotypic cell populations, which are necessary for organogenesis in developing organisms. The concept of organogenesis has been discussed previously. To what extent these interactions persist after embryonic development is not clear, but in the skin at least, it has been suggested that dermal fibroblasts are necessary for complete maturation of keratinised squames and cornified envelope formation (Fusenig, 1986;1989).

The biochemical identity of the signalling molecules which are believed to mediate epithelial-mesenchymal interactions have been the subject of intensive investigation, since the initial speculation by Grobstein in 1975 that matrix macromolecules may be involved. Adult bladder epithelium was induced to differentiate into glandular epithelium after recombination with urogenital sinus mesenchyme (Cunha et al, 1983), suggesting these cells could retain a certain responsiveness to embryonic mesenchymal induction. A similar study by Neubauer et al, (1983) showed that epithelial cytodifferentiation was associated with expression of prostate specific antigens and androgen receptors, suggesting that the inductive influence of the urogenital sinus mesenchyme had reprogrammed both the morphological and functional characteristics of the epithelium. This event appears to be tissue specific, since only urogenital sinus mesenchyme stimulates prostatic bud formation, with heterotypic mesenchyme failing to do so, and hormone dependent, as pretreatment of the mesenchyme with androgens

enhanced prostatic bud formation (Laznitski and Mizuno, 1979). In the same way, epithelial cells from BALB/c testicular feminisation mutant (tfm) mice, which lack nuclear androgen-binding sites, when combined with wild-type stromal cells (which are androgen-receptor positive), results in prostatic development, suggesting that stromal cells have a direct role in androgen-mediated differentiation in the prostate (Cunha, 1984). Normal epithelia, however, do not respond to tfm stroma.

The loss of appropriate cell-cell interactions, whether due to a deletion of a glycopeptide on the cell surface (Hynes, 1976; Vaheri and Mosher, 1978; Sherbert et al, 1982), or induced by the tumour stroma (Gross et al, 1983) may result in tumours receiving the wrong inductive signals from the stroma. Eventually this breakdown in communication could lead to a loss of differentiated function and ultimately neoplasia.

1.5.3 Polarity

In epithelial cells, the expression of complete differentiation requires the development of polarity within the cell (Sattler et al, 1978; Chambard et al, 1982). Polarity involves correct cellular orientation in space. Chambard et al (1981) have shown the importance of collagen gels on the orientation of polarity of epithelial thyroid cells in culture. Cells cultured on the surface of the gel formed a monolayer with the apical pole in contact with

the culture medium and the basal membrane attached to the the substratum. Cells embedded within the gel matrix organised within 8 days into follicles, with the basal pole in contact with the collagen and the apical pole orientated towards the interior of the follicular lumen. The same group in 1983, this time using thyroid cells growing in a filter well assembly, showed the basal surface generated receptors for thyroid stimulating hormone and secreted triiodotyrosine, while the apical surface released thyroglobulin. From these observations, it seems likely that a cell which is correctly polarised will be capable of regulating gene expression. This, in fact, has been demonstrated by Ben-Ze'ev et al (1988), who showed that hepatocytes cultured on dried rat tail collagen had a flattened morphology and expressed high levels of cytoskeletal mRNA's but low levels of liver specific mRNA's. Conversely, cells growing on a hydrated gel matrix formed spherical aggregates with a low cytoskeletal mRNA and a high liver specific mRNA, thus conforming more closely to the gene expression seen in the whole animal. The relevance of polarity to epithelium differentiation in lung will be discussed in a later chapter.

1.5.4 Soluble factors

Fibroblasts are the principal cell type in the mesenchymal matrices. Despite their widespread distribution and relative ease of culture in vitro, they still remain a poorly defined group of cells, commonly identified on the basis of such non-specific

attributes as a spindle shaped morphology, the presence of vimentin intermediate filaments and the synthesis of interstitial collagens and fibronectin. Nevertheless, they have been shown to be responsible for the synthesis and secretion of a number of soluble factors which influence neighbouring epithelial sheets in a paracrine fashion (Stoker and Pennyman, 1986), notably PDGF or a PDGF-like molecule (Bowen-Pope et al, 1984; Nimian et al, 1984).

The role of soluble factors as differentiating agents will be discussed below.

1.6 COMMONLY USED DIFFERENTIATING AGENTS

Over the past several years, cells, both normal and transformed have been shown to be capable of undergoing differentiation in response to many phenotypic inducers. Commonly used inducers can be divided into 2 main categories:

1. Non-physiological inducers

2. Physiological inducers

1.6.1 Non-physiological inducers

Non-physiological inducers such as planar polar solvents e.g. dimethylsulphoxide (DMSO) and N-methlyformamide (NMF) have been

used for a number of years, and have proved effective differentiating agents for many cell types, both normal and transformed (Spremulli and Dexter, 1986; Reiss et al, 1986; Marks et al, 1987). DMSO has been reported to stimulate differentiation in a human amelanotic melanoma, measured by a decrease in proliferation and an increase in melanisation (Siracky et al, 1985). Dexter and colleagues (1982), showed a growth inhibition in 2 human colon cancer xenografts following treatment with dimethylformamide (DMF) and its analogue NMF. It has been suggested that in the induction of differentiation by polar solvents in the HL-60 leukaemic cell line, the potency of a potential differentiating agent can be predicted on the basis of its molecular size (Langdon and Hickman, 1987).

Other non-physiological inducers which have been used successfully to induce differentiation include sodium butyrate, a 4 carbon fatty acid (Reiss et al, 1986). Sodium butyrate has been shown to induce morphological cell polarity in clones of the colonic cell line HT-29, as well as the formation of domes and the secretion of mucus (Augeron and Laboisse, 1984). A study by Chung et al (1985), using colorectal cell lines, showed that sodium butyrate was capable of increasing the amount of brush border membrane associated enzymes, particularily alkaline phophatase, which is a marker of differentiation in colonic cells.

1.6.2 Physiological inducers

Physiological inducers include substances such as growth regulatory factors and hormones. Haematopoietic regulatory proteins (cytokines) e.g. G-CSF and GM-CSF have been found to enhance proliferation and differentiation of granulocyte-macrophage precursors and induce differentiation in cultured leukaemic cells (Reiss et al, 1986; Begley et al, 1987). Staying with the haematopoietic system, interferon,(IFN), hailed as the cancer wonder drug in the early eighties, has proved to be a useful differentiating agent for some leukaemias, particularily the rare hairy cell leukaemia, where alpha-IFN is highly effective (Quesada et al, 1984; Sigaux et al, 1987). Similarily, tumour necrosis factor (TNF) is also effective towards leukaemic cells (Sugarman et al, 1985). In the same way, Geissler et al (1989) have recently demonstrated that cytokines such as recombinant human TNF-alpha and gamma-IFN have can induce differentiation in vitro in blast cells derived from patients suffering from acute myeloid leukaemia.

Retinoic acid, a vitamin A analogue (Lotan, 1980; Bloch, 1984) is another physiological inducer, although it has been used in tumour studies at non-physiological concentrations. Retinoids, especially retinol acetate, have been shown to reduce the growth and invasive capacity of A549 cells, accompanied by a significant decrease in type IV collagenase activity (Fazely et al, 1988). Similarily, retinoids can induce transglutaminase expression in HL-60 cells, an enzyme associated with the induction of a group of retinoid

regulated genes which are linked to cellular differentiation (Davies et al, 1988).

Hormones, particularily steroids, have been reported to be differentiation inducers in a number of cells. The glucocorticoid, dexamethasone, has been particularily well studied, and has been found to inhibit both cell growth and DNA synthesis in glioma (Guner et al, 1977; Mackie et al, 1988), lung carcinoma cell lines, (Jones et al, 1978; McLean et al, 1986) and in breast (Osborne et al, 1979). In the same way, a rat fibrosarcoma cell line cultured in the presence of glucocorticoids became flattened in shape, lost bipolarity, extended in size and showed arrest of growth at confluence (Steffen et al, 1988). Other effects of glucocorticoids will be discussed more fully in a later chapter.

Whilst the mode of action of many of the non-physiological inducers is unclear, the action of steroid hormones is fairly well documented. Steroids diffuse passively through the plasma membrane and translocate to the nucleus. There they bind to empty receptors where interaction with the chromatin is mediated by a nuclear receptor complex (Alberts et al, 1989).

1.7 MODELS FOR DIFFERENTIATION

Haemopoietic tumours have proved good models for the induction of differentiation by pharmacological and biological agents for two

reasons. First of all, they are comparatively easy cells to culture and secondly (and most importantly), their differentiation pathways are fairly well characterised, making it easy to see at which stage of the differentiation pathway the blockage is occuring. For example, tumours such as the murine Friend erythroleukaemic cell line has been shown to undergo terminal differentiation in response to DMSO (Friend et al, 1971), and the human promyelocytic cell line, HL-60, has been reported to differentiate terminally in response to agents such as purine and pyrimidine analogues (Bodner et al, 1981) and polar organic solvents such as NMF (Langdon and Hickman, 1987).

1.8 NORMAL LUNG DEVELOPMENT

1.8.1 Epithelial-mesenchymal interactions

As with many developing organs, the appearance of structure (organogenesis) in the embryonic lung is strongly dependent on bi-directional interactions between epithelium and mesenchyme (Taderera, 1967; Smith and Fletcher, 1979). The initial lung bud develops from the ventral surface of the endodermal foregut and gives rise to the epithelium of the respiratory tract. As it grows and branches repeatedly, it is surrounded by a loose mesenchyme, which will ultimately give rise to the pulmonary interstitium, smooth muscle, cartilage and endothelium. The branching pattern of the endoderm is strongly influenced by the surrounding mesenchyme,

since organ explants of the lung bud (endoderm and mesoderm) continue to branch *in vitro*, but if the mesenchyme is removed, branching ceases (Wessels, 1970). This effect on the mesenchyme is organ specific and also region specific as bronchial mesenchyme will induce the lateral tracheal epithelium, which does not normally bud, to do so, and, conversely, paratracheal mesenchyme will inhibit budding of the more peripheral airway epithelium (Wessels, 1970). Thus the mesenchyme is responsible for conferring specificity.

1.8.2 Cytodifferentiation of type II cells

Stages of human foetal lung development are divided into three main stages:

1. glandular (5-16 weeks)
2. canalicular (17-24 weeks)
3. saccular (25 weeks onwards)

During the glandular phase of embryonic lung development, the walls of the lung primordium are lined with undifferentiated columnar epithelial cells. In late glandular and early canalicular phase, these undifferentiated cells start to differentiate into prospective respiratory (alveolar) epithelium (Cutz *et al*, 1982). This begins with the formation of acinar tubules, which are lined with cuboidal epithelium, composed of cells expressing phenotypic

features of type II cells (Post and van Golde, 1988). The alveolar respiratory system develops further by budding of the acinar tubules, which leads to the development of primitive saccules (alveoli) (Cutz et al, 1982). This results in a rapid proliferation of the type II cells, with an increase in size and number of cytoplasmic multilamellar bodies, the intracellular storage sites for pulmonary surfactant (Post and van Golde, 1988). The role of pulmonary surfactant will be discussed in greater detail in Chapter 3.

1.9 LUNG CANCER

Lung cancer is one of the leading causes of death from malignant disease and it's incidence is increasing alarmingly throughout the world. The main causative agent for lung cancer is inhaled tobacco smoke, and, not surprisingly, cigarette smokers show at least a 25-fold increase in the incidence of lung cancer. Although a strong positive correlation between inhaled tobacco smoke and the risk of developing lung cancer has been established, other carcinogenic substances that cause lung cancer have also been identified. These include atmospheric pollution, chemical products and industrial hazards such as asbestos.

Lung cancer can be divided into 2 main categories. Non small cell lung cancer (NSCLC) accounts for approximately 75% of all lung carcinomas, with small cell lung cancer (SCLC) making up the

remaining 25%. However, these 2 types differ in many respects and this is summarised in Table 1.

TABLE 1 PROPERTIES OF LUNG CANCER CELL LINES

PROPERTY	NSCLC	SCLC
MORPHOLOGY	Epithelioid	Floating aggregates
SUBSTRATE ADHESION	Present	Absent
POPULATION DOUBLING	Short	Long
CLONING EFFICIENCY	High	Low
*APUD CELL PROPERTIES	Absent	Present
POLYPEPTIDE HORMONE SECRETION	Occasional	Frequent
CREATINE KINASE LEVELS (BB ISOSYME)	Low	High

*APUD (amine precursors uptake and decarboxylation) cell properties refer to neuroendocrine properties associated with SCLC cell lines and include secretion of 4 biochemical markers; L-DOPA decarboxylase (DDC), bombesin, neurone specific enolase and the BB enzyme of creatine kinase, CK-BB (Pearse, 1968).

Previously, it was thought that SCLC and NSCLC were of different origins, the cell of origin in SCLC being the Kulchitsky neuroendocrine cell, while the more heterogeneous NSCLC had epithelial origins. However, it has recently been proposed that a common stem cell may exist for all types of lung cancer (Carney, 1987). Mixed NSCLC/SCLC tumours occur in up to 40% of patients with SCLC at autopsy, suggesting that one cell type e.g. SCLC may

undergo differentiation to another e.g. adenocarcinoma (Carney, 1987).

NSCLC represents a heterogeneous population of tumours with distinct but overlapping histologic appearances, clinical course and biological behaviour. For this reason, NSCLC is further subdivided into:

(a) squamous carcinoma	35%
(b) adenocarcinoma/alveolar carcinoma	25%
(c) large cell carcinoma	15%

Unlike SCLC, which is generally responsive to chemotherapy on initial presentation (although it frequently relapses and becomes more resistant), NSCLC is normally resistant from the outset, and, other than by surgical excision, is very difficult to treat successfully.

There is, therefore,a pressing need for new regimes of therapy in the treatment of solid tumours, since these tumours are generally evasive to conventional cytotoxic techniques, if not from the outset, then after acquiring drug resistance.

1.10 RATIONALE FOR THE PRESENT WORK

The rationale behind this study was based on observations made by Smith in 1979. Smith was able to demonstrate that foetal lung fibroblasts could produce, in response to glucocorticoids, a factor, fibroblast pneumocyte factor (FPF), which accelerated lung maturation in foetal rats. This was measured by the phospholipid profiles in treated and untreated animals. When foetal rats were injected on day 17 of gestation with 1ug of FPF, there was evidence, on day 20, of accelerated lung maturation. A later study (Smith and Sabry, 1983) looked at the effects of cortisol and triiodothyronine (T3), on 20 day foetal rat lung cultures. They proposed that glucocorticoid was inducing FPF production in fibroblasts which in turn induced cAMP (a second messenger) in epithelia, resulting in enhanced production of saturated phoshatidylcholine. The glucocorticoid and T3 were having a synergistic effect on the expression of differentiated epithelial functions. In cultured type II cells, FPF was shown to stimulate the production of surfactant lipids in foetal rat lung (Post and Smith, 1984).

The essential role of FPF on pulmonary surfactant production by alveolar type II cells was demonstrated by Post et al (1984), who developed a monoclonal antibody against FPF which blocked the stimulatory effect of FPF in type II cells. Similarily, when embryonic chicks were injected on day 15 of incubation with the monoclonal, they showed biochemical evidence of delayed lung

maturation, as compared to controls. This confirmed that in normal lung development, the production of a fully mature type II cell is mediated by glucocorticoids which act on fibroblasts rather than on lung epithelium directly, causing the production of FPF, which in turn stimulates pulmonary surfactant production, an indicator of lung maturity.

If this is the case in normal lung development, can such a system be applied to neoplastic cells? After all, tumours often assume a quasi-foetal phenotype, expressing foetal markers such as carcinoembryonic antigen, and might be expected to re-express differentiated functions if given the correct embryonic physiological stimulation(s).

Fibroblasts have been implicated previously in the control of cell proliferation and differentiation of epithelial tumours. Human fibroblasts have been shown to cause degenerative changes in KB carcinoma cells, and this effect was enhanced by interferon (Imanishi et al, 1983). This event was believed to be mediated by factors produced by the fibroblasts, because the supernatant from fibroblast cultures caused the same degenerative changes in KB cells as the coculture with fibroblasts did. Another study has shown that medium conditioned by normal human fibroblasts could cause growth inhibition and stimulate differentiation in human salivary adenocarcinoma cells (Shirasuna et al, 1988). Again this was mediated by soluble factors. The role of fibroblasts in epithelial differentiation will be discussed in greater detail in

Chapter 4, but it is clear that their involvement in epithelial differentiation extends to malignant epithelium.

As discussed previously above, normal epithelial differentiation requires the support of the mesenchyme during organogenesis (Auerbach and Grobstein, 1958; Taderera, 1967). The importance of the mesodermally derived component of prostate and lung has been demonstrated by Cunha et al (1983), and Post et al (1984), and it has also been shown that interaction with the correct temporal phase at the correct site can inhibit the tumourigenic potential of melanoma and neuroblastoma (Podesta et al, 1984). By recreating the correct cellular environment, it may be possible to to increease the differentiation and repress malignancy of lung carcinoma.

1.11 MODEL SYSTEM

The present study examines the importance of fibroblasts for the in vitro and in vivo differentiation of alveolar carcinoma cells, A549, and their response to glucocorticoids.

Reiss et al (1986) outlined 3 optimum criteria which should be considered when devising experimental models to test the potential of differentiation inducing agents. Firstly, mature cells should be able to be distinguished from the tumour cell phenotype by biochemical and morphological markers. With many epithelial cells, this is often a problem, since they do not possess well

characterised differentiation markers, whilst in others, they are easily measured, e.g. melanin production in melanocytes and melanoma. While the precise differentiation pathway is not well established, in the cell line selected for the bulk of this study, A549, biochemical and morphological markers do exist. These include synthesis and secretion of pulmonary surfactant and the presence of multilamellar bodies in the cell cytoplasm (Lieber et al, 1976; Smith, 1977; Shapiro et al, 1978, McLean et al, 1986). Secondly, the cells should have the potential to be cloned in vitro and their tumourigenic capacity should be able to be measured in vivo. These cells have a good plating efficiency in vitro, and grow well as xenografts. Finally, every in vitro model should have an in vivo counterpart with measurable parameters of maturation. A549, although originally derived from a human tumour, may be successfully grown as xenografts in nude mice, allowing such determinations to be made.

CHAPTER TWO

MATERIALS AND METHODS

This chapter will deal with the main methodology employed in this thesis. Specific details will be dealt with separately within the relevant Chapters.

2.1 GENERAL CELL CULTURE METHODS

2.1.1 Maintenance of cell lines

The origins and characteristics of the principle cell lines used is shown in Table 2.

All cell lines used grew as monolayers and were maintained in either Nunclon 80cm^2 or Falcon 75cm^2 flasks in a 1:1 mixture of Hams F10: DMEM (NBL;Flow) containing 2mM glutamine (Gibco) and 0-10% foetal calf serum (FCS) as indicated in the text. Sodium bicarbonate (Gibco) was added in equilibrium with 2% CO_2 or 5% CO_2 giving 8mM and 23mM respectively. Cells were passaged weekly, after trypsinisation, with a seeding concentration of 10^3 or 10^4 cells/ml. Briefly, the medium was aspirated and replaced with phosphate buffered saline (PBS) containing 1mM EDTA for 30 seconds. This was removed and replaced with 0.25% trypsin (Gibco) for a further 30 seconds. Following removal of trypsin, the monolayer was incubated at 37^{0}C for 10-15 minutes until the cells had detached, and then dispersed into a single cell suspension.

Medium used for most of the experiments described in Chapter Four contained a mixture of penicillin/streptomycin at 100 units/ml. Otherwise, all cultures were maintained free of antibiotics.

TABLE 2: ORIGINS AND CHARACTERISTICS OF THE MAIN CELL LINES USED

CELL LINE	SOURCE	ORIGIN	CELL TYPE	PATHOLOGY
A549[a]	ATTC, Maryland	Human lung	Type II alveolar, epithelial	Alveolar carcinoma
MOG-LF113	Medical Oncology Glasgow	1st trimester human foetal lung	Fibroblast	Normal
WIL	Haddow Labs. Sutton	Human lung	Epithelial	Adenocarcinoma
A2780[b]	NCI Bethesda	Human ovary	Epithelial	Adenocarcinoma
MOG-LT-34	Medical Oncology Glasgow	Human lung	Epithelial	Adenocarcinoma
NCI-H69V	NCI Betheseda	Human lung	Small cell lung carcinoma	Derivative of NCI H69

[a]Giard et al, 1972; Lieber et al, 1976

[b]Tsuruo et al, 1986; Van der Blick et al, 1988

2.1.2 Cell freezing

To reduce the chances of genetic drift, all cell lines were replaced from frozen stocks every 3 months. Frozen stocks were maintained in liquid nitrogen. Cells for freezing were trypsinised and suspended in culture medium at a concentration of at least 10^6/ml, and 10% DMSO (BDH) was added as a preservative and the suspension aliquotted into 1ml vials (Nunclon). They were frozen with a cooling rate of approximately 1^0C/minute to -70^0C and retained at -70^0C overnight before being transferred to liquid nitrogen.

Cells were thawed by rapid warming of the vial in a covered container with water at around 35^0C, and then transferred to sterile 25cm^2 flasks (Nunclon), and diluted 1:20 by the slow addition of fresh culture medium.

2.1.3 Mycoplasma testing

Cultures were checked for the presence of mycoplasma every month. Cells were fixed with 25% glacial acetic acid in methanol and incubated with the fluorescent DNA stain Hoescht 33258 for 15 minutes at room temperature (Chen, 1977). Cultures which contained extranuclear DNA in a characteristic punctate or filamentous pattern were indicative of mycoplasma infection. All experiments were carried out on cells which were free of mycoplasma.

2.1.4 Aseptic technique

All aseptic manipulations were carried out in a class II microbiological safety cabinet with vertical air flow. All glassware was dry heat sterilised at 160^{0}C for a minimum of one hour and heat stable solutions autoclaved prior to use. Heat labile solutions were either purchased sterile or filtered through 0.2um sterile filters (Millipore).

2.1.5 Cell counting

Cell counts were performed using a Coulter counter, model ZB_1 (Coulter Electronics, Luton, Beds.) previously calibrated for these cell lines. The cell suspension was diluted 1:50 in PBS and 0.5ml of the diluted suspension drawn through the orifice tube (100um) on the counter. As the cells pass through the orifice, they change the resistance to the electrical current flowing through the orifice by an amount proportional to cell volume. This generates a pulse which is amplified and counted. The final cell count on the readout was multiplied by 100 to give the cells/ml in the original suspension.

2.1.6 Growth curves

Growth curves were performed using A549 and LF113 and terminal cell densities and population doubling times determined from the data. Cells were seeded at a concentration of 10^4 cells/ml ($2x10^3$ cells/cm^2) for A549 and $5x10^4$/ml ($1x10^4$/cm^2) for LF113. The cells were allowed to attach overnight, then the medium was removed and replaced with fresh culture medium with or without the particular variable under study. Triplicate samples were counted every 2 days, and the cells fed as necessary depending on the pH of the spent medium.

2.2 PREPARATION OF FOETAL MOUSE LUNG

(Adapted from Douglas and Teel, 1976)

19 day mouse foetuses were aseptically removed from timed-pregnant AKR females, freed of attending membranes and decapitated. The lungs were dissected out under aseptic conditions and washed in chilled Hams F10 culture medium, then chopped into $1mm^3$ pieces with crossed scalpel blades. The pieces were transferred to a universal container (Sterilin) which contained 10ml of 0.25% trypsin, and left overnight at room temperature to allow the cells to dissociate. The trypsin was aspirated and 15ml of F10 medium added to the cells, which were then filtered through nylon bolting cloth to remove cell clumps. The cell suspension was centrifuged at 260 x g for 5 minutes then the pellet was resuspended in Hams F10:DMEM

containing 10% FCS. After another centrifugation step, the pellet was incubated with the supernatant for one hour at 37^0C, since it is believed this allows synthesis of matrix macromolecules which are essential for cell reaggregation (Douglas and Teel, 1976). The pellet was resuspended in fresh F10:DMEM containing 10% FCS, 100 units/ml of penicillin and 100ug/ml of kanamycin, and 1ml aliquots were transferred to 25cm^2 flasks. The number of viable cells, estimated by trypan blue exclusion was always greater than 95%, and the cell concentration always exceeded 10^6 cells/ml

2.3 CHROMOSOME ANALYSIS

Cells in exponential growth were incubated in the presence of colcemid for 4 hours at 37^0C to arrest dividing cells in metaphase. Following incubation, the medium was removed and replaced with 5ml of hypotonic buffer (0.04M KCl/0.025M NaCitrate). After 20 minutes at 37^0C, the metaphase cells were collected by gently pipetting the buffer over the monolayer. The metaphase cells were then transferred to plastic test tubes (Sterilin) and 5ml of ice cold, freshly prepared acetic acid/ethanol fixative (1 part glacial acetic acid/3 parts ethanol) was added dropwise with constant mixing. The tubes were centrifuged at 2000g for 2 minutes at 4^0C (IEC-centra), and the supernatants discarded. The fixing process was repeated twice more. The final pellets were resuspended in approximately 100ul of fixative and a few ul dropped onto a glass slide. The preparations were air dried and stained with Giemsa

(BDH). Chromosomes were counted by eye or photographed under oil immersion using a Polyvar microscope equipped with an automatic camera.

2.4 PROTEIN DETERMINATION

All protein measurements were carried out using the Bradford protein assay (BioRad), which is based on the differential colour change of a dye, Coomassie Blue, in acidic solution, in response to various concentrations of protein (Bradford, 1976). When binding to the dye occurs, the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465-595nm. Depending on the amounts of protein present, the assay was performed using either standard assay or microassay conditions. Protein was solubilised in 100mM NaOH. When using standard assay conditions, (20-140ug/ml of protein), 100ul aliquots were dispensed into test tubes and 5ml of Bradford reagent, diluted 1:4 with distilled water was added and the tubes vortexed. Colour was allowed to develop for up to 1 hour. Absorbance was measured on a Gilford 250 spectrophotometer at 595nm.

For the microassay, (less than 20ug of protein), 200ul aliquots of solubilised protein were mixed with 50ul of undiluted Bradford reagent in microtitre plates (Flow Laboratories) and the plates read on a microtitration plate reader 600nm (BioRad ELISA, model 2550).

Using bovine serum albumin (BSA) standards (Sigma) of known concentrations (0-1000ug/ml), a standard curve was performed each time, and from this the protein content of the unknown solution was determined.

2.5 MEASUREMENT OF PULMONARY SURFACTANT

2.5.1 Secreted pulmonary surfactant

Pulmonary surfactant was measured using a modification of the technique of Smith (1977). Cells were plated out in either $25cm^2$ flasks or in filter wells at a cell density of 5×10^4 per ml (flasks) or 8×10^4 per ml (filter wells), and allowed to achieve confluence. At confluence, the medium was aspirated and the cells washed twice with serum free medium, then incubated for the appropriate times with the compound under investigation. The cells were labelled with 0.1uCi/ml (76Ci/mmol) of [methyl-^{3}H] choline chloride (Amersham) for 24 hours. After the labelling period, the medium was removed and the cells washed 3 times with serum free medium. A 3ml portion of serum free medium containing 1.0mM isoproterenol (Sigma) was added to the cells plus 20ul of L-3 phosphatidylcholine, 1,2-di[1-^{14}C] palmitoyl (117mCi/mmol; approximately 2000cpm; Amersham) for 15 minutes at room temperature. The ^{14}C-DPPC acts as an internal standard for calculating subsequent recoveries. This medium was collected and mixed with 9ml of a 2:1 mixture of chloroform:methanol (BDH) and

1.0mg of DL-alpha-phosphatidylcholine dipalmitoyl (Sigma) and the sample vortexed. The two resulting layers were allowed to partition, and the top aqueous phase removed by suction. The organic phase was dried under a stream of nitrogen or in a vortex evaporator (Buchler), then reacted with 0.5ml of a 6.2mg/ml solution of osmium tetroxide (Sigma) for 15 minutes at room temperature. The tubes were allowed to stand at room temperature for a further 30 minutes to ensure the reaction had reached completion, then the samples evaporated as before.

The remaining cell monolayer was dissolved in 100mM NaOH and assayed for protein content (see section 2.4).

2.5.2 Cell associated pulmonary surfactant

Cell associated surfactant was measured as follows. Cells were incubated with or without 0.25uM dexamethasone for 48 hours then labelled with 0.1uCi/ml ^{3}H-methyl choline chloride (Amersham; see 2.5.1) for 24 hours. Following the labelling period, the medium was removed and the cells fixed with 5ml of methanol (BDH). These cells were scraped from the flasks and transferred to glass tubes and the lipids extracted with 5ml of 100mM KCl (BDH) and 10ml of chloroform (BDH). 1.0mg of dipalmitoylphosphatidylcholine, a "cold" carrier was added, along with approximately 2000cpm of ^{14}C-dipalmitoylphosphatidylcholine, an internal standard for calculating recoveries. The tubes were vortexed and the aqueous and organic layers allowed to separate. After removing the top aqueous

phase, the lower organic phase was dried under nitrogen, or in a vortex evaporator. The remaining protocol was identical to that described in section 2.5.1.

In both cases, disaturated phosphatidylcholine (DPPC) was purified by column chromatography according to the method of Mason et al (1976). Columns were prepared by plugging a 145mm glass Pasteur pipette with 0.08g of glass wool, then layering 0.8g of neutral alumina on top (BioRad). Samples were redissolved in 0.5ml of a 20:1 (v/v) mixture of chloroform:methanol and applied to the columns (which were pre-equilibrated with chloroform:methanol). To elute neutral lipids, 15ml of the 20:1 chloroform:methanol mixture was run through. The bound DPPC, which is acidic, was eluted with 8ml of a 70:30:2 (v/v) solution of chloroform:methanol:7M ammonia. Eluted DPPC was collected in scintillation vials and evaporated to dryness, then 10ml of scintillant (Ecoscint) added.

Samples were counted on a double channel scintillation counter (Beckman or Packard), and the results calculated by the channels ratio method using the following formula.

1. channel 1 = ^{3}H

   channel 2 = $^{3}H + ^{14}C$

   ^{14}C = channel 2 - channel 1

2. % spillover from ^{14}C into ^{3}H = channel 2 - channel 1/channel 2 = X%

3. Total ^{3}H count (cpm):

   (channel 2 - channel 1) x X = Y

   Total ^{3}H count = channel 2 - Y

4. To correct for recovery,

   cpm channel 2 - cpm channel 1/cpm ^{14}C added x 100 = Z = % recovery ^{14}C

5. ^{3}H-choline (surfactant),

   Total ^{3}H cpm x 1/Z x 100 = R (surfactant)

6. Expressed as pulmonary surfactant/mg of protein or pulmonary surfactant/10^{6} cells.

2.6 PREPARATION OF CONDITIONED MEDIUM

Bulk quantities of conditioned medium were prepared by Dr Gierish Shah, Glaxo Group Research, Greenford, Middx. according to the following protocol.

Foetal lung fibroblasts were grown to confluence in either 175cm^{2} flasks (Nunclon) or 850cm^{2} roller bottles (Corning). The medium was aspirated and the cell monolayers washed twice with serum free culture medium, then incubated with serum free medium containing 25uM dexamethasone (dexamethasone sodium phosphate; Merck Sharp and Dohme) for a further 24 hours. At the end of the incubation period, the cell monolayers were washed twice with serum free medium, then

incubated with serum free medium for 6 hours at 37^{0}C. The medium was collected, centrifuged at 1000g to remove cell debris, and either used immediately or stored at -70^{0}C until required.

2.7 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF CONDITIONED MEDIUM TO DETECT DEXAMETHASONE

HPLC analysis of both conditioned medium and extracted LF113 cells was performed, using a modification of the technique of Derendorf et al (1986), to measure dexamethasone levels.

Samples of conditioned medium were collected six hours after dexamethasone removal. In addition, intact cells were scraped from the flasks at various predetermined time points and resuspended in a small volume of PBS (1-2ml). To release cell contents, the cells were disrupted using a blender (Ultra Turrax) for 2 minutes. Both conditioned medium and cell extracts were mixed with 30ul of internal standard (20mg/l 6-alpha-methylprednisolone (Sigma) in methanol (BDH)) and extracted twice at pH 1.0 by shaking for 15 minutes with 3ml portions of ethyl acetate (James Burrough), then centrifuged at 5000rpm for 5 minutes (Jouan). The samples were dried in a vortex evaporator (Buchler), then resuspended in 50ul of mobile phase. The mobile phase consisted of acetonitrile-0.05M phosphate buffer, pH 2.0, 30:70 v/v, both HPLC grade. For the assay, the following instruments were used: a high pressure pump (Altex, model 110A) equipped with a "Promis" autosampler with a variable injection volume (Milton Roy), a 10um u-Bondapak C18 column 24cmx4mm internal diameter (Phase Sep) and a U.V. detector

(Altex Hitachi) set at 230nm. All integration was performed on a JCL 6000 chromatography data system (Jones Chromatography). The flow rate was 2ml/minute and the injection volume was 30ul.

2.8 PROTEIN PURIFICATION FROM CONDITIONED MEDIUM

2.8.1 Ammonium sulphate precipitation

Aliquots of conditioned medium were cooled to 4^0C on ice and 20% of $(NH_4)_2SO_4$ (w/v) added. The medium was left for 30 minutes on ice with constant stirring. Following incubation, the medium was centrifuged in a Sorvall for 20 minutes at 10 000 x g. The supernatant was retained and precipitated to 40% with $(NH_4)_2SO_4$ as above. Further fractions were collected at 60 and 80% (w/v) using the same protocol. The precipitates were resuspended in a small volume of ice-cold PBS (no more than 2ml) and dialysed extensively overnight in Sigma dialysis tubing against PBS at 4^0C. The dialysed samples were either used immediately or stored at -70^0C until required.

2.8.2 Isoelectric focusing (IEF)

IEF is a relatively gentle non-denaturing and high resolution technique whereby mixtures of proteins in solution can be separated, usually without loss of biological activities. IEF in

free solution has the added advantage that the separated proteins can be recovered easily once they are focused - they do not need to be eluted from a solid gel matrix. Separation of proteins by IEF is based on the fact that all proteins have a pH dependent net charge. This is determined by both the amino acid sequence of the protein and the pH of the environment. When a protein is electrophoresed through an established pH gradient, it will migrate until it reaches the point at which the net charge is zero; at this point will stop migrating and is said to be focused.

Conditioned medium was dialysed against Tris-HCl, pH 7.4 overnight at 4^{0}C. Before focusing began, the dialysed solution was mixed with 1% ampholyte solution to establish a pH gradient (BioRad; pH range 3-10). The sample was loaded into the focusing chamber of a Rotofor Cell (BioRad) linked to a recirculating water bath at 4^{0}C and allowed to run for 4-6 hours at 12W constant power until the voltage reached plateau. At the end of the run, all 20 samples were harvested simultaneously under vacuum and assayed directly.

2.8.3 Large scale purification of conditioned medium by ion exchange chromatography

A glass column, 8cm X 25cm and fitted with a scintre was packed with 200ml of S-Sepharose (Pharmacia), a cation exchanger, to give a gel bed of approximately 5cm. A filter paper disc was placed on top of the gel bed to allow a more even flow of liquid through the

column. The column was linked to a UV absorbance monitor set at 280nm (Uvicord; LKB) and a chart recorder. The column was equilibrated with 50mM MES (2-[N-morpholino]ethanesulphonic acid), pH 6.0 at 4^{0}C overnight. Aliquots from a 20 litre batch of conditioned medium were diluted 1:4 with equilibration buffer just prior to loading and the diluted medium allowed to run under gravity at 4^{0}C for 4 days. Flow rate was between 10 and 20ml/minute. At the end of the run, proteins were eluted from the column using 1M NaCl in 50mM MES, pH 6.0

2.8.4 Gel filtration using fast protein liquid chromatography (FPLC)

200ul of protein solution was loaded onto a Superose 12 gel filtration column linked to a fully automated FPLC system (Pharmacia). The run took 40 minutes, after which the samples were collected every 2 minutes in 1ml aliquots in PBS.

2.9 POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE was performed routinely after each purification stage under denaturing conditions according to Laemmli (1970) as follows. A 12.5% running gel was prepared by mixing 10ml of 1.5M Tris-HCl pH8.8 containing 0.4% SDS with 12.5ml 1.07% bis, 40% acrylamide (Sigma/BDH), 17.2ml of distilled water and 12ul of N,N,N',N'-

tetramethyl-ethylenediamine (TEMED; Sigma). This solution was degassed for 5 minutes, then 300ul of a 10% solution of ammonium persulphate (BDH) added. The gel was cast between 2 clean glass plates and allowed to photopolymerise. The interface between the running and the stacking gels was made level by the addition of a few drops of water saturated n-butanol - this was poured off before the addition of the stacking gel.

The stacking gel was prepared by mixing 2.5ml of Tris-HCl pH 6.8 containing 0.4% SDS with 1.0ml of bis/acrylamide (1.07%/40%), 6.42ml of distilled water and 10ul of TEMED. After degassing, 75ul of a 10% solution of ammonium persulphate was added and the gel applied on top of the preformed running gel.

Protein samples were diluted with appropriate amounts of sample buffer. This consisted of 1.25ml of 1M Tris-HCl pH 6.8, 0.1ml of glycerol (BDH), 2ml of 10% SDS (Sigma), 0.1ml of beta-mercaptoethanol (Gibco) and 0.55ml of distilled water. Samples plus buffer were boiled for 4 minutes to denature the proteins and applied to the gel in 50ul aliquots with a Hamilton syringe. Running buffer consisted of 0.25M Tris/1.92M glycine, diluted 1:10 before use with the addition of 10ml of 10% SDS per litre.

SDS-PAGE was performed using either a BioRad or a BRL system at 50V (constant voltage) overnight.

2.10 GEL STAINING

All gels were silver stained, since silver staining techniques are

generally 100-fold more sensitive than Coomassie Blue and can detect nanogram quantities of protein. The gels were fixed in 30% ethanol:10% acetic acid, v/v, (James Burroughs; BDH) for one hour at room temperature, then stained using either an Amersham or Sigma silver stain kit according to the manufacturers instructions.

2.11 IMMUNOBLOTTING OF PROTEINS (WESTERN BLOTTING)

To try to identify further the partially purified peptide, a series of western blots were carried out using a panel of antibodies to growth factors. Western blotting depends on the transfer of proteins from polyacrylamide gels to nitrocellulose membranes using an electric current. The bound protein can be detected using various probes, the most common being double antibody techniques. Immediately after electrophoresis, gels were soaked in degassed transfer buffer for 30 minutes. This contained 20mM Tris buffer, 150mM glycine (both Sigma) made up in 20% methanol v/v. Nitrocellulose and filter paper (both BioRad) used to make up the gel sandwich were equilibrated overnight in the same buffer. Blotting was carried out for up to 4 hours at 150V in a BioRad Trans-Blot according to the manufacturers instructions. At the end of the run, the nitrocellulose was soaked for 10 minutes in Tris buffered saline (TBS; 500mM NaCl, 20mM Tris-HCl, pH 7.4), then blocked for 1 hour at room temperature with TBS buffer containing 3% EIA grade gelatin (BioRad).

To check for successful transfer of the proteins, the gel was

stained with Coomassie Blue and transfer was always at least 95%. For specific blotting details, refer to Chapter 5.

2.12 FILTER WELL CULTURES

2.12.1 General culture methods

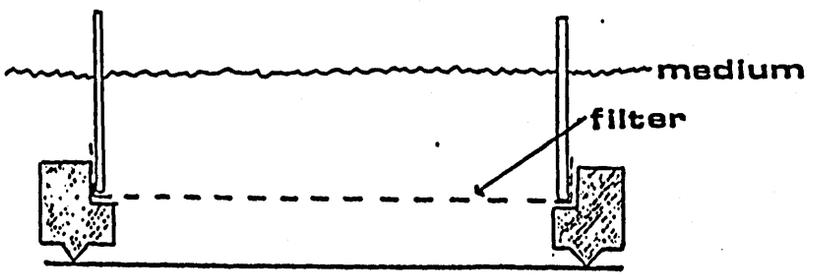
Filter wells (Costar Transwells; 24.5mm) were used for most coculture experiments. This permitted culture of a second cell type independently in the lower chamber, thus allowing cell-cell interactions to take place without the need for cell-cell contact. A diagram of a filter well can be seen in Figure 2. The filter wells used were fitted with a 10um thick polycarbonate membrane (Nucleopore) with a pore size of 0.4um. Routinely, cells were seeded at a concentration of 5 x 10^4cells/ml. The total volume of culture medium used was 3.5ml; 2.5ml in the dish which housed the filter well, and 1ml (in which the cells were suspended) above the filter membrane.

2.12.2 Preparation of filter wells for electron microscopy

Electron micrographs were prepared by Mr Graham Ainge and Mr John Bowles, Glaxo Group Research, Ware, Herts. using the following protocol. A549 cells growing on filter wells were fixed for a minimum of 90 minutes in 4% formaldehyde/2% glutaraldehyde,

FIGURE 2

Diagram of a filter well.



followed by a postfix in 1% osmium tetroxide for 1 hour. The cells were dehydrated in graded alcohols, then washed in acetone/epoxy resin before being embedded in the resin. Ultrathin sections were cut using a Diatome diamond knife, collected on copper grids, and then stained with uranyl acetate (15 minutes at 60^0C) and lead citrate (5 minutes at 20^0C) in an LKB ultrastainer. The stained grids were placed in a Philips CM10 electron microscope and photographed on Ilford 83x102mm E.M. film.

2.13 CLONING EXPERIMENTS

2.13.1 Monolayer cloning

Cells were diluted with fresh culture medium to a concentration of 100 cells/ml and 5ml aliquots seeded into 6cm petri dishes (Nunclon). These were incubated for 10 days at 37^0C in a humid incubator with 2% gas phase to allow the formation of colonies. After incubation, colonies were pre-fixed with a 1:1 mixture of 5ml of PBS/methanol for 10 minutes. This was removed and replaced with 5ml of methanol for a further 10 minutes. The dishes were air dried overnight, stained with 0.1% crystal violet (BDH) for 10 minutes, then rinsed extensively with running tap water until the water ran clear. The final rinse was in distilled water. Colonies greater than 0.5mm were counted using an Artex colony counter.

2.13.2 Suspension cloning

A suspension of 1000 cells/ml in culture medium supplemented with 10% FCS containing 0.3% agar (Gibco) was poured onto a preformed layer of 1% agar in culture medium in 35mm petri dishes (Nunclon). Thereafter, the cells were cultured for 14 days at 37^0C in a humid 2% CO_2 incubator. Colonies were stained with 5mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT;Sigma), for four hours at 37^0C in the dark. MTT is taken up by live but not dead cells (see MTT assay later). Colonies containing more than 20 cells were counted by eye using a binocular dissecting microscope.

2.14 IN VIVO STUDIES

2.14.1 Experimental animals

For most experiments, 6-8 week old male MF1/NuNu/Ola/Hsd immunodeficient nude mice were used. For experiments using the ovarian tumour cell line, A2780, female BALB/c nude mice were used. The athymic nature of these mice is the result of a genetic mutation and subsequent inbreeding to produce the homozygous nude mouse. Nude mouse production is achieved by mating heterozygous Nu^+ females with homozygous NuNu males. A hareem system is used with one male for every two females. Nude mice are weaned at 21 days.

All experimental animals were maintained on sterile rat and mouse number 3 diet (Special Diet Services) and sterile tap water ad libitum. They were housed in transparent MT1 polycarbonate boxes within a laminar flow hood (Bassair) at a temperature of 27^0C and a humidity of 40-60%. A light cycle of 12 hours light, 12 hour dark was used.

2.14.2 Xenograft preparations

Mice received a subcutaneous (s.c.) injection of at least 10^6 cells/ml in PBS. The animals were left for three weeks, after which time the cells had formed a tumour. The tumours were removed aseptically and cut into 5mm pieces. These pieces were transplanted into the appropriate hosts under anaesthetic (Hypnorm), and left for a further two weeks to allow them to become established. At this point the experiment was started.

2.14.3 Xenograft histology

At the end of each experiment, xenografts were excised aseptically and processed for light microscopy. Each tumour piece was fixed in 10% formol saline for at least 24 hours, then dehydrated through graded alcohols. The pieces were then embedded in wax (Paraplast), and 5um sections were cut on a Leitz rotary microtome, then transferred to a clean glass slide (Chance Propper). The sections

were then rehydrated through graded alcohols and stained with haematoxylin and eosin.

2.15 PHOTOGRAPHY

Phase contrast photographs of live cells were taken using an Olympus OM camera fitted to an Olympus CK-2 inverted microscope. Photographs of tissue sections were taken using a Reichart-Jung Polyvar microscope equipped with an automatic camera using Kodak Ektachrome 160 ISO Tungsten or Kodacolour Gold 35mm film (except where stated otherwise). Exposure was set automatically.

2.16 STATISTICAL ANALYSIS OF DATA

All statistical analysis was performed using the Student's t-test (unpaired test, two tailed analysis) using the following formula:

$$S_c^2 = \frac{[\Sigma X_1 - (X_1)^2/N_1 + \Sigma X_2^2 - (X_2)^2/N_2]}{N_1 + N_2 - 2}$$

$$t = \frac{X_1 - X_2}{S_c \ (1/N_1 + 1/N_2)}$$

(where t has $(N_1 + N_2 - 2)$ degrees of freedom.

CHAPTER THREE

GENERAL PROPERTIES OF A549 ALVEOLAR CARCINOMA AND LF113 FIBROBLASTS IN CULTURE

This chapter will deal with some of the more general aspects of the cell lines used in the study with particular emphasis on their response to steroid and the induction of pulmonary surfactant secretion.

INTRODUCTION

As already discussed in the General Introduction, alveolar type II epithelial cells undergo rapid proliferation during the saccular phase of lung development. In preparation for extrauterine life, the foetal lung must produce sufficient quantities of pulmonary surfactant for correct post-natal functions.

Pulmonary surfactant is a phospholipid rich material which reduces surface tension and maintains alveolar stability, thereby reducing the chances of alveolar collapse during the expiratory phase (Goerke, 1974). It is generally accepted that surfactant purified from bronchoalveolar lavage is composed of approximately 90% lipid, 5-10% protein with trace amounts of carbohydrates (Post and van Golde, 1988). Almost 75% of surfactant is composed of phosphatidylcholine, and disaturated phosphatidylcholine accounts for approximately 60% of the total surface lipid (Goerke, 1974; King, 1979). Floros et al (1986) have recently isolated cDNA clones for the 35kDa major protein component of pulmonary surfactant, and southern blot analysis has revealed that this is encoded on chromosome 10 (Bruns et al, 1987). The composition of surfactant is shown in Table 3.

TABLE 3 COMPOSITION OF PULMONARY SURFACTANT

DIPALMITOYLPHOSPHATIDYL CHOLINE	60%
MONOENOIC PHOSPHATIDYL CHOLINES	11-20%
PHOSPHATIDYL ETHANOLAMINE	5-10%
PHOSPHATIDYL GLYCEROL	<10%
NEUTRAL LIPIDS	5-13%

Surfactant is synthesised and secreted by alveolar type II cells of the lung, one of the two epithelial cell types which line the alveolar wall. The other cell type is the alveolar type I cell. Type I cells (also known as membraneous pneumocytes), are thin squamous cells which cover most of the alveolar wall and represent the site where gaseous exchange takes place. Type II cells are cuboidal cells, located in the corners of the alveoli (hence they are sometimes referred to as 'corner' cells). They contain the characteristic multilamellar bodies, concentric osmiophilic rings within which pulmonary surfactant is stored (Mason et al, 1977). Surfactant is believed to be synthesised in the endoplasmic reticulum, then modified by the Golgi cisternae, before being transported to the lamellar bodies for storage and secretion (Mason et al, 1977). Much of the evidence concerning surfactant has come from studies using fetal tissue, since one of the leading causes of

mortality in premature human neonates is respiratory distress syndrome, which is caused by an immaturity in the mechanisms controlling the synthesis and secretion of surfactant by the type II cells of the lung (Snyder et al, 1981). However it now appears that the onset of surfactant production and consequent lung maturation can be promoted by the administration of exogeneous glucocorticoids. Torday (1980), showed that foetal rabbit lungs were dependent on the presence of cortisol for differentiation and pulmonary surfactant secretion. Similarily, dexamethasone has been shown to elevate levels of surfactant in human foetal lung in organ culture (Odom et al, 1988), by increasing the numbers of beta-adrenergic receptors, the action of which stimulated pulmonary surfactant secretion (Maniscalco and Shapiro, 1983). In addition, Liley et al, (1987) showed that dexamethasone could induce synthesis of the major surfactant-associated protein, SP 28-36.

Primary cultures of type II epithelial cells have been reported to secrete phospholipids in response to exogeneous stimuli other than glucocorticoids, including beta-adrenergic agonists (Brown and Longmore, 1981), phorbol esters and cytochalasins (Rice et al, 1984a,b). Supplementing culture medium with linoleic acid increased the phospholipid composition of A549 (Smith, 1982). However, glucocortocoid or glucocorticoid analogues such as dexamethasone appear to be the most widely used substances for the acceleration of lung maturation and concomitant secretion of pulmonary surfactant.

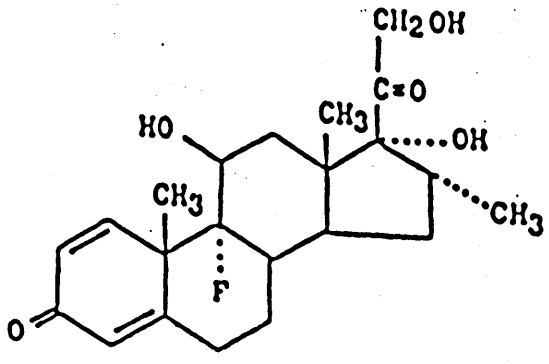
A549, the main cell type examined in this study, was initiated in 1972 by Giard et al from an explant culture of pulmonary adenocarcinomatous tissue from a 58 year old Caucasian male. This cell line displays the morphological features associated with pulmonary alveolar type II cells i.e multilamellar bodies, and actively synthesises phosphatidylcholine, a large proportion of which is disaturated (Lieber et al, 1976). A549 is therefore assumed to be of type II origin.

Production of pulmonary surfactant in cultured A549 cells has been reported by a number of groups (Lieber et al, 1976; Smith, 1977; Shapiro et al, 1978; Mason and Williams, 1980). This is a major characteristic of lung maturity and the principal differentiated function of type II pneumocytes.

Dexamethasone (9-alpha-fluoro-16-alpha-methylprednisolone) is a synthetic glucocorticoid analogue. (For structure see Figure 3). Glucocorticoids stimulate gluconeogenesis, the synthesis of glucose from non-carbohydrate precursors. Synthesis of glucocorticoids occurs in the adrenal cortex under the control of adrenocorticotrophic hormone (ACTH) which stimulates the conversion of cholesterol to pregnenolone, the rate limiting step in steroid hormone synthesis.

Glucocorticoids have been shown to have a number of effects both on tissues and cultured cells. This is reflected by the fact that receptors for glucocorticoid hormones have been found in a variety

FIGURE 3 STRUCTURE OF DEXAMETHASONE



DEXAMETHASONE.

of tissues and cells, including normal and pathological white cells, liver, placenta, kidney, prostate, lung and colon of most vertebrates (Munck and Leung, 1977). However the phenotypic response varies from one tissue to another, and this will be discussed below. In some tissues, glucocorticoids induce de novo synthesis of a number of enzymes, whereas in others, a catabolic effect has been observed. These hormones have been shown to exert an anti-proliferative effect in many tumour systems (Crabtree et al 1978; Jones et al, 1978; Freshney et al, 1980; McLean et al, 1986). Although the duration and magnitude of this anti-proliferative response has been found to be both dexamethasone dose- and glucocorticoid receptor-dependent (Braunschweiger et al, 1983), the actual levels of glucocorticoid receptors in cells of a particular type of cancer do not correlate well with their overall clinical chemosensitivity.

As well as having important anti-proliferative effects, glucocorticoids have also been used as differentiating agents in many different cell culture systems. Dexamethasone has been shown to stimulate differentiation in a mouse myeloid leukemia cell line (Sachs, 1978), as well as in a breast cancer cell line, ZR-75 (Osborne et al, 1979) and Steffen et al (1988) showed that a cloned rat mammary tumour cell line could become more differentiated with less aggressive in vivo behaviour and partially reduced growth after treatment with dexamethasone.

Glucocorticoids have been reported to modify the cell surface of human glioma cells. Mackie et al (1988), showed that steroid treated cultures of human glioma cell lines expressed elevated levels of heparan sulphate whilst hyaluronic acid levels decreased in response to steroid.

The glucocorticoid induction of the enzyme tyrosine aminotransferase (TAT), the first enzyme in the metabolism of tyrosine in the homogentisic acid pathway, has been known for many years, since Granner et al (1968) demonstrated that dexamethasone could stimulate TAT synthesis in cultured hepatocytes. This observation has since been verified and extended by Dow et al (1983), who showed TAT induction by cortisol in chick embryo mixed cultures, and Chou et al (1988), have shown that in hepatocytes, administration of glucocorticoid could induce a series of events associated with normal hepatocyte maturation, notably induction of albumin, transferrin and TAT, and inhibition of foetal alphafetoprotein secretion.

In human glioma cell lines, McLean et al (1986) have shown that glucocorticoids could almost completely abolish the activity of the proteolytic enzyme, plasminogen activator (PA). The same group also showed that the same was true in human NSCLC cell lines, where the effect was found to be time dependent, with the majority of the reduction induced by dexamethasone in the first 25 hours of culture. Whether this was due to decreased synthesis of PA or increased synthesis of an inhibitor was not established.

Glucocorticoids have also been shown to regulate EGF receptors in HeLa-S_3 cells (Fanger et al, 1983). This effect was both reversible and cell cycle dependent, with a lack of steroid response in the G_2/M and early G_1 phases. They have also been shown to stimulate glutamyl synthetase, a differentiation marker in the embryonic retina (Moscona and Piddington, 1966), and in certain lymphoid cell lines, these hormones have a cytocidal effect (Blewitt et al, 1983).

Perhaps one of the best documented glucocorticoid actions is their ability to reduce swelling and inflammation. Consequently, this property has been well utilised in the clinical environment, particularily in the management of brain tumours.

3.1 CONFIRMATION OF THE IDENTITY OF LF113 AND A549

3.1.1 Chromosome analysis

Both cell lines were grown to mid-log phase in 75cm^2 flasks (Falcon) and colcemid (Sigma), at a final concentration of 0.1uM added directly to the medium to arrest the cells in metaphase, according to the Materials and Methods. An average of 20 typical chromosome spreads were counted for each cell line and this confirmed that LF113 had the normal human diploid chromosome number of 46. A549, as expected, was aneuploid and heteroploid, with a

modal chromosome number of 59. Histograms of the chromosome spreads are shown in Figure 4.

3.1.2 Lactate dehydrogenase isoenzymes

Lactate dehydrogenase (LDH) occurs as 5 distinct isoenzymes in the tissues of all vertebrates. Each species has a chacteristic LDH pattern when separated by electrophoresis on cellulose acetate or agarose gels. LDH catalyses the same overall reaction:

$$\text{LACTATE} + NAD^{+} \xrightarrow{\text{LDH}} \text{PYRUVATE} + NADH^{+} + H^{+}$$

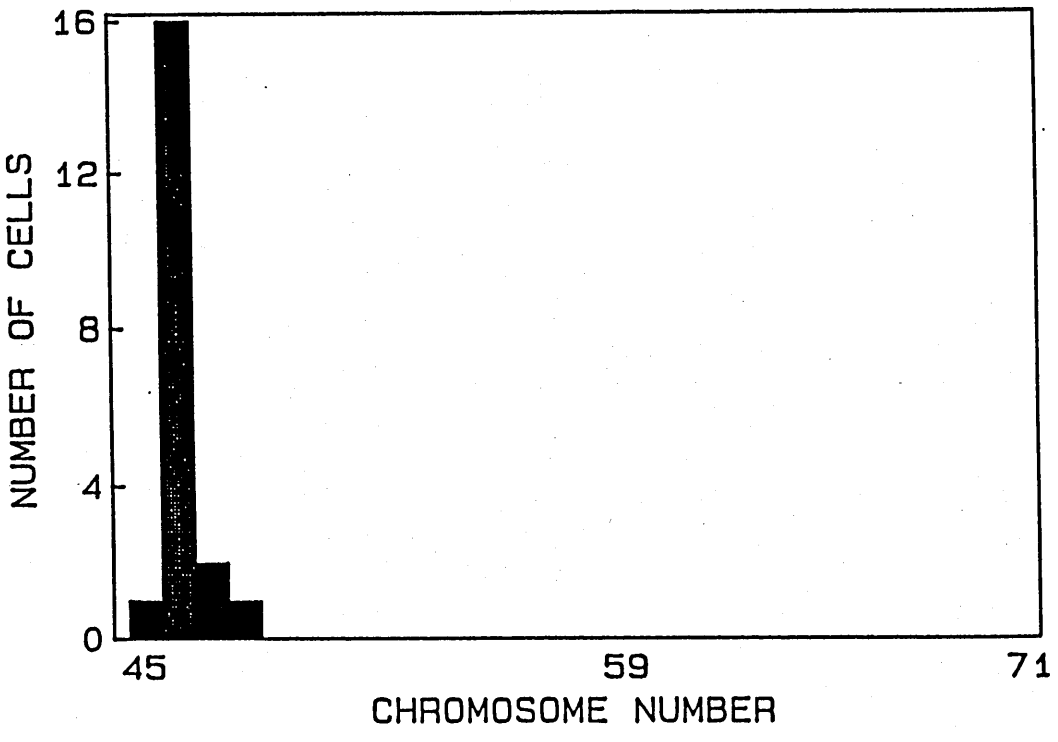
Confluent cultures of A549 and LF113 plus one known non human cell line, STO (mouse embryo fibroblasts) were scraped from their flasks and resuspended in a small volume of PBS (<2ml) in plastic tubes. The samples were then frozen and thawed twice to release cellular contents. Cells were frozen rapidly in a mixture of dry ice/methanol and thawed in a water bath at $37^{0}C$. An LDH kit (Sigma) was used for separation and identification of LDH isoenzymes, Briefly, 2x1ul samples were applied to the wells of a precast agarose gel (Ciba-Corning) and run at 12V/cm for 30 minutes in LDH electrophoresis buffer (50mM Tris, 12mM citrate and 25mM barbital). A sample of human LDH, provided in the kit, was used as a standard. The areas of enzyme activity were then visualised by histochemical procedures according to the reactions shown in Figure 5.

FIGURE 4

A549 and LF113 chromosome spreads.

a LF113 spread

b A549 spread


16
12
8
4
0
NUMBER OF CELLS
45
59
71
CHROMOSOME NUMBER

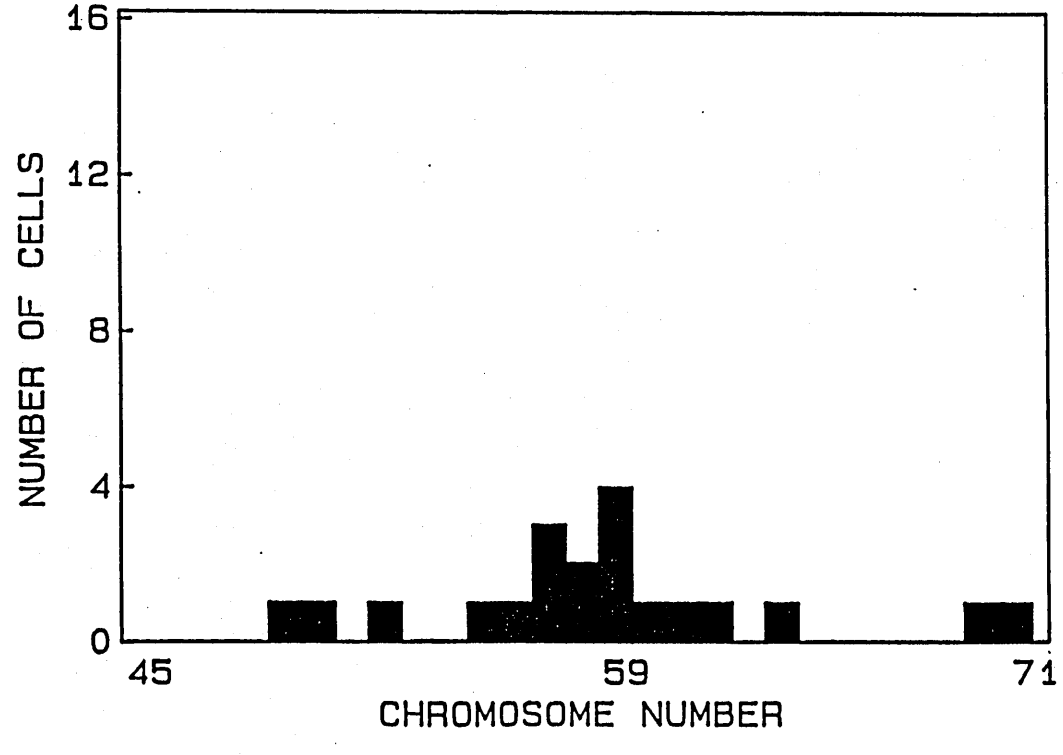

16
12
8
4
0
NUMBER OF CELLS
45
59
71
CHROMOSOME NUMBER

FIGURE 5

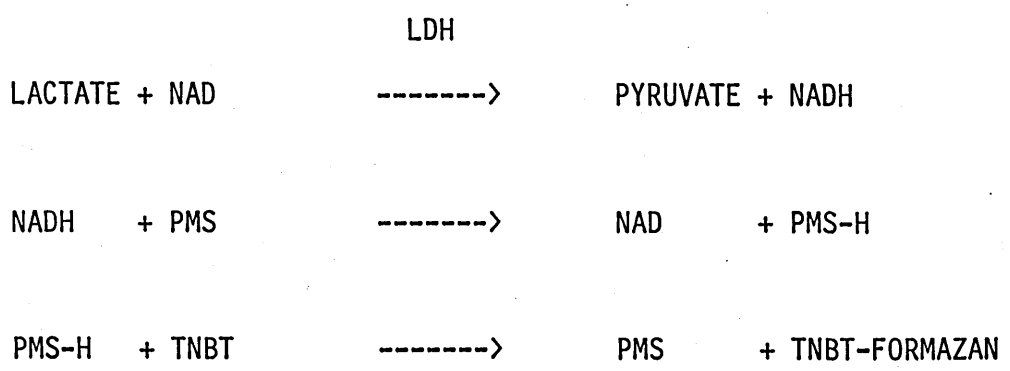
Abbreviations:

NAD nicotinamide adenine dinucleotide

NADH " " " (reduced form)

PMS phenazine methosulphate

TNBT tetranitroblue tetrazolium



The TNBT-formazan which is highly coloured and insoluble, localises in the electrophoretic zones of LDH activity.

Photographs of LDH profiles are shown in Plate 1 and both A549 and LF113 possess a characteristic human profile. STO (mouse) has quite a different profile. This, and the chromosome data, confirm the identity of the cell lines.

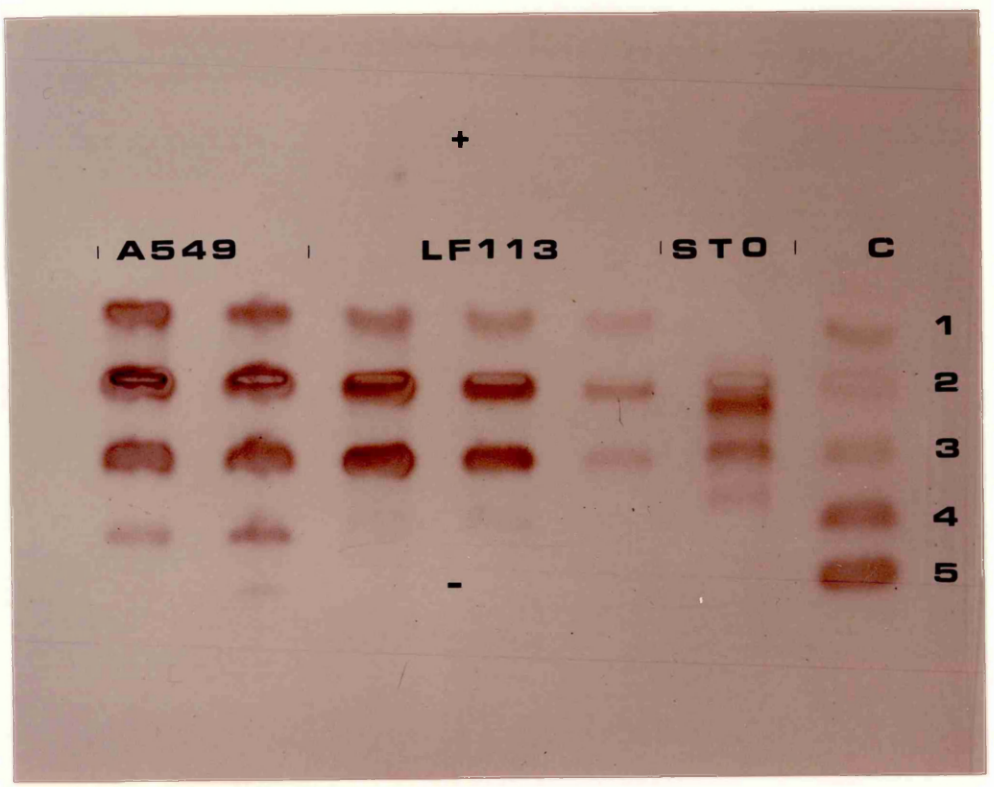
PLATE 1: LDH GEL OF A549, LF113 AND STO.

Samples were electrophoresed and stained as described in the Materials and Methods.

C = Human LDH standard

Numbers 1-5 refer to the five LDH bands present in the sample.

The differences in intensity in the three LF113 samples are due to different volumes of sample loaded.


+
A549
LF113
STO
C
1
2
3
4
5
-

3.2 EFFECT OF DEXAMETHASONE ON THE GROWTH OF A549 AND LF113.

Growth curves were performed using each cell line and terminal cell densities determined from the data.

Dexamethasone was found to be cytostatic to A549 resulting in a 30% reduction in the terminal cell density (Figure 6). With LF113, however, the result was different (Figure 7). At a concentration of 0.25uM, dexamethasone appeared to have a mitogenic effect on the treated cultures of fibroblasts. This increased the terminal cell density by some 12%. These results are summarised in Table 4.

TABLE 4 EFFECT OF DEXAMETHASONE (0.25uM) ON TERMINAL CELL DENSITY IN A549 AND LF113

CELL LINE	TERMINAL CELL DENSITY (CELLS/cm^2 x 10^4)			
	+FCS		-FCS	
	+DX	-DX	+DX	-DX
A549	8.9±0.15	12±0.2	2.85±0.41	4.7±0.43
LF113	6.1±0.5	5.4±0.4	1.45±0.39	1.7±0.41

FIGURE 6

Semi-logarithmic plot of the effect of dexamethasone on the growth of A549 cells in the presence and absence of serum.

A549 cells growing in 24 well plates were exposed to steroid, ± FCS as described in the Materials and Methods, and triplicate wells were counted once every two days.

Each data point represents the mean of three observations ± SE of one representative experiment out of a series of three.

a Cells incubated in the presence of serum ± 0.25uM DX

b Cells incubated in the absence of serum ± 0.25uM DX

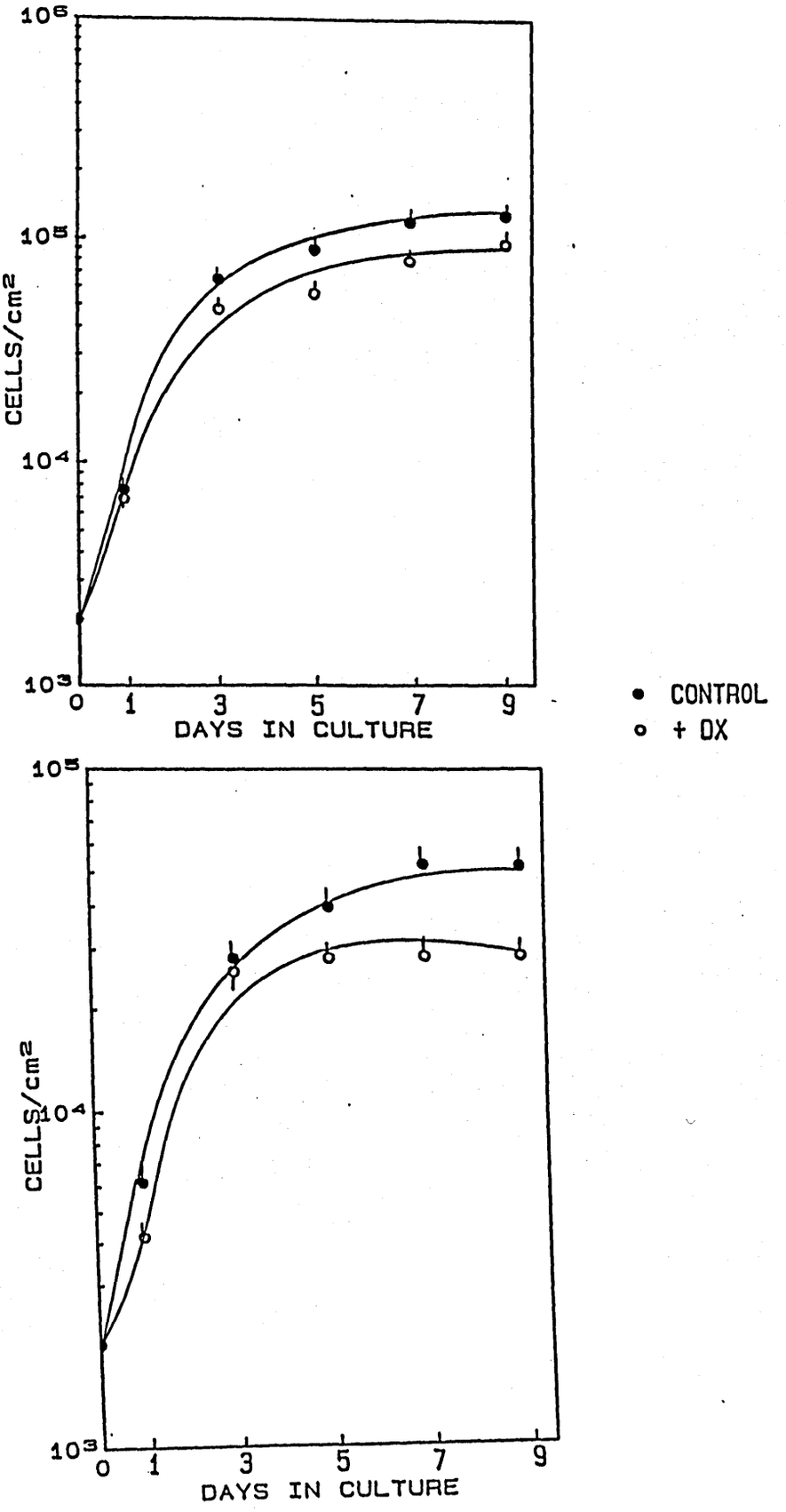

10^6
10^5
10^4
10^3
CELLS/cm²
0 1 3 5 7 9
DAYS IN CULTURE
● CONTROL
○ + DX
10^5
10^4
10^3
CELLS/cm²
0 1 3 5 7 9
DAYS IN CULTURE

FIGURE 7

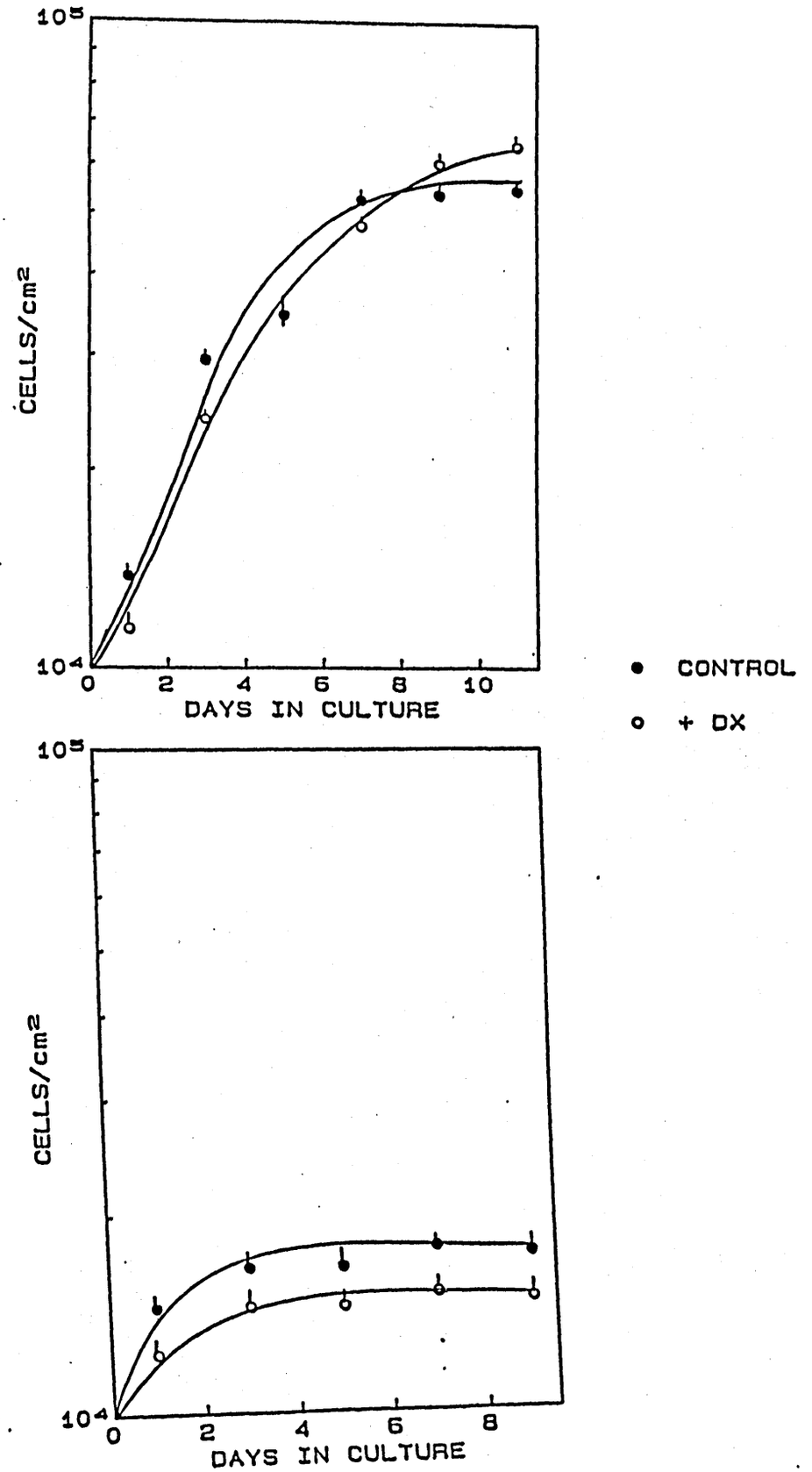
Semi-logarithmic plot showing the effects of dexamethasone on MOG-LF113 in the presence or absence of serum.

Cells growing in 24 well plates were exposed to dexamethasone, ± FCS as described in the Materials and Methods, and triplicate wells counted once every 2 days.

Each data point represents the mean of three observations ± SE

a Cells incubated in the presence of serum ± 0.25uM DX

b Cells incubated in the absence of serum ± 0.25uM DX


10^5
10^4
CELLS/cm²
0
2
4
6
8
10
DAYS IN CULTURE
CONTROL
+ DX
10^5
10^4
CELLS/cm²
0
2
4
6
8
DAYS IN CULTURE

3.3 DOSE RESPONSE TO DEXAMETHASONE

3.3.1 Optimum concentration required for maximum cytostasis

Mid log phase cultures of A549 growing in 25cm^2 flasks (Falcon) were incubated with dexamethasone (Decadron; Merck, Sharp and Dohme) at various concentrations ranging from 0-25uM in serum free culture medium. After 6 days in the presence of steroid, the cells were trypsinised and counted. The results are shown in Figure 8.

Dexamethasone at a concentration of 0.25uM was cytostatic for A549 without being cytotoxic to the cells.

3.3.2 Optimum concentration to achieve maximum pulmonary surfactant synthesis

A549 was incubated in 6-well plates (Nunclon) with various concentrations of dexamethasone (0-25uM) for 48 hours and assayed for pulmonary surfactant synthesis as described in the Materials and Methods.

As shown in Figure 9, dexamethasone caused a rise in the production of pulmonary surfactant, resulting in an approximately two-fold

FIGURE 8

Dexamethasone dose response curve for maximum cytostasis.

Log phase cultures of A549 growing in $25cm^2$ flasks were incubated in the presence of different concentrations of dexamethasone for four days. At the end of the incubation period the cells were trypsinised and counted electronically.

Each data point represents the mean of four individual experiments $\pm$ SE.

Cell density at start of experiment = $5 \times 10^4/cm^2$

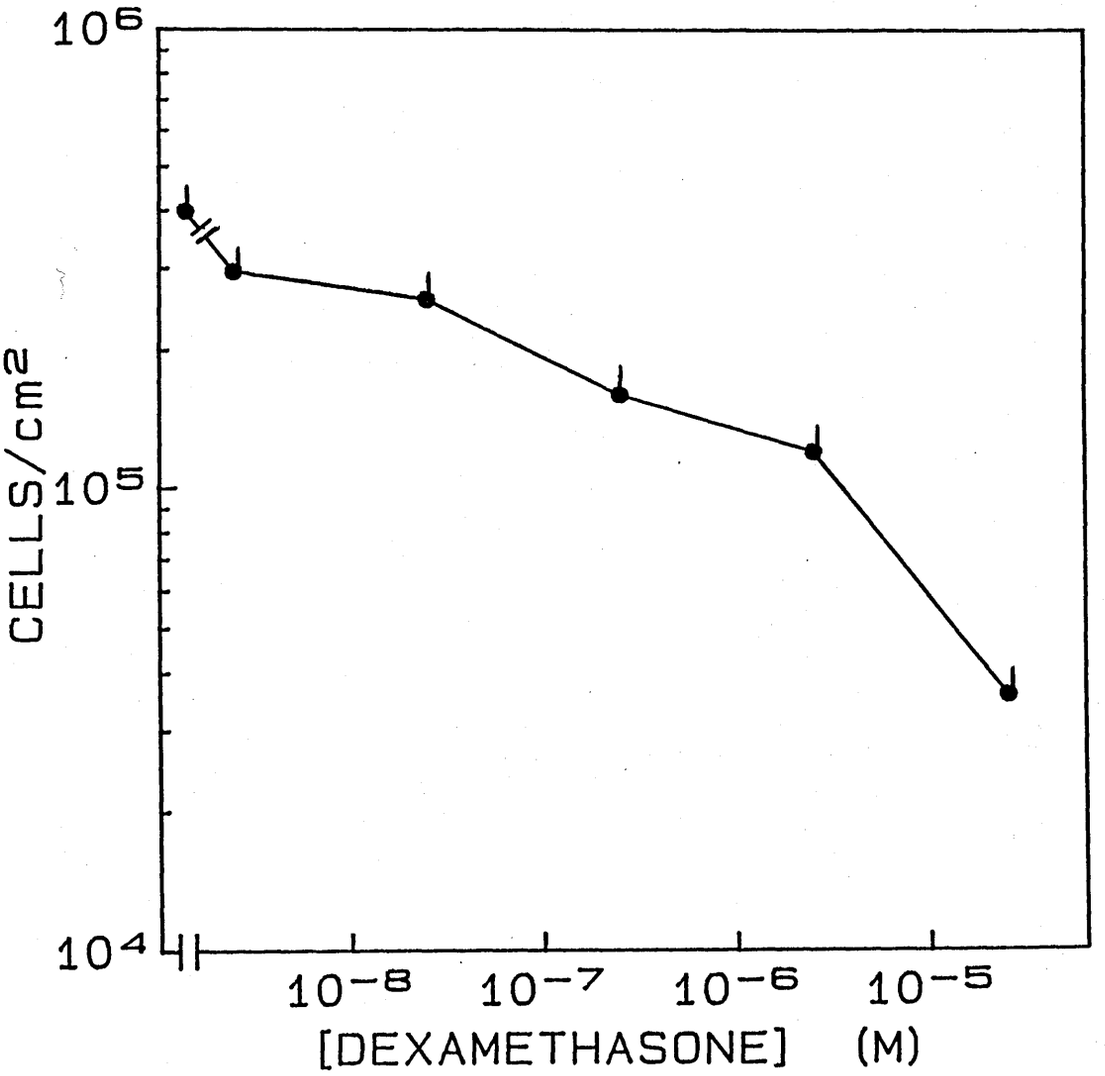

10^6
10^5
10^4
CELLS/cm^2
10^-8
10^-7
10^-6
10^-5
[DEXAMETHASONE] (M)

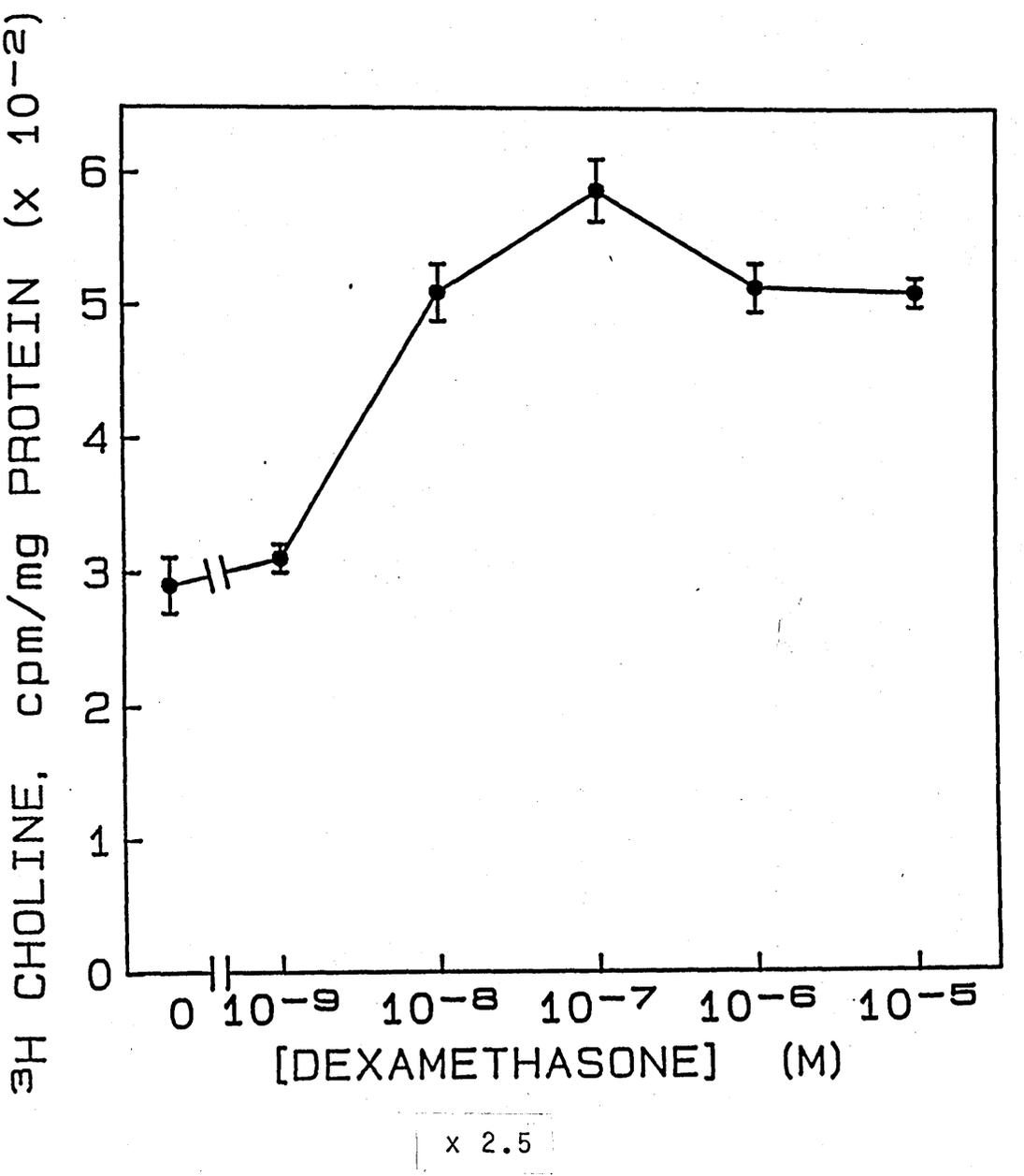
FIGURE 9

Dose response of induction of pulmonary surfactant production in A549 cultured on plastic by dexamethasone.

A549 cells growing in $25cm^2$ flasks were treated with various concentrations of dexamethasone for 48 hours and the ability of the drug to stimulate pulmonary surfactant was measured as described in the text.

Each data point represents the mean of three observations $\pm$ SE of one representative experiment out of a series of three.

Cells were in late log phase at the start of the experiment.


^{3}H CHOLINE, cpm/mg PROTEIN (x 10^{-2})
0
1
2
3
4
5
6
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
[DEXAMETHASONE] (M)

x 2.5

increase with a concentration of 0.25uM. Since this concentration was also found to have a cytostatic effect on the cells (section 3.3.1), it was used for all subsequent experiments.

3.4 PULMONARY SURFACTANT SPECIFICITY

As mentioned in the introduction to this Chapter, pulmonary surfactant is composed largely of disaturated phosphatidylcholine. However, this phospholipid is also an integral part of the cell membrane so it must be determined whether there is any difference between surfactant production between A549 and other cell lines. In addition, cell associated surfactant was measured, but in A549 only, to determine specificity for surfactant synthesis and release.

3.4.1 Specificity of pulmonary surfactant synthesis and release

To determine specificity, cell-associated and secreted surfactant was measured in cultures of A549, as descibed in the Materials and Methods.

A549 control cultures produced a low level of surfactant in culture, but this was stimulated some 50% by the addition of dexamethasone. Induction of surfactant production can be seen in Figure 10 with the greatest effect seen in the secreted component.

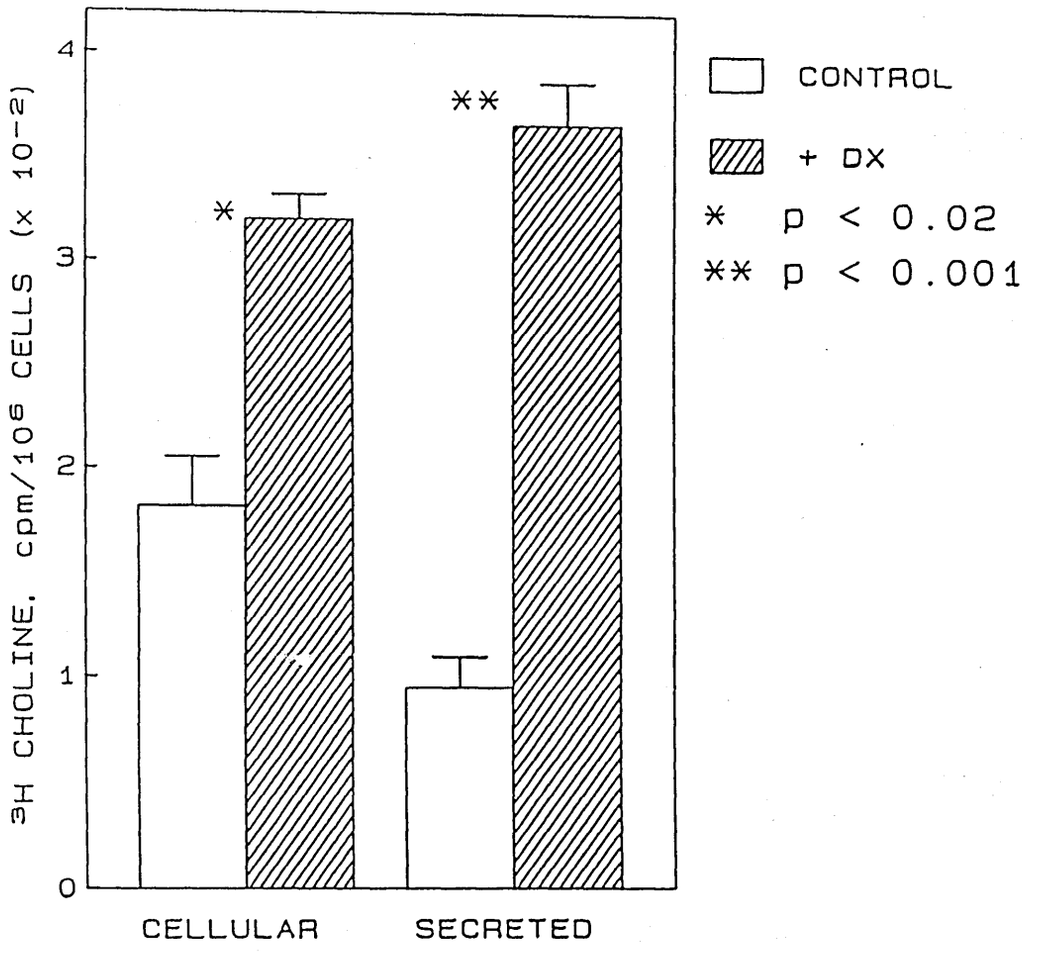
FIGURE 10

Effect of dexamethasone on cellular and secreted pulmonary surfactant production in A549.

Late log phase cultures of A549 cells growing in $25cm^2$ flasks were incubated with 0.25uM dexamethasone for 48 hours, as described in the Materials and Methods and then assayed for cell-associated and secreted pulmonary surfactant.

Each data point represents the mean of three observations $\pm$ SE of one representative experiment out of a series of three.

Cell density at start of experiment = $5 \times 10^4/cm^2$


4
3
2
1
0
^{3}H CHOLINE, cpm/10^6 CELLS (x 10^{-2})
*
**
CELLULAR
SECRETED
CONTROL
+ DX
* p < 0.02
** p < 0.001

3.4.2 Specificity for steroid response

A549 plus 4 other lung carcinoma cell lines, WIL, H125 (both adenocarcinomas), L-DAN (a mixed large cell/squamous carcinoma) and SK-MES (a squamous cell carcinoma) were seeded into 6-well plates (Nunclon) at a cell density of $2.5x10^4$ cells/cm^2 and grown to pre-confluence. The medium was aspirated and the cells washed twice with serum free culture medium, then incubated with or without 0.25uM dexamethasone for 48 hours. Cells were assayed for secreted pulmonary surfactant according to the protocol described in the Materials and Methods chapter. As is shown in Figure 11, WIL, SK-MES and H125 showed only very low levels of surfactant secretion, both in control and in steroid treated cultures. L-DAN however showed a greater secretion, and appeared to be stimulated by dexamethasone, although this measurement was not significant. the highest levels of pulmonary surfactant were found in A549.

DISCUSSION

This Chapter set out to define the characteristics of the main cell lines used in this study, and to determine their response to steroid.

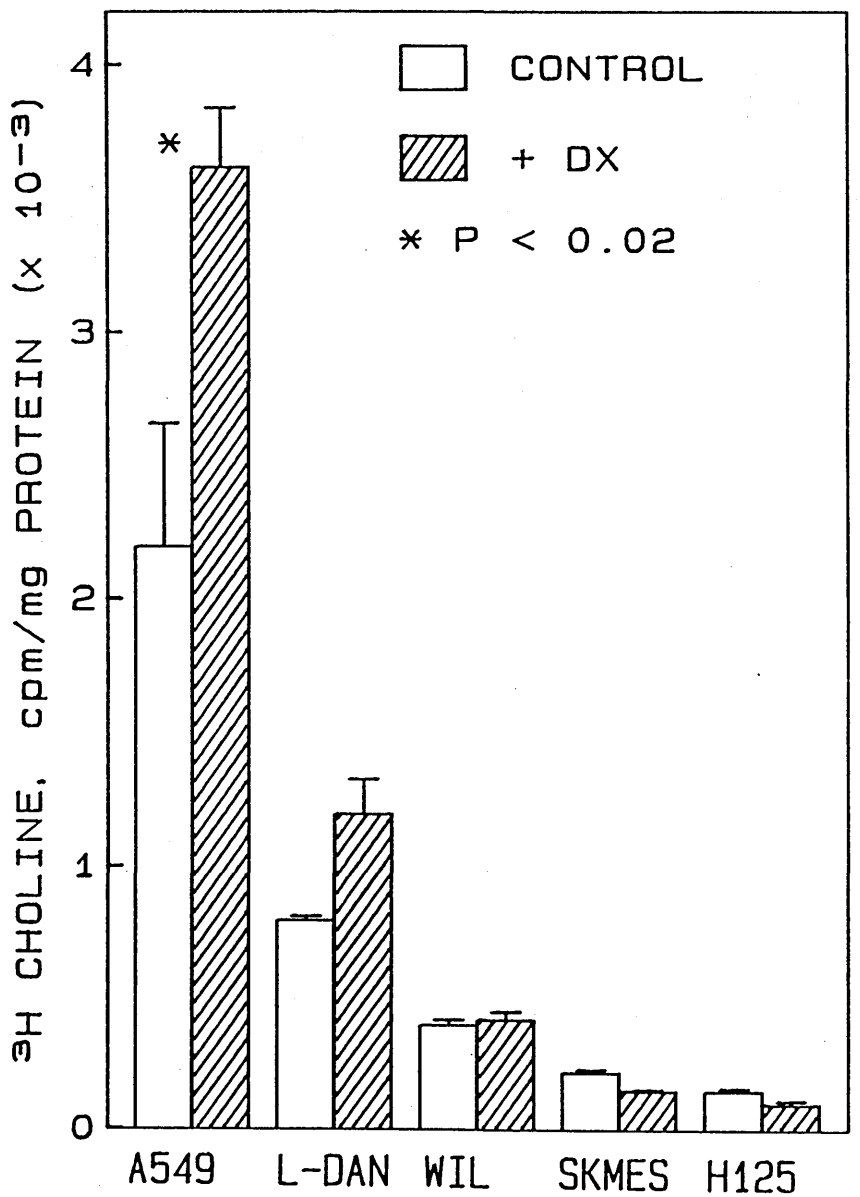
Dexamethasone was found to have differing effects on cell proliferation and its actions seemed to be dependent both on cell density and cell type. With the transformed line, A549, the

FIGURE 11

Effect of dexamethasone on pulmonary surfactant production in A549, L-DAN, SKMES and WIL.

All five cell lines were grown to late log phase in $25cm^2$ flasks, then treated with 0.25uM dexamethasone in serum free medium for 48 hours. Pulmonary surfactant production was determined as described in the text.

Each data point represents the mean of three observations $\pm$ SE of one representative experiment out of a series of three.


CONTROL
+ DX
* P < 0.02
3H CHOLINE, cpm/mg PROTEIN (x 10-3)
0
1
2
3
4
*
A549
L-DAN
WIL
SKMES
H125

cytostatic effect of dexamethasone was observed. These results are in agreement with the observations of McLean (1986), who showed that dexamethasone caused a significant reduction in the growth rates af a number of NSCLC cell lines in a dose dependent manner, with an accompanying increase in the plating efficiency. In 1978, Jones et al reported similar cytostatic effects on A549, although with a different steroid concentration (0.04uM as opposed to 0.25um used in this study). In breast, dexamethasone has also been shown to have similar effects with a decrease in growth rates and saturation densities in several cell lines (Osborne et al, 1979; Dabre and King, 1987). In glioma, whilst glucocorticoids were cytostatic at high cell densities, they were actually mitogenic at clonal cell densities. In the absence of serum, nanomolar concentrations were mitogenic (McLean et al, 1986) in regular monolayer culture.

The effect of dexamethasone on LF113 however, was different. The presence of steroid was seen to cause a cytostatic effect at low cell densities, but as the cells approached confluence, the effect was mitogenic. This observation is perhaps not quite as unusual as it may first appear, since observations by Grove et al (1977) have shown that DNA synthesis and cell growth in the foetal lung fibroblast cell lines WI-38 and WI-26, were both stimulated by by hydrocortisone.

The proliferative response of WI-38 cells to glucocorticoid has recently been found to be attributed to stimulatory factor(s)

present in the culture medium, suggesting an autocrine response (Finlay et al, 1985; Finlay and Cristofalo, 1987). The stimulatory effect of WI-38 conditioned medium was mediated by secondary factors present in the medium conditioned by cells in the presence of hormone. Preliminary attempts to characterise the the activity revealed a low molecular weight, heat stable, dialysable factor (Finlay et al, 1985). More recently, Finlay and Cristofalo (1987), have shown that the generation of stimulating factors seemed dependent on the culture conditions, since confluent or low density quiescent cultures failed to produce media stimulatory to cell growth. The factor was protease resistant with a molecular weight of less than 10kDa. The autocrine stimulation of the glucocorticoid conditioned medium was attributed, at least in part, to a low molecular weight factor(s) present in the medium. Moreover, production of the factor was dependent on the presence of fresh serum containing medium, making it difficult to determine whether the activity was actually synthesised by the cells and subsequently secreted into the medium, or if it was a serum component which was altered to a more mitogenic form by a cell derived product such as a growth factor (Finlay and Cristofalo, 1987).

Dexamethasone alone was found to increase both cell associated and secreted pulmonary surfactant in A549 in agreement with observations previously reported (Smith, 1977; Shapiro et al, 1978; McLean, 1986). Since initial observations by Liggins (1969), that accelerated foetal lung maturation followed the injection of cortisol into foetal lambs, many authors have suggested that there

is a role for glucocorticoids in the regulation of type II pneumocyte differentiation and subsequent surfactant synthesis and secretion (de Lamos et al, 1970; Kotas and Avery, 1971; Torday, 1980). However, these studies have dealt with normal lung, which contained mixed cell types, so it is difficult to say whether or not the steroid was having a direct effect on the type II cells or acting indirectly through the mesenchyme (this point will be discussed in much greater detail in a later chapter). It appears that dexamethasone can act directly on A549 cells, inducing a more differentiated phenotype.

The fact that L-DAN was capable of synthesising and secreting pulmonary surfactant after dexamethasone treatment was surprising. Although L-DAN is thought to be a mixed large cell/squamous carcinoma, it is possible that it may have some secretory properties, or perhaps a small population of multipotent stem cells are present. However, disaturated phosphatidylcholine is present in the cell membrane and the presence of dexamethasone may just be altering the membrane phospholipids somewhat, with a resultant increase in phosphatidylcholine. Compared with the other lung lines studied, there does seem to be a degree of specificity in this induction, with greatest specificity confined to the secretory component in A549.

It was difficult to quantify the effects of the steroid on cell-associated surfactant, because of the fact that pulmonary surfactant is composed mainly of disaturated phosphatidylcholine,

which is also an integral component of the cell membrane. Since the assay measured total cellular surfactant, it was impossible to say whether the labelled choline was due either to membrane cholines or to surfactant-associated choline. A possible way round this would have been to isolate the multilamellar bodies, the intracellular storage sites for surfactant, from the cells, thus giving a more accurate estimation of cell-associated surfactant. This technique has been reported by a number of groups including Snyder et al, (1981), Crecelius and Longmore, (1983) and Schalme et al, (1988), and the major advantage is that it isolates membrane phosphatidylcholine from surfactant-associated phoshatidylcholine.

To summarise, glucocorticoids have a direct effect on A549 cells, causing a cytostatic effect as well as stimulating pulmonary surfactant secretion. This suggests that these cells possess the appropriate glucocorticoid receptors, and in fact, receptor binding studies have shown this to be the case both in normal lung (Ballard, 1977) and in A549 (McLean, 1986). The induction of pulmonary surfactant appears to be specific for A549, since other NSCLC cell lines studied failed to produce any appreciable levels. The effect of glucocorticoids on fibroblasts whilst cytostatic at low cell densities, was mitogenic at high cell densities.

CHAPTER FOUR

INTERACTION BETWEEN A549 AND FIBROBLASTS

A549 was shown to produce surfactant when stimulated with steroid. This is not a new finding and has been reported elsewhere. However, the main aim behind this work was to determine if fibroblasts were implicated in the response of A549 to steroid as demonstrated by Post et al (1984) in normal lung. The following chapter will describe the effect of such interactions as well as optimisation of culture conditions.

All experiments described in this Chapter were performed my myself with the exception of the EM preparation, which was done by Mr. Graham Ainge and Mr. John Bowles, Glaxo Group Research, Ware, Herts.

INTRODUCTION

An important aspect to be considered when dealing with cultured cells is whether or not the establishment of polarity is necessary for expression of the correct differentiated functions. Polarity is a requisite function in both tissues and cultured normal cells, although the extent to which polarity is required in transformed cells in culture is as yet unclear.

In the case of normal lung, cells seeded onto tissue culture grade plastic substrates lose their ciliated and secretory cell morphology and adopt a squamous cell appearance within 72 hours of seeding (Wu et al, 1985). There are 2 possible explanations for this. Firstly, when a cell is growing on a plastic substrate, there is little chance of the cell being able to become polarised correctly, as, depending on how the cell adheres to the plastic, it may find itself incorrectly orientated or have the wrong shape for the correct expression of differentiated functions. Polarity has been shown to be necessary for the secretion of thyroxine by thyroid epithelium in culture (Chambard et al, 1983) and it has also been shown that rat pheochromocytoma cells when cultured on cellulosic filters exhibit a polarity not seen by the same cells cultured on a conventional plastic substrate (Van Buskirk et al, 1988).

Secondly, and most importantly in the case of cells of lung origin, it may be necessary to have the cells growing at or near to the

air-liquid interface for the correct expression of differentiated features (Van Scott et al, 1986). Under normal circumstances, respiratory epithelial cells are situated at the air-liquid interface, so to culture a cell of respiratory origin submerged in culture medium does not relate to the in vivo environment.

Whitcutt and his colleagues (1988) developed their own culture chamber, the Whitcutt chamber, which permitted the maintenance of polarity and differentiation in cultured respiratory tract epithelium. The chamber consisted of a moveable transparent permeable gelatin membrane which could be used either submerged in the culture medium, thereby feeding the cells in the traditional immersion method, or raised to the surface of the culture medium, which enabled the apical surface of the cells to come in contact with air and provide nutrients from the basal surface only. Thus, polarity or asymmetrical expression of cellular functions appears to be a prerequisite for full expression of differentiated function, not only in vivo but in vitro as well, for many epithelial cell types.

The cell line A549, used in this study, is reputed to be of type II origin (Smith, 1977) and although transformed, it may be possible that it will respond better to various exogeneous stimuli when it has the opportunity to become polarised or be close to the air-liquid interface.

A third important point to be considered is the relationship between tumour cells and cells of the mesenchyme. The implications of such a relationship have been discussed at length in the general introduction.

There are several reports supporting the concept of stromal-associated effects in mammary tissues (McGrath, 1983, Haslam, 1986). In mice, mammary fibroblasts directly stimulate the division of mammary epithelial cells (Enami et al, 1983) whilst augmenting their response to oestrogens (McGrath, 1983). In the developing tooth, intimate close-range interactions between mesenchymal cell processes, matrix vesicles and the outer surfaces of the responding epithelium appear to be crucial determinants of tooth morphogenesis (Slavkin et al, 1977; Mackenzie, 1984).

Age specificity has been demonstrated by Fukamachi et al (1979), who showed that isolated mouse stomach mesenchyme could only modify embryonic development in embryos of less than 10.5 days. They postulated that epithelium from later embryos had the ability to undergo self-differentiation.

There are two main types of epithelial-mesenchymal interactions. Firstly, instructive interactions, whereby the mesenchyme influences the epithelium to differentiate into a particular phenotype, e.g. dental ameloblasts which produce enamel ECM constituents. The second type of interaction is the permissive interaction which maintains or stabilises a committed epithelial

phenotype e.g. mediators of production of pulmonary surfactant by type II cells (Smith, 1977).

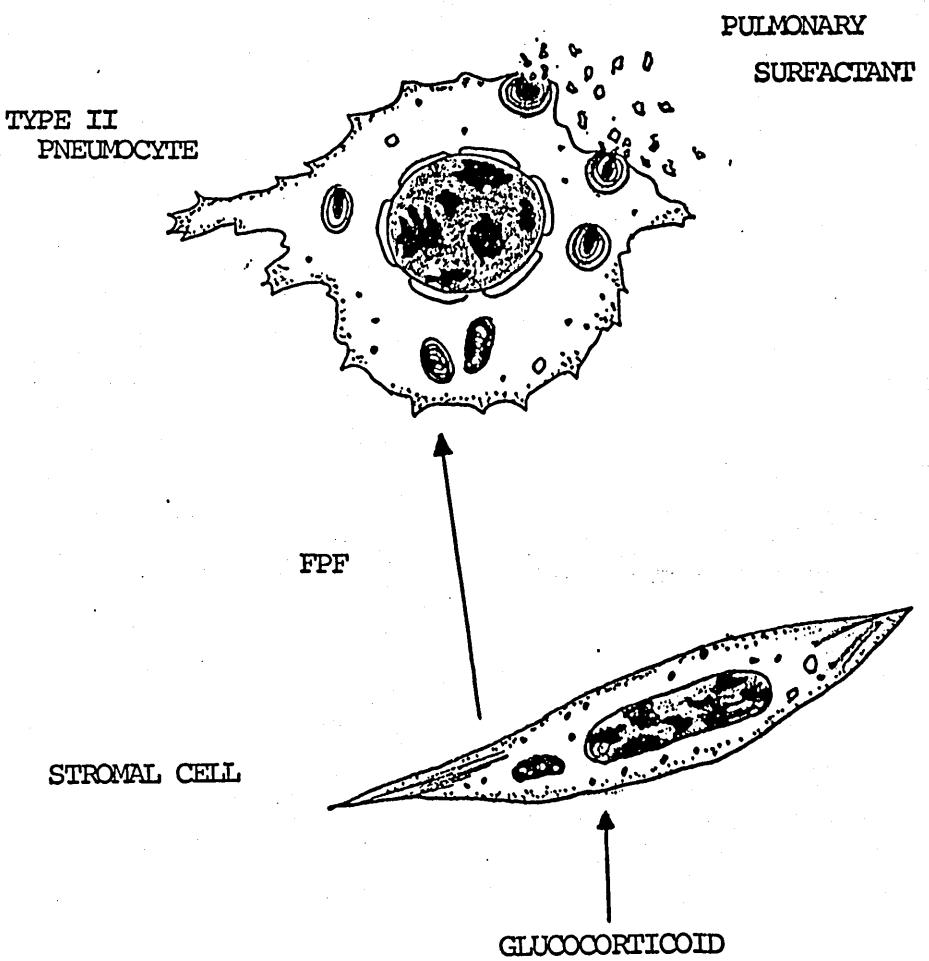
In the female reproductive tract, stroma plays a crucial role in the regulation of epithelial morphogenesis, cytodifferentiation, hormone responses and function, where neonatal uterine and vaginal stromal cells have been shown to express normal inductive functions when reassociated with the appropriate epithelium and grown in vivo (Cooke et al, 1987). More relevant to this study, in the lung, Smith and Fletcher, 1979, showed that a factor transferred from mesenchymal to epithelial cells accelerates lung maturation in foetal rodents.

Similarily, experiments with lung type II pneumocytes have shown that production of pulmonary surfactant is dependent on the steroid binding to receptors in the stroma which then releases a peptide which activates the type II cells (Post et al, 1984 - see also Figure 12).

Normal skin stroma can induce basal cell carcinoma to express a more normal phenotype, charactersised by growth and differentiation (Cooper and Pinkus, 1977), and in vitro studies with rat tracheal epidermal cells have demonstrated that differentiation factors produced by these cells could inhibit the growth of carcinogen-induced epithelial cells (Terzaghi-Howe and McKeown, 1986).

FIGURE 12

Schematic representation of pulmonary surfactant induction in type II pneumocytes by glucocorticoid induced fibroblast pneumocyte factor.



These observations raise some interesting questions regarding the possibility of stimulating the differentiation of lung carcinoma cells in vitro. Perhaps the tumour-associated stroma is defective in some way, possibly through interaction with the tumour cells, or production of tumour cell factors which override inductive signals from the stroma, such that it may be incapable of "instructing" the epithelium. Thus, if the tumour cells were cultured, under the correct conditions, with normal stroma capable of supplying the correct signal, they may respond. Also, as discussed in the general introduction, there is good evidence to suggest that the activity of the stroma is hormone dependent (Lasnitski and Mizuno, 1979; Cunha, 1984; Post et al, 1984). Taken together, these observations suggest that given the correct inducing environment, with the appropriate hormonal stimulus, it may be possible to alter the phenotype of A549. These questions will be addressed in this chapter.

4.1 CULTURE CONDITIONS: FILTER WELL VERSUS PLASTIC

A549 is reputed to be of type II origin, therefore under in vivo conditions, it would be situated in the alveolar wall of the lung, close to the air-liquid interface. It may be that these cells will respond better when cultured near the air-liquid interface rather than completely submerged, so to try to achieve this and allow for the development of polarity, A549 cells were grown both on a conventional plastic substrate or raised from the bottom of the dish by means of a filter well.

A549 was seeded either into 6-well plates (Nunclon) or into filter wells (Costar) at a cell density of 2.5×10^4 cells/cm^2 and assayed for secreted pulmonary surfactant as previously described after exposure to 0.25uM dexamethasone.

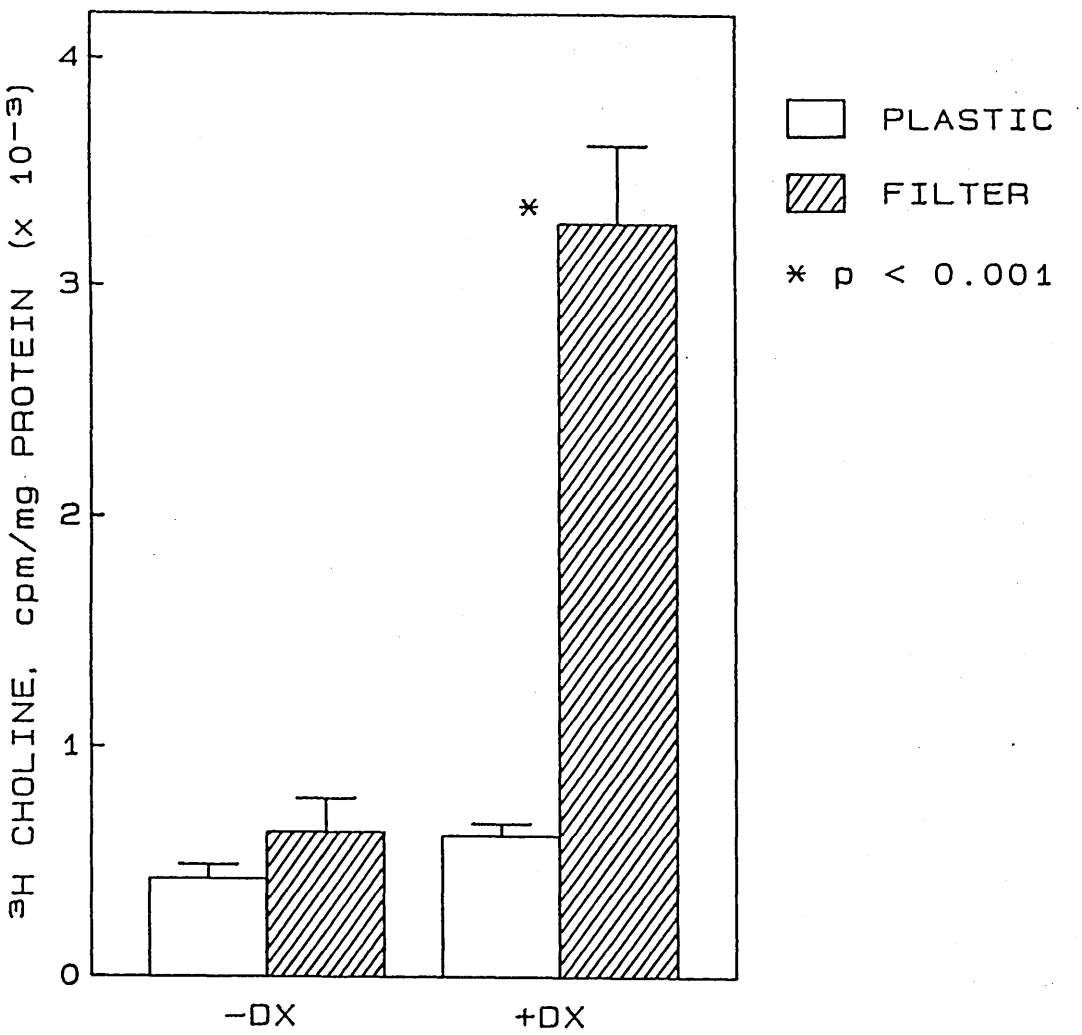
The results can be seen in Figure 13 and it is clear from the graph that cells growing on the filter well appeared to respond better to steroid, resulting in a 5-fold increase in pulmonary surfactant secretion compared with only a 1.5-fold increase when the cells were grown on plastic. Since culturing the A549 cells in filter wells seemed to enhance surfactant secretion, all subsequent assays involving surfactant measurement were performed under these conditions.

FIGURE 13

Effect of culture in filter wells on pulmonary surfactant production in A549.

A549 were cultured on the base of the dish or in filter wells in the presence and absence of steroid (0.25uM dexamethasone) and pulmonary surfactant production measured as described in the Materials and Methods.

Each data point represents the mean of four separate experiments $\pm$ SE.


4
3
2
1
0
3H CHOLINE, cpm/mg PROTEIN (x 10-3)
*
-DX
+DX
PLASTIC
FILTER
* p < 0.001

4.2 EFFECT OF STEROID ON MOUSE FOETAL LUNG CULTURES

It has been shown that A549 can produce pulmonary surfactant in culture, but it was interesting to compare this with normal lung. Since normal human lung is difficult to obtain, foetal mouse lung was used instead.

Cultures of foetal mouse lung were prepared according to an adaptation of the method of Douglas and Teele (1976), as described in the Materials and Methods and cultured on either 6 well plates (Nunclon) or in filter wells (Costar). Cells were assayed for surfactant following treatment with or without dexamethasone as previously described.

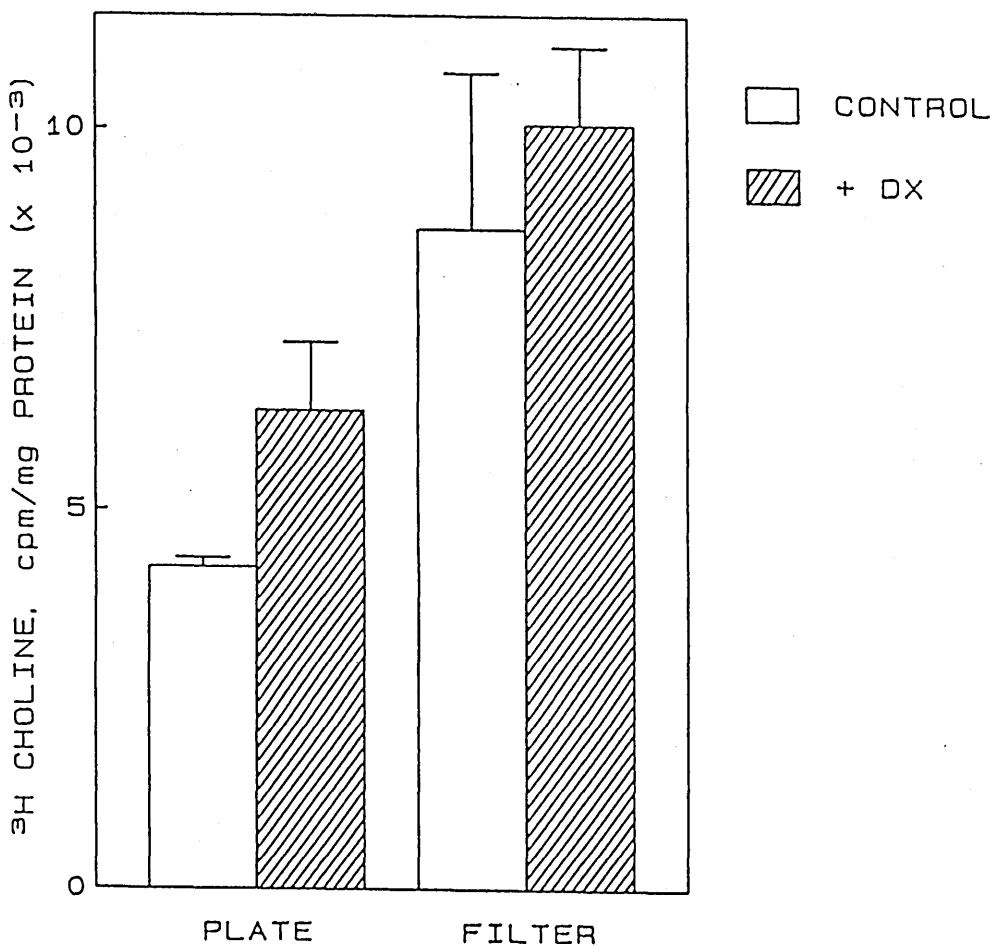
The results are shown in Figure 14. Dexamethasone increased incorporation of ^{3}H-choline into disaturated phosphatidylcholine by approximately 1.5 fold, regardless whether the cell were growing on plastic or on a filter well. Overall, the cells cultured on the filter expressed higher levels of surfactant than parallel cultures incubated on plastic. However, it must be remembered that these cultures not only contained epithelial cells, but fibroblasts as well, and this point will be discussed later.

FIGURE 14

Pulmonary surfactant production by early passage cultures of foetal mouse lung and the effect of dexamethasone.

Cultures of foetal mouse lungs were prepared and assayed for pulmonary surfactant production when grown on plastic and in filter wells in the presence and absence of steroid as described in the Materials and Methods.

Each data point represents the mean of three observations $\pm$ SE


^{3}H CHOLINE, cpm/mg PROTEIN (x 10^{-3})
10
5
0
PLATE
FILTER
CONTROL
+ DX

4.3 MORPHOLOGICAL INTERACTIONS

A549 cells are fairly typical epithelial cells with a fairly regular shape and forming a pavement-like appearance in culture. LF113 have a typical fibroblast morphology, assuming a spindle shape and producing characteristic parallel arrays of cells at confluence. As the main aim of this work was to determine the effect of fibroblasts on epithelial phenotype, the first logical step was to see how the cells reacted at the morphological level when confronted in coculture.

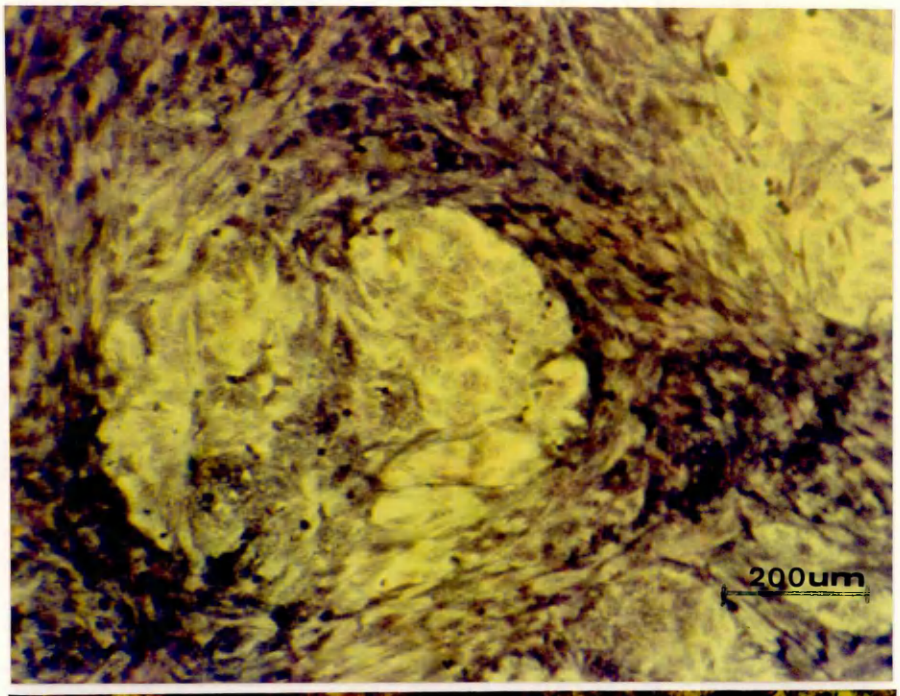
LF113 were grown to confluence on glass coverslips with a diameter of 16mm (Chance Propper Ltd), in 24 well plates (Nunclon), then transferred to non- tissue culture treated petri dishes (Sterilin). A549 ($5x10^4/cm^2$) was added to the dish containing the coverslip and left to attach overnight. After 24 hours, distinct interactions were observed between the fibroblasts and A549 which had plated down on top of the fibroblast coverslip, with evidence of A549 cells invading the fibroblast monolayer. This resulted in a "pulling back" of the fibroblasts to form circular structures. These interactions persisted for up to 10 days in culture (Plate 2a,b). In addition, circular holes appeared in the A549 monolayer which was growing separately from the coverslip, on the non-tissue culture plastic, with evidence of chromophilic material in the spaces so formed. This did not occur in the absence of fibroblasts (Plate 2c).

PLATE 2: PHENOTYPIC EFFECT OF COCULTURING A549 AND LF113.

Plate 2a: Structural reorganisation in A549/LF113 cultured in direct contact.

Plate 2b: Effect of a fibroblast coated coverslip on A549 growing on non tissue culture grade plastic. No direct contact.

Plate 2c: Control cultures of A549 growing on non-tissue culture grade plastic.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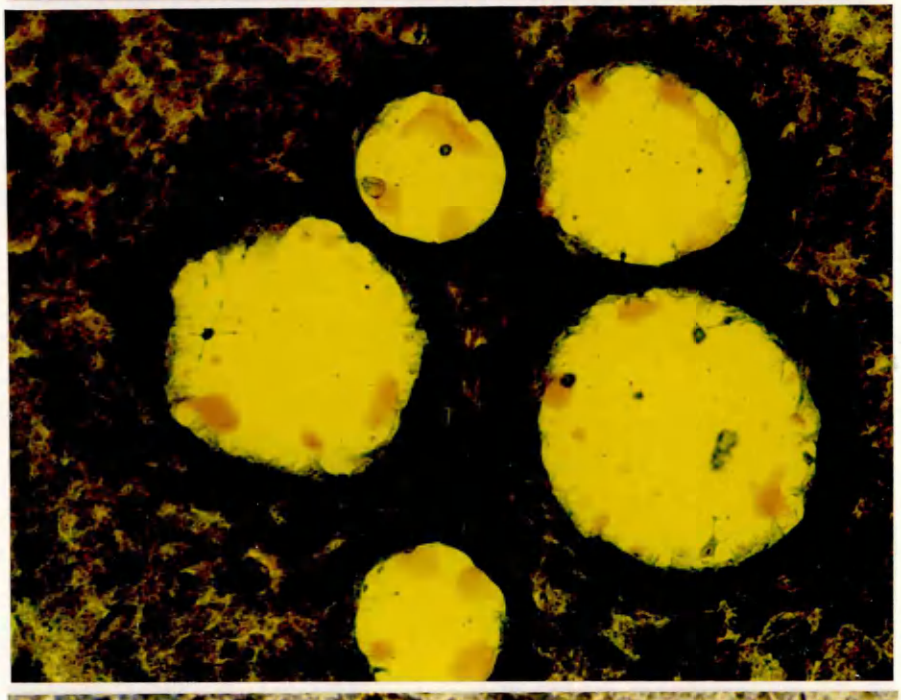
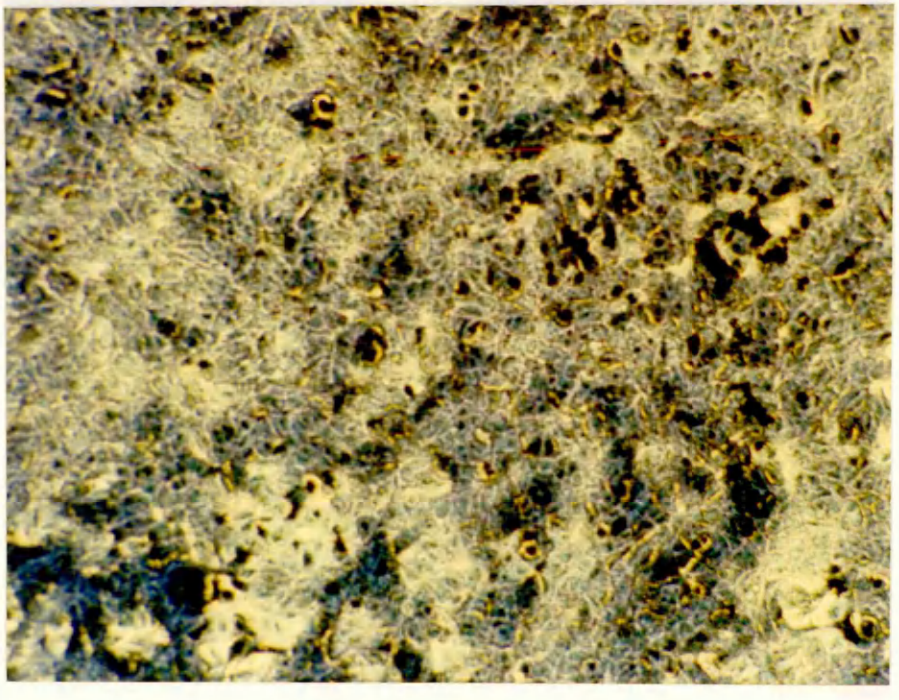




Clearly there were interactions going on between the 2 cell types, suggesting that biochemical estimation of surfactant production should be investigated.

4.4 MEASUREMENT OF PULMONARY SURFACTANT USING COCULTURED CELLS

Fibroblasts were seeded on to a Costar filter well membrane and grown to confluence. At confluence, $2.5x10^4$ A549/cm^2 were plated on top. The cells were cocultured in this fashion for 2 days before the addition of 0.25uM dexamethasone in serum free medium. Parallel cultures were also set up as controls, and secreted pulmonary surfactant was measured as before. The draw back of this type of experiment is in the difficulty of expressing pulmonary surfactant as specific activity, due to the contribution of the fibroblasts to the total cellular protein. In the coculture experiments, when the cells were solubilised to release total protein, it was impossible to determine A549 associated protein separate from LF113 associated protein. So, not only did the measurements account for A549 associated protein, but fibroblast associated protein as well. In an attempt to get around this, fibroblast cultures alone were subjected to exactly the same experimental conditions as the cocultures and the protein values were obtained after the value for fibroblast-associated protein had been subtracted from the coculture values. This assumed, of course, that cells cultured independently had the same growth rates as cells which were growing in coculture. The results of the experiment are shown in Figure 15.

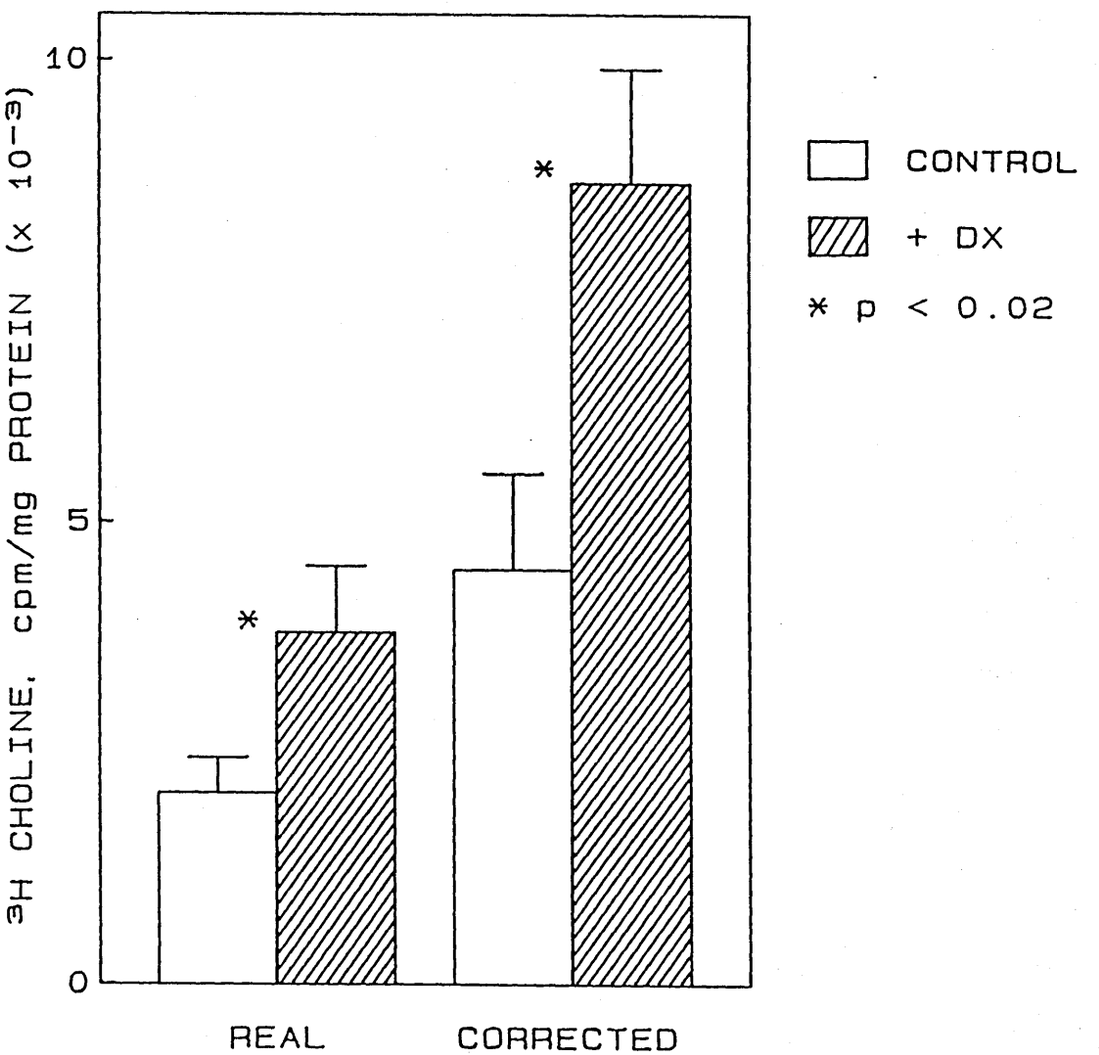
FIGURE 15

Effect of coculture with MOG-LF113 on pulmonary surfactant production in A549.

A549 were cocultured in filter wells with MOG-LF113 in direct contact in the presence or absence of steroid and assayed for pulmonary surfactant production. The cells were cocultured for 3 days before the addition of steroid, and then cocultured for a further 48 hours in the presence of 0.25 uM dexamethasone.

Real = observed value, corrected = value obtained after accounting for fibroblast-associated protein. For explanation, refer to text.

Each data point represents the mean of five separate experiments $\pm$ SE.


10
5
0
^{3}H CHOLINE, cpm/mg PROTEIN (x 10^{-3})
*
*
REAL
CORRECTED
CONTROL
+ DX
* p < 0.02

In both the observed values and the values corrected for fibroblast- associated protein, there was an approximate doubling in surfactant secretion in the presence of steroid. Relative to the control values in the previous graph (Figure 13), the addition of fibroblasts caused a three-fold increase in pulmonary surfactant in the presence of steroid, and a seven-fold stimulation in the absence of steroid.

4.5 TRANSFILTER COCULTURE

To avoid the contribution of the fibroblasts to the total cell protein, and, more importantly, to test whether cell-cell contact was required, the cells had to be separated in some way from A549. The use of filter wells permitted this, allowing a separate cell type to be cultured in the lower chamber of the filter well holder (Figure 16).

Fibroblasts were grown to confluence on the bottom of the dish which housed the filter well and A549 cells plated onto the filter membrane. The cells were grown in serum free medium with or without steroid 3 days, then assayed for surfactant secretion as before. The specific activity of secreted surfactant relative to cell protein could now be calculated more accurately, and the highest levels of surfactant were recorded (Figure 17), a 5-fold increase in the absence of steroid and a further 4-fold increase in the presence of steroid (relative to control values).

FIGURE 16

Diagram of A549 and LF113 cells cultured under transfilter conditions.

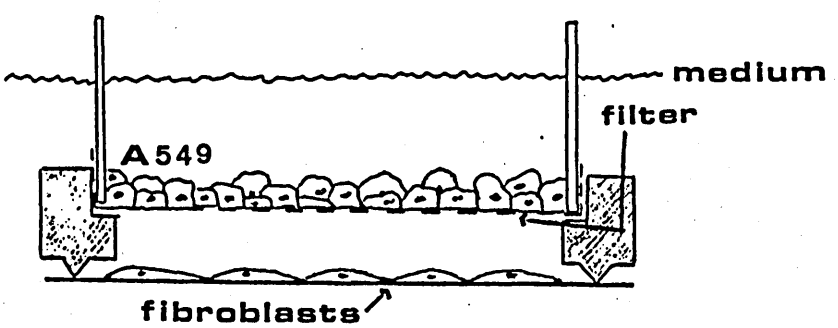
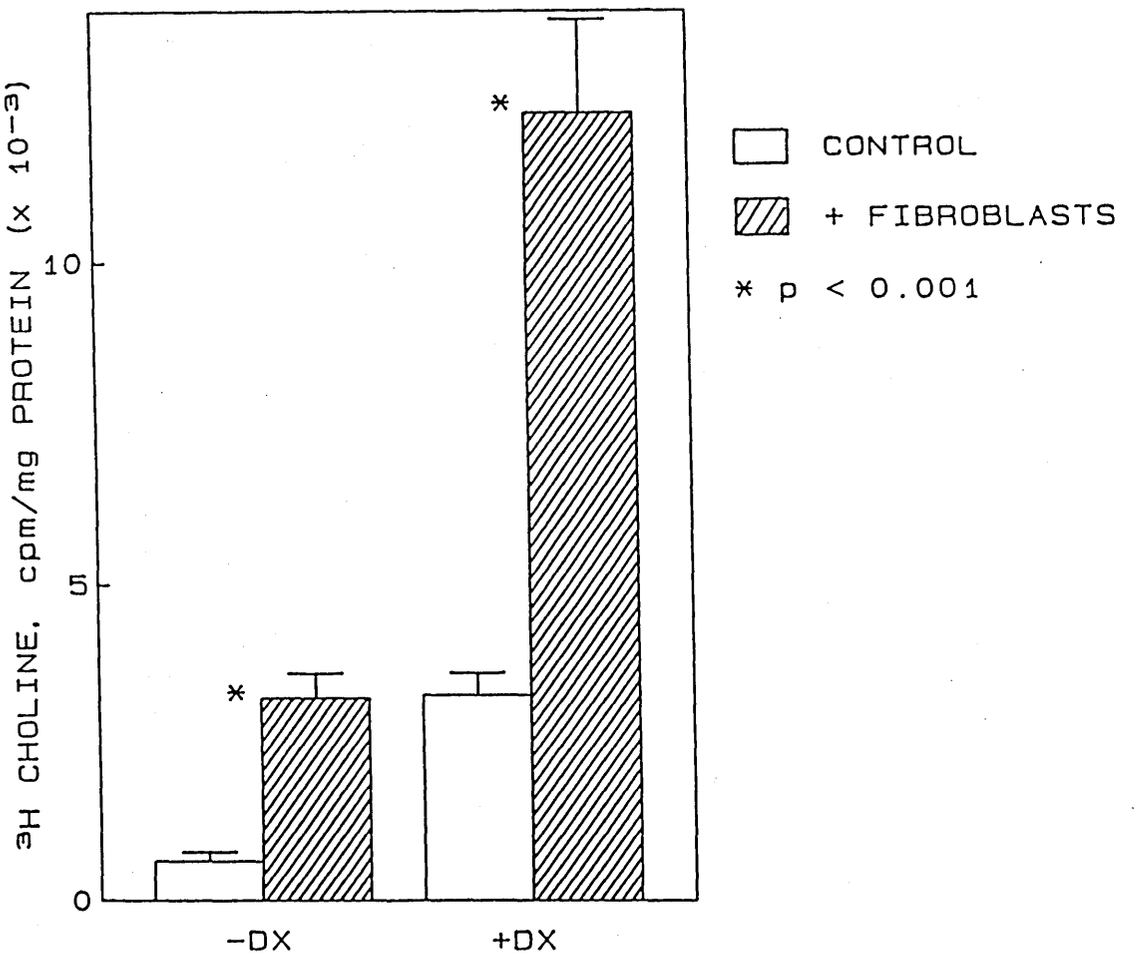


FIGURE 17

Stimulation of pulmonary surfactant production in A459 by the addition of MOG-LF113 transfilter.

A549 cells were cultured in a filter well and MOG-LF113 cultured on the bottom of the dish, which housed the filter well, in the presence and absence of dexamethasone, and assayed for pulmonary surfactant production as described in the text.

Each data point represents the mean of four separate experiments $\pm$ SE.


^{3}H CHOLINE, cpm/mg PROTEIN (x 10^{-3})
0
5
10
*
*
-DX
+DX
CONTROL
+ FIBROBLASTS
* p < 0.001

4.6 EFFECT OF FIBROBLAST CONDITIONED MEDIUM

4.6.1 Effect on pulmonary surfactant

Conditioned medium was prepared from LF113 cultures exactly as described in the Materials and Methods section. A549 cells were grown to confluence in filter wells and then treated with steroid treated conditioned medium, diluted 1:1 with an equal volume of fresh serum free culture medium, for either 24 or 48 hours, and then assayed for surfactant as usual. The results are shown in Figure 18.

There was no significant difference between control and treated cultures after 24 hours in culture in the presence of conditioned medium, but after 48 hours there was an approximate doubling in pulmonary surfactant secretion. The actual levels achieved were lower than the transfilter values (Figure 17). This point will be discussed later.

The surfactant data is summarised in Table 5.

4.6.2 Effect on cell growth

To determine the effects on cell growth, A549, LF113 and Swiss 3T3 fibroblasts were treated with various concentrations of fibroblast conditioned medium and assayed for mitogenicity, as described in the Materials and Methods. The results are shown in Figures 19 and

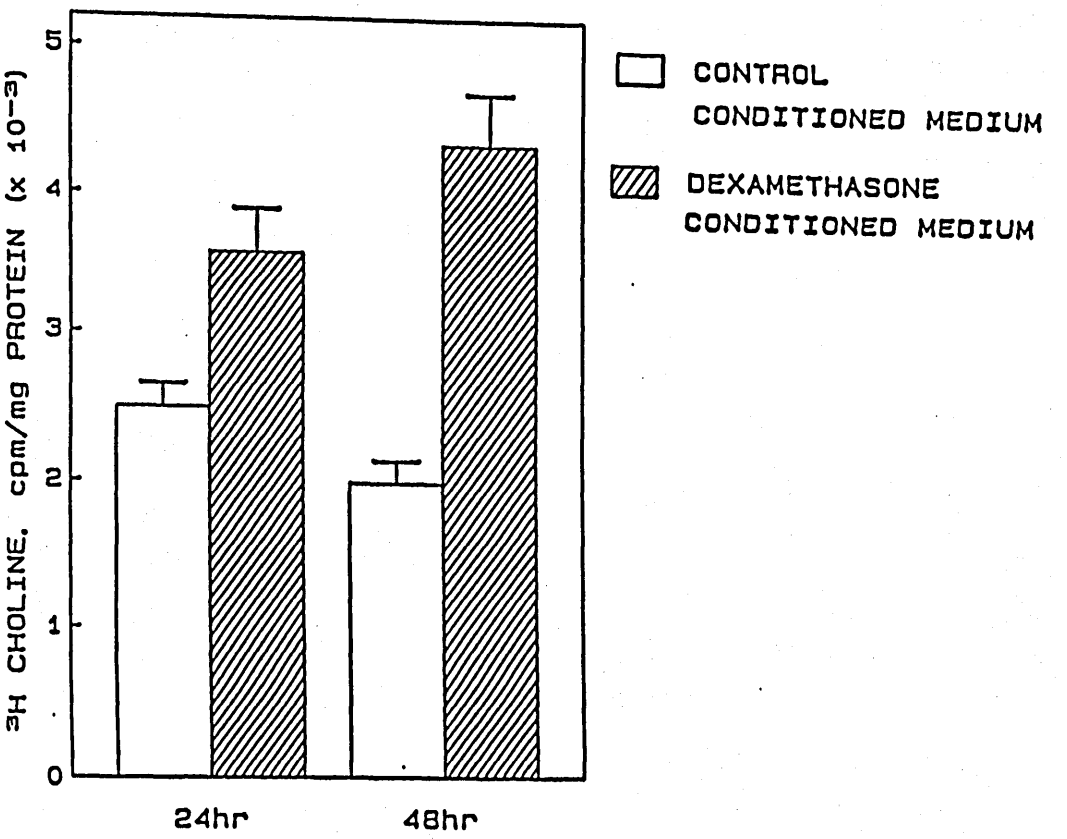

^{3}H CHOLINE, cpm/mg PROTEIN (x 10^{-3})
0
1
2
3
4
5
24hr
48hr
CONTROL CONDITIONED MEDIUM
DEXAMETHASONE CONDITIONED MEDIUM

FIGURE 18

Effect of fibroblast conditioned medium on production of pulmonary surfactant in A549.

A549 cells growing in filter wells were exposed to conditioned medium for either 24 or 48 hours and then assayed for pulmonary surfactant production as previously described.

Each data point represents the mean of three observations $\pm$ SE of one representative experiment out of a series of three.

TABLE 5: SUMMARY OF PULMONARY SURFACTANT DATA

EXPERIMENTAL CONDITIONS	cpm/mg PROTEIN ± SE			
	+DX		-DX	
A549 ALONE FILTER	3273.87	345.30	637.14	144.09
LF113 TRANSFILTER	12375.67	1487.92	3222.66	385.83
COCULTURE (real)	3818.48	709.60	2079.71	381.84
COCULTURE (corrected)	8670.14	1239.39	4493.41	1036.44
CONDITIONED MEDIUM (24hrs)	3501.17	350.15	3008.01	200.00
CONDITIONED MEDIUM (48hrs)	4350.17	359.12	1950.09	350.11
FOETAL MOUSE (filter)	10062.81	1018.95	8717.43	1812.92
FOETAL MOUSE (plate)	6319.42	904.62	4243.93	122.96

Each value represents the mean of at least three separate experiments ± the standard error. Pulmonary surfactant is expressed as ^{3}H-choline incorporation/mg of total cell protein.

FIGURE 19

Effect of MOG-LF113 conditioned medium on the growth of MOG-LF113 and A549 in vitro.

Both cell lines were incubated with 0-100 percent of MOG-LF113 conditioned medium for 48 hours and the effect on cell growth monitored by a modification of the MTT assay as explained in the Materials and Methods.

Each data point represents the mean of three observations ± SE of one representative experiment out of a series of three.

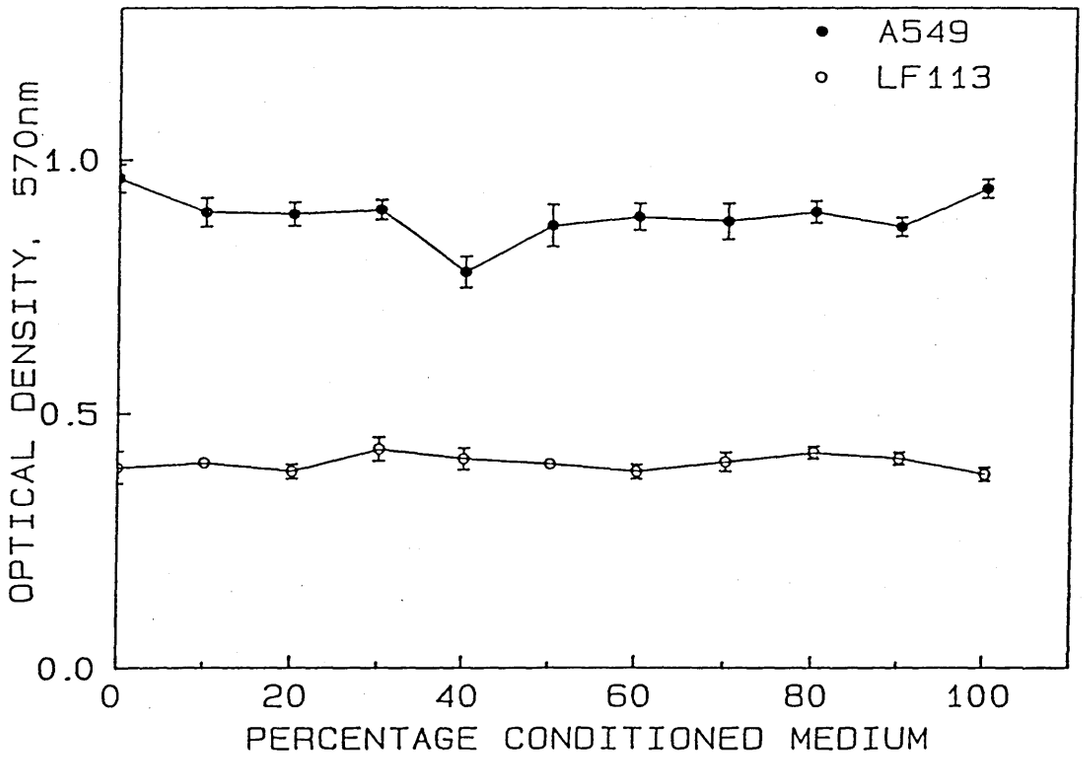

A549
LF113
OPTICAL DENSITY, 570nm
1.0
0.5
0.0
0
20
40
60
80
100
PERCENTAGE CONDITIONED MEDIUM

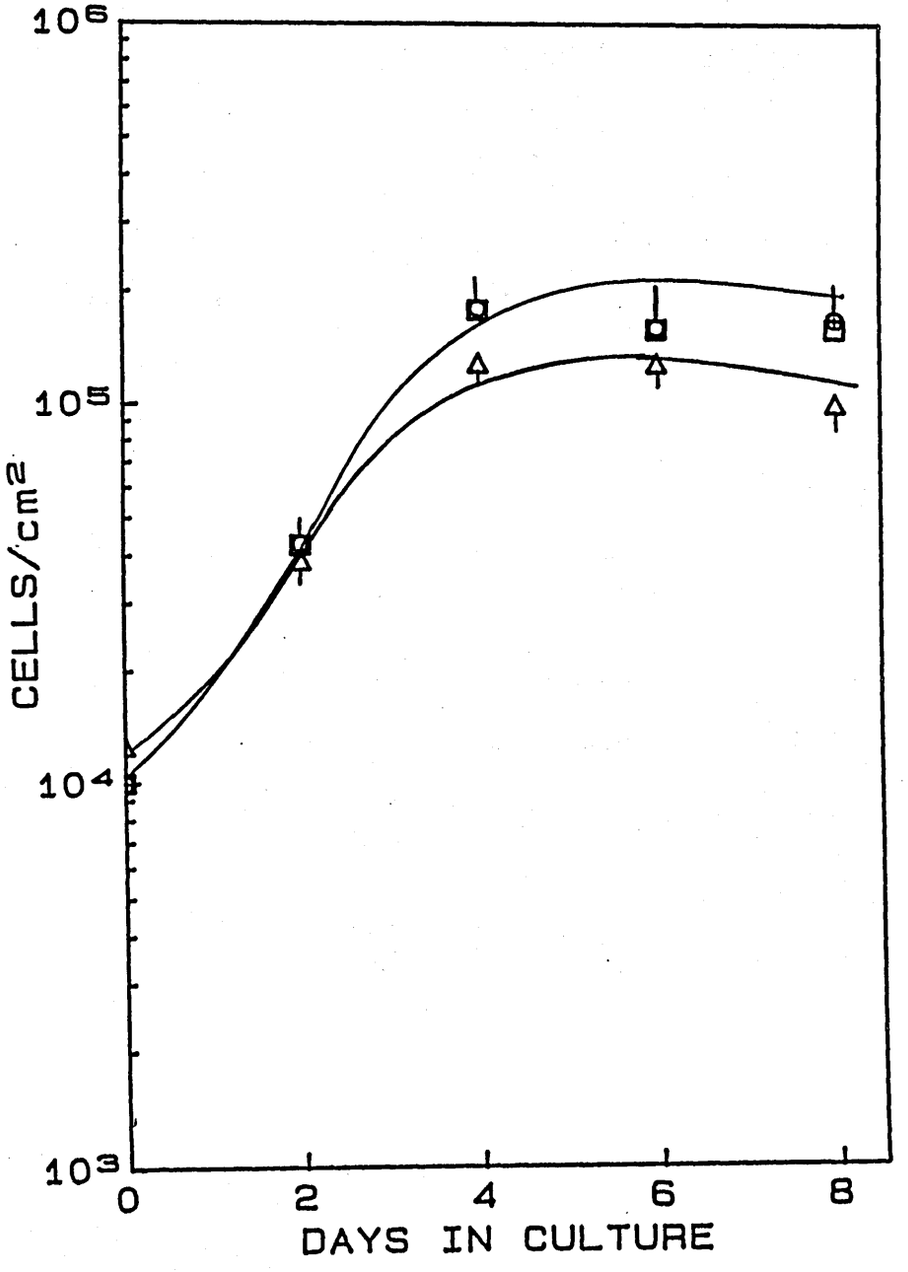
FIGURE 20

A549 growth curve in the presence of fibroblast conditioned medium.

A549 was treated with either MOG-LF113 conditioned medium or diluted MOG-LF113 conditioned medium and the effect on cell growth measured by direct cell counts as described in the text.

Each data point represents the mean of three observations ± SE of one representative experiment out of a series of three.

Circles = control (serum free medium)
Squares = steroid treated fibroblast conditioned medium (undiluted)
Triangles = steroid treated fibroblast conditioned medium (diluted 1:1 with fresh serum free medium).


10⁶
10⁵
10⁴
10³
CELLS/cm²
0
2
4
6
8
DAYS IN CULTURE

20.

As can be deduced from the graphs, conditioned medium had no effect on the growth of A549, either by direct cell counting or using an adaptation of the MTT assay. Similarily, there was no effect on LF113, the cell line from which the conditioned medium was derived. However, the medium was stimulatory to Swiss 3T3 fibroblasts (Figure 21), resulting in a threefold increase in optical density when the cells were treated with 100% conditioned medium.

4.7 HPLC ANALYSIS OF CONDITIONED MEDIUM

It was possible that dexamethasone still remained in the culture medium after the 6 hour recovery period, due to release of bound steroid from receptors. Obviously, if traces of steroid still remained, these could account for the increases in pulmonary surfactant synthesis seen after treatment with conditioned medium, since dexamethasone alone was found to stimulate surfactant synthesis. To determine whether or not dexamethasone was still present, HPLC analysis of both conditioned medium and intact cells was performed, using a modification of the technique of Derendorf et al (1986) - see Materials and Methods.

Figure 22 shows the chromatographs obtained from the conditioned medium and confirms that dexamethasone levels fell to 0.7nM 6 hours after steroid removal. With the cells, there was no steroid detected after the 6 hour recovery period (Table 6a,b). This

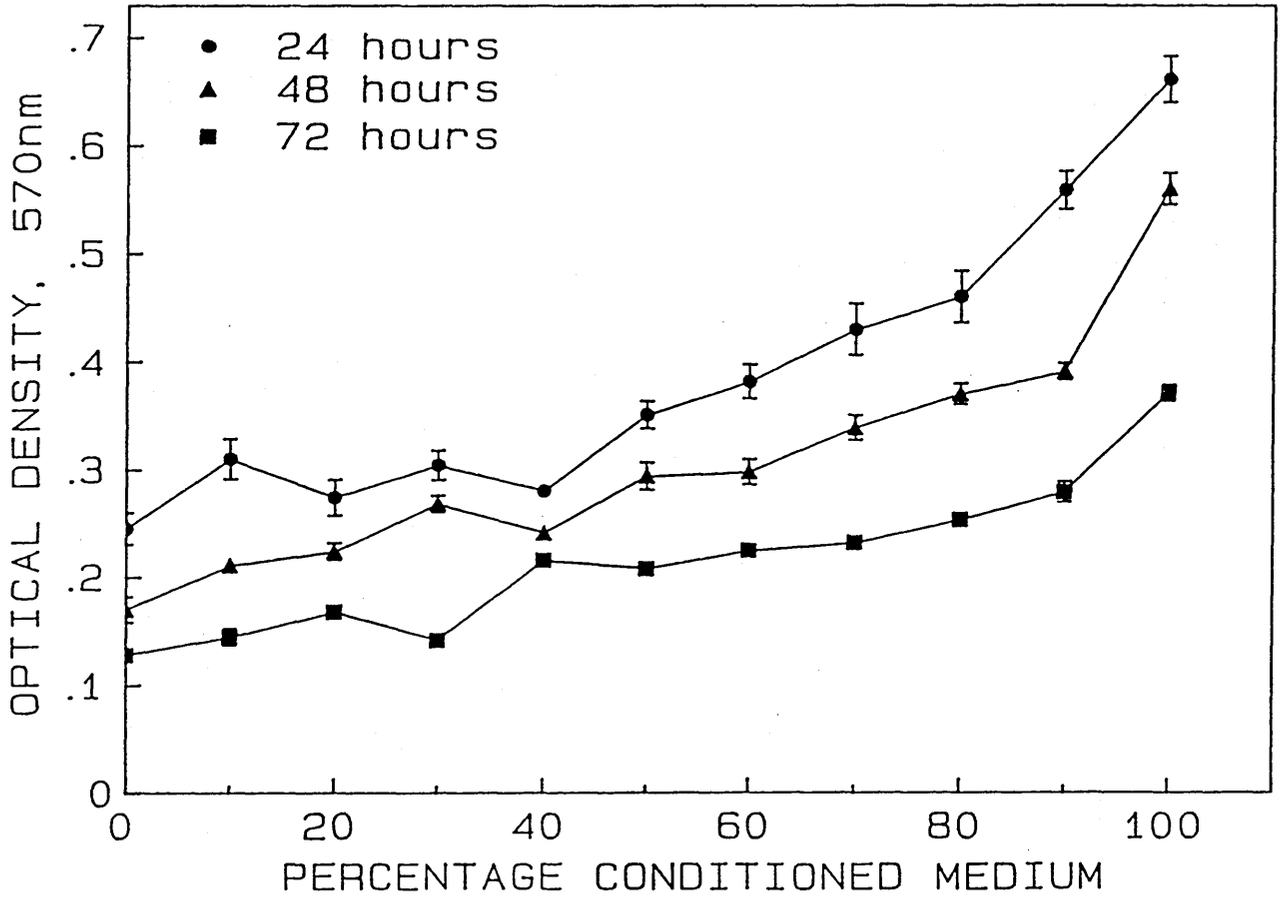

24 hours
48 hours
72 hours
OPTICAL DENSITY, 570nm
0
.1
.2
.3
.4
.5
.6
.7
0
20
40
60
80
100
PERCENTAGE CONDITIONED MEDIUM

FIGURE 21

Effect of MOG-LF113 conditioned medium on the growth of Swiss 3T3 fibroblasts.

Quiescent cultures of Swiss 3T3 fibroblasts were treated with 0-100% fibroblast conditioned medium and the effect on cell growth measured by MTT reduction as outlined in the text.

Each data point represents the mean of three observations $\pm$ SE of one representative experiment out of a series of three.

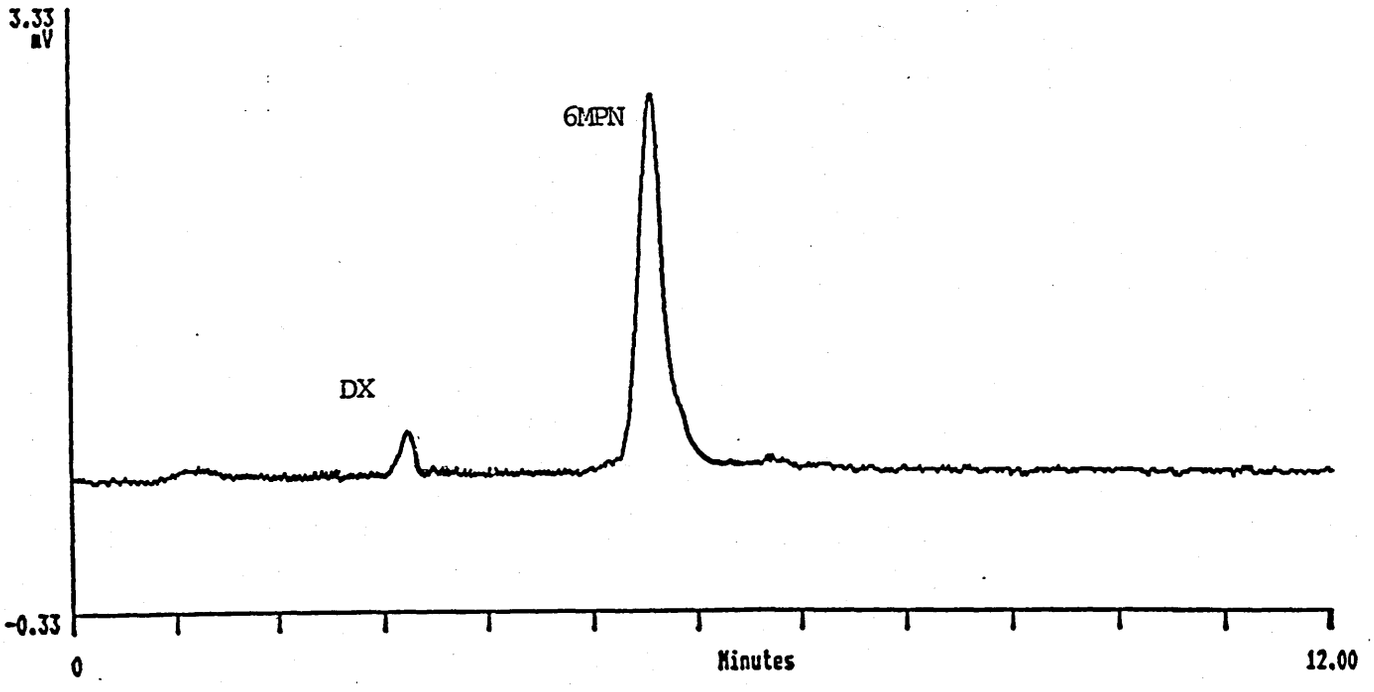

3.33
mV
6MPN
DX
-0.33
0
Minutes
12.00

FIGURE 22

HPLC chromatograph.

HPLC determination of conditioned medium was performed as described in the Materials and Methods.

DX = dexamethasone peak

6MPN = methylprednisolone internal standard for calculating dexamethasone recovery.

TABLE 6a: HPLC ANALYSIS OF STEROID TREATED FIBROBLAST EXTRACT.

TIME AFTER STEROID REMOVAL	DEXAMETHASONE DETECTED ($ug/10^6$ cells)
0	0.157
1 minute	0.11
5 minutes	0.101
10 minutes	0.059
30 minutes	0.026
2 hours	not detectable
4 hours	" "
6 hours	" "

TABLE 6b: HPLC ANALYSIS OF FIBROBLAST CONDITIONED MEDIUM.

TIME AFTER STEROID REMOVAL	DEXAMETHASONE DETECTED (ug/ml conditioned medium)
0	10.51
6 hours	0.289

confirms that the effects seen using the conditioned medium must have been due to a constituent(s) in the medium, since there was no significant carryover of dexamethasone.

4.8 ELECTRON MICROSCOPY

Electron microscopy was performed on A549 cells treated with either fibroblast conditioned medium or dexamethasone according to the Materials and Methods to see if there were any ultrastructural changes indicative of differentiation, particularily evidence of multilamellar body formation, a characteristic feature of type II cell maturity.

E.M. photographs of both the treated and control cells are shown in Plates 3 and 4, and there was a clear difference between control and treated cultures. Controls showed extensive surface microvilli, and the cells appeared rounded. There was little evidence of multilamellar body formation. Conversely, the treated cultures were much flatter, with little sign of surface microvilli, and the treated cells showed large numbers of multilamellar bodies within the cell cytoplasm.

PLATE 3: ELECTRON MICROGRAPH OF A549 CELLS GROWING ON A FILTER WELL.

Print magnification = 13 520X

A = apical surface

F = filter well membrane

Arrow = microvilli

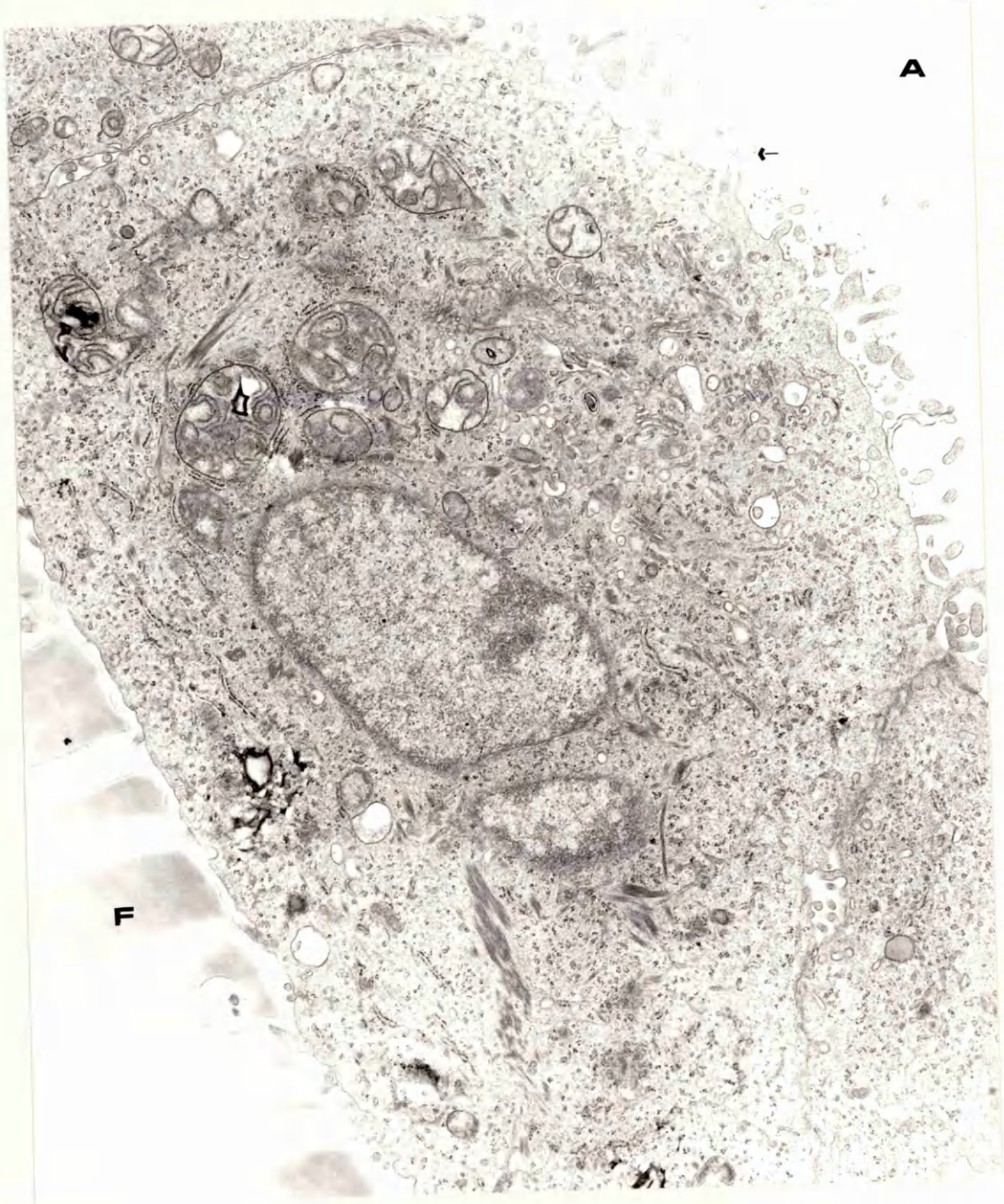

A
F

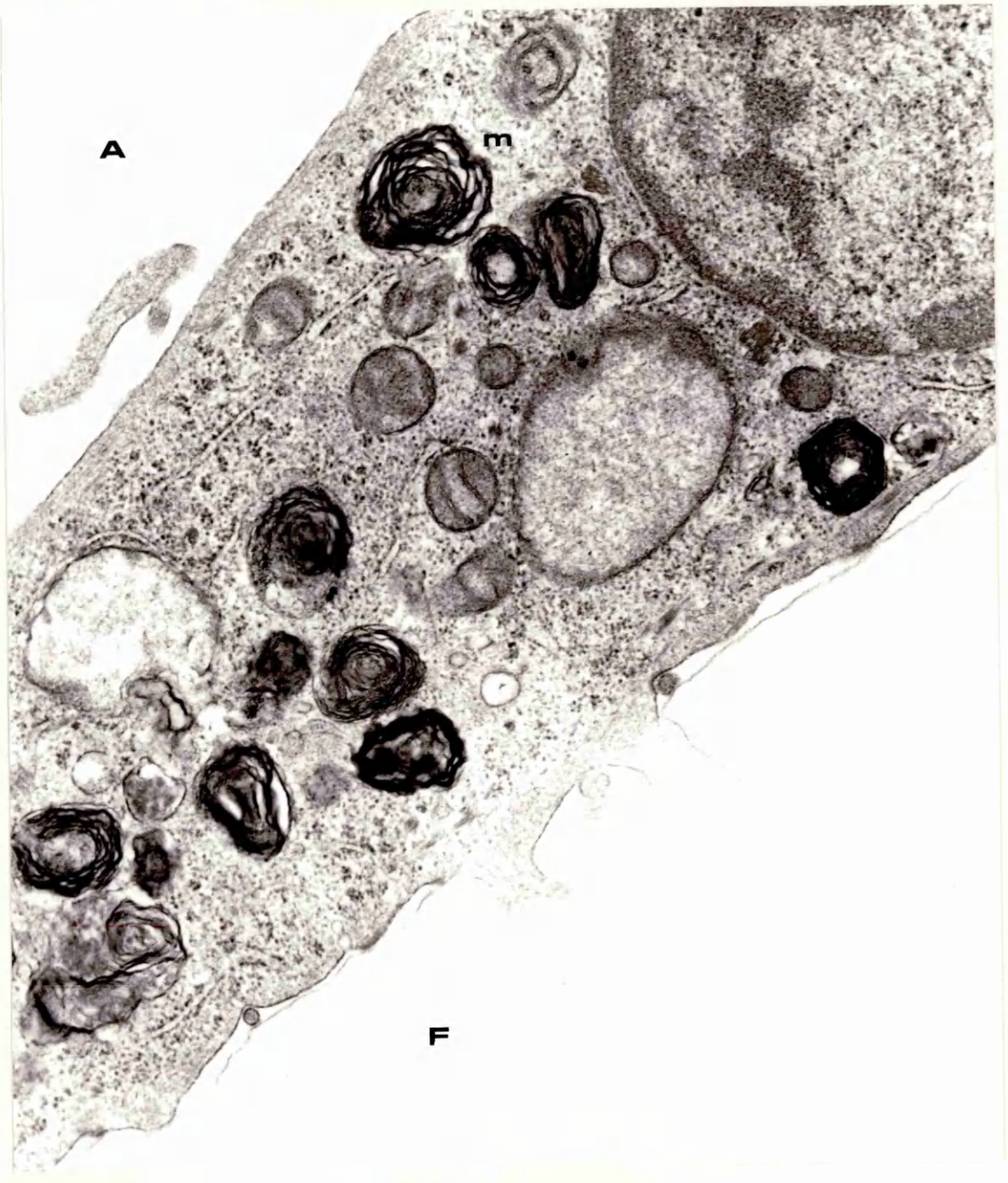
PLATE 4: EFFECT OF FIBROBLAST CONDITIONED MEDIUM ON A549 MORPHOLOGY BY ELECTRON MICROSCOPY.

Print magnification = 34 100X

m = multilamellar body.

A = apical surface

F = filter well membrane


A
m
F

DISCUSSION

The main objective of this Chapter was to determine if fibroblasts were important in the response of A549 cells to steroid, and to optimise the culture conditions permissive for differentiation in A549.

Induction of surfactant was maximised when A549 was cultured on a filter membrane rather than on a conventional plastic substrate. Other investigators have looked at the effects of different culture systems on surfactant biosynthesis. Doucet et al, (1987), using foetal rat lung, found that in their system, growth in semi-solid medium was preferred. They postulated that this may be due to better oxygenation of the cultures or improved biophysical properties of the substrate. It is also possible that culture in semi-solid media could improve cell shape or bring about the onset of polarity. Earlier, Douglas and Teel (1976), again using foetal rat lung, found the cell could reorganise to form alveolar-like structures when cultured on a 3-dimensional gelatin-sponge matrix. This did not happen on plastic. Zimmermann, (1987), has described an organoid culture system whereby lung cells were cultured at high density at the air-liquid interface. This permitted epithelial-mesenchymal cell sorting, the formation of an alveolar-like lumen and the formation of a basal lamina, all features which point to a more differentiated phenotype.

Relative to control values, the addition of fibroblasts to A549 cells caused a stimulation in pulmonary surfactant secretion, a 2.5-fold increase in the presence of steroid, and a 5-fold increase when steroid was absent. However, these observations were corrected for fibroblast-associated protein and were based on the assumption that fibroblasts cultured alone have the same growth characteristics as fibroblasts cocultured with A549. Attempts were made to try to separate the two cell populations using Percoll density gradient centrifugation, but it proved impossible to separate them cleanly (results not shown). Perhaps fortunately, an even better stimulation was observed when both cell types were cultured under transfilter conditions, demonstrating that surfactant secretion in A549 was not dependent on direct cell-cell contacts, and suggesting the involvment of a diffusible factor.

Cultures of foetal mouse lung could produce pulmonary surfactant in response to dexamethasone in agreement with earlier observations (de Lamos et al, 1970; Kotas and Avery, 1971; Torday 1980). The results obtained were comparable with the pulmonary surfactant values achieved for LF113/A549 cocultures. As with A549, the actual levels of surfactant were enhanced when the cells were cultured on a filter well. However, on the filter, production of surfactant did not seem to be much affected by the addition of steroid, suggesting the importance of the correct culture environment for expression of differentiated function. The effects of culture conditions on the growth of foetal mouse lung cultures has been reported by Hilfer et al (1988), who showed that cells grown at the

air-liquid interface in serum free medium ramified, a characterstic consistent with normal lung development, and contained multilamellar bodies, the intracellular storage sites for pulmonary surfactant. In submerged cultures, this did not occur, with a large central lumen forming instead of a branched tree.

However, the foetal lung cultures contained mixed cell populations, with stroma already present. The cultures were derived from 19 day mouse embryos; in the mouse, full term is 21 days, so it is possible that by 19 days, the fibroblasts were already "switched on" and pulmonary surfactant production was maximised. In this respect, it would be useful to look at fibroblasts from different developmental ages to see at which point they become switched on to induce pulmonary surfactant production in lung epithelium.

Another possibility is that differentiation of foetal mouse lung could be controlled by autocrine and paracrine factors intrinsic to the developmental program of embryonic lung, and independent of exogeneous hormonal controls. Such a proposal has also been put forward by Jaskoll et al (1988), who noticed that long term cultures of embryonic lung primordial cells cytodifferentiated and expressed pulmonary surfactant-associated proteins in the absence of exogeneous hormones or growth factors.

In the literature, two different schools of thought appear to have evolved regarding contact mediated epithelial-mesenchymal interaction versus diffusible epithelial-mesenchymal interactions,

and there appears to be some disagreement as to whether direct cell-cell contacts are necessary for epithelial-mesenchymal interactions. These points, and how they are related to the results of this study, will be discussed below.

Firstly, contact mediated responses. Adamson and King (1986), have shown that rats born to mothers injected 2 days before birth with steroid showed not only accelerated lung development, but also a greater frequency of epithelial-mesenchymal cell contacts, supporting the concept that regulatory messages are passed directly from the foetal fibroblast to specific epithelial cell types, initiating the onset of pulmonary surfactant production. Moving away from lung, cultures of human hepatocytes again maintained a more differentiated phenotype when grown in coculture with diploid human fibroblast feeder layers (Michaelopoulos et al, 1979). They suggested that the close proximity of the fibroblasts may allow reciprocal exchange of the products of metabolism between the two cell types. Perhaps the most convincing piece of evidence came from Kedinger et al (1987), who showed that glucocorticoids could influence structural differentiation of intestinal endodermal cells. This only happened under coculture conditions with fibroblasts, suggesting that there was a mesenchymal dependence on the hormonal response, and that this required direct cell-cell contact.

In the same way, primary cultures of adult hamster pancreatic cells could be maintained on feeder layers of irradiated

fibroblasts (Malick et al, 1981). The pancreatic cells retained many differentiated properties, notably insulin production. The preponderance of mitotic figures observed in the pancreatic cell cultures indicated that epithelial-fibroblast interactions were beneficial for growth and proliferation of the pancreatic cells, and retarded the process of dedifferentiation and resultant loss of differentiated functions.

Similarily, mammary epithelial cells cocultured with irradiated Swiss 3T3 fibroblast feeder layers showed that direct epithelial-mesenchymal contacts permitted the mammary epithelium to undergo hormone dependent differentiation, with secretion of casein into the culture medium, after a brief period in coculture (Levine and Stockdale, 1985).

Equally, there are examples in which differentiation does not appear to be mediated by direct cell-cell contacts. In 1953, Grobstein showed that isolated mouse mesenchyme could prevent the induction of morphogenesis, when both cell types were cultured on opposite sides of a Millipore filter. Similarily, Wolters and Van Mullen (1977), cultured epithelium and mesenchyme from 20-day old rat embryo tooth germs on opposite sides of a Millipore filter. Although the cells did not touch, EM studies revealed that cell processes from both cell populations penetrated into the pores of the filter, but failed to make contact. Nevertheless, the epithelial cells underwent differentiation, implying that cell contact was unnecessary in this system. More recently, Fusenig,

(1986;1989) has demonstrated that full epidermal keratinisation of skin in vitro is only possible when dermal and epidermal components are cultured together, not in direct contact, but separated by a layer of collagen. This implies the intervention of a diffusible "message".

Bohnert and colleagues (1986) carried out an elaborate series of experiments to determine the effects of both mesenchyme and substratum on epidermal cell differentiation. Cells cultured on plastic showed incomplete maturation whilst the same cells cultured on a floating collagen raft displayed improved cell orientation and tissue organisation. Why should this be so? When a collagen raft is detached from the base of a culture vessel, it shrinks, so it is possible that this permits the epithelial cells to change shape, thereby establishing the correct morphology for the expression of a differentiated phenotype. The same group (Bohnert et al, 1986), then looked at the consequences of adding mesenchymal elements to the system. On floating collagen plus mesenchyme, the cells were very well differentiated, with an organised epithelium. Similar results were obtained when the cells were cultured with a layer of collagen interposed between the epithelial and mesenchymal elements. This implied that direct cell contact was not necessary for the onset of differentiated functions.

In cancerous breast, Adams et al (1988a,b), showed that conditioned medium from human breast fibroblasts affected the in vitro growth and 17 beta-oestradiol dehydrogenase activity in MCF-7 cells in

culture. Moreover, they showed that fibroblasts derived from normal breast tissue could secrete factors which could inhibit the growth of MCF-7, whilst fibroblasts from malignant breast tumours secreted factors which actually stimulated growth. Therefore, breast stromal tissues have a strong paracrine effect on the surrounding epithelial cells, not only on growth but also on intracellular enzyme activities as well. Purification of fibroblast conditioned medium has revaled a trypsin sensitive polypeptide, which eluted from a Sephadex G-75 column with a molecular weight of around 50kDa. The observed paracrine activity, at least in part, has been attributed to secretion of this factor (Adams et al, 1988b).

In the same way, Post et al (1984), using rat type II epithelial cells showed that the induction of surfactant could be influenced not only by hydrocortisone in the presence of lung fibroblasts, but also by conditioned medium from steroid treated lung fibroblasts. Inhibition of this induction was possible using a monoclonal antibody raised against a factor purified from fibroblast conditioned medium.

The observations made here showed that direct cell-cell contact was unnecessary for the induction of a more differentiated phenotype in A549 cells by glucocorticoid treated fibroblasts. How does this fit in with regard to the observations described above?

It could be argued from the results of Post et al (1984), and Bohnert et al (1986), cited above, that in the lung, direct cell-

cell contact is not required for the induction of differentiation. However, the results of Adamson and King (1986), would appear to refute this claim. One possible explanation may be a difference between in vitro and in vivo response to embryonic induction. In vitro, Adamson and King (1986), noted an increase in epithelial-mesenchymal cell contacts following steroid administration to pregnant rats. In vivo, it would make good sense to pass regulatory substances directly from one cell type to another. This would reduce the chances of the substance being lost e.g. in the bloodstream, or being degraded by e.g. macrophages. By having direct cell-cell contacts, this would significantly reduce the chances of losing such information. In vitro, one is dealing with a contained environment, and any regulatory molecule which is produced cannot escape, so it has a greater likelihood of reaching the epithelium.

Another possibility is the differences between normal and transformed cells. However, all the lung examples cited dealt only with normal cells. In the breast system (Adams et al, 1988a,b), where a paracrine factor was implicated, transformed cells were used, and this was also the case in the present study. There appears to be no clearcut reason why some cells need contact for the expression of differentiated function, whilst others do not, and it may be that different cell lineages have different requirements at different times. Indeed, the possibility that both contact and diffusible factors are necessary for optimum differentiation cannot be ruled out.

Most of these examples, (with the notable exception of breast), have been drawn from studies using normal lung epithelium not with a transformed line such as A549, and it is perhaps surprising that this cell line, which is released from the normal constraints of growth, can respond in such a way.

One possible explanation maybe the re-establishment of polarity. This is a normal condition in vivo for many epithelial cell types. In vivo, concentration gradients of nutrients and growth regulating factors are maintained across basement membranes and cell layers. Cells may attach to the basement membrane on the basal surface and feed there, whilst showing more specialised differentiated features at their apical surface. Many studies have shown the influence of the substratum on the survival and growth of epithelial cell types (Emerman and Pitelka, 1977; Lillie et al, 1980 and Chambard et al, 1981). This can affect their response to specific growth factors and the concomitant expression of differentiated functions (Lee et al, 1985). Indeed A549 cells were able to respond better when cultured on a filter well membrane than when growing on a conventional plastic substrate, therefore the possibility of polarity being important cannot be discounted. The development of polarity may also be dependent on the degree of differentiation of a cell. A549 is a reasonably well differentiated tumour, so might be expected to respond better when given the opportunity to become polarised.

Another interesting aspect of the requirement for direct cell-cell contacts is the apparent sex-related differences in the fibroblast response to steroid, and subsquent production of pulmonary surfactant. Sex-related differences in lung structure (Adamson and King, 1984a,b) and surfactant synthesis (Torday et al, 1981) have been identified in normal lung development, and it appears that the reason for the difference lies with the fibroblast (Torday, 1984). It has been shown that female fibroblasts have greater oxidoreductase activity, which is necessary for the conversion of cortisone to cortisol. Moreover, female fibroblasts secrete higher levels of fibroblast pneumonocyte factor, especially in response to cortisol (Torday, 1984). Similarily, Adamson and King (1986), have shown there to be greater numbers of epithelial-interstitial cell contacts in female fibroblasts, and more of the epithelial cells contained lamellar bodies.

LF113 fibroblasts were derived from a foetus, the sex of which, was unknown. It is interesting to speculate that the responses seen here may have been enhanced because the fibroblasts were actually female derived.

Fibroblast conditioned medium was also found to be stimulatory to A549 cells, although it has to be said, much less so than the presence of fibroblasts. HPLC analysis of the conditioned medium showed little evidence of steroid carryover, so it is probable that the observations made were due entirely to a factor(s) present in the conditioned medium. The reduction in stimulatory capacity

relative to coculture and transfilter results may be attributed to 3 possible explanations. (1) Although there was no direct cell-cell contact when the cells were cultured under transfilter conditions, it is possible that there may be reciprocal cell-cell communications, via, e.g. growth factors or differentiation factors. Perhaps one or both cell types could respond to a factor(s) produced by the other cell type, which in turn may "switch on" surfactant synthesis in A549. (2) Any factor(s) being produced from the steroid treated fibroblasts may have a very short half life in culture, or it may even require to be alkalified or acidified before it becomes active, as is the case with TGF-beta (Sporn et al, 1987). (3) In the conditioned medium experiments, to avoid the possibility of nutrient exhaustion, the medium was diluted 1:1 with fresh culture medium, so if the factor concentration is limiting, further dilution will reduce the activity.

Conditioned medium was neither stimulatory nor inhibitory to the growth of A549 in vitro, either by direct cell counting or by the MTT assay. This suggests that the factor(s) present in the medium is having a direct effect on surfactant synthesis rather than on growth regulation. The medium had no effect on LF113 cells either, so it can be assumed that the factor(s) is not an autocrine growth regulator for the cells from which it was derived. Interestingly, the medium was stimulatory to Swiss 3T3 fibroblasts in the MTT assay in adose dependent manner, resulting in a 3-fold increase in optical density (which is proportional to cell number), when the

cells were treated with 100% conditioned medium. Swiss 3T3 cells are known to respond to a number of exogeneous growth factors, notably bombesin, which can induce DNA synthesis and cell division in these cells (Woll and Rosengurtz, 1988). Indeed bombesin-like peptides are abundant in foetal lung (Wharton et al, 1978; Yamaguchi et al, 1983) and the mRNA for bombesin is maximally expressed at 16-30 weeks (Spindel et al, 1987). The LF113 cell line used here was derived from a first trimester foetus, corresponding to a gestational time of 12-24 weeks, so it is certainly possible that the stimulatory activity of the medium could be attributed to bombesin (although bombesin is usually derived from epithelial cells, not fibroblasts). However, this is entirely speculative at this stage, and arguments for and against bombesin will be discussed in a later chapter.

As well as changes at the biochemical level, there were alterations in cell morphology both at the light microscope and electron microscope level. A549 cells, when cocultured with fibroblasts, and examined under a light microscope seemed to form circular holes in the monolayer, with evidence of chromophilic material in the spaces so formed. From the surfactant results, one could speculate that the chromophilic material could possibly be surfactant, secreted by the A549 cells into putative "alveolar" structures. A549 coplated on top of the fibroblasts also showed a structural reorganisation, although, because of the density of the two cell populations, no evidence of chromophilic material could be seen.

In the absence of fibroblasts, A549 did not show any structural reorganisation. These cells were growing on non-tissue culture grade plastic, and would not really have been expected to have grown at all. Certainly, the cell growth was patchy, but the cells themselves looked like fairly typical A549 cultures. The probable reason for the growth is because the cells are transformed, they are less dependent on the substratum.

E.M. studies showed untreated A549 cells to have widely dispersed endoplasmic reticulum throughout the cell cytoplasm. Tonofilament bundles were abundant, a feature typical of epithelial cells. In control cultures, microvilli were evident, suggesting metabolic activity. Cells treated with conditioned medium generally had a more elongated shape and the cells tended to form a layer which was only 1-2 cells thick whilst the control cultures tended to pile up. In addition, there was no evidence of microvilli. Large multilamellar bodies were evident in the cytoplasm of most treated cells, suggesting surfactant synthesis and the potential for secretion. This concurs with the observation that conditioned medium actually stimulated surfactant production in A549, and the morphological observations would seem to point to a better differentiated cell.

Scanning electron micrographic observations of a number of cell lines, both tumour derived and cells tranformed in culture, have led several investigators to suggest that neoplastic cells show increased membrane activity in the form of ruffles, blebs and

microvilli (Pugh-Humphreys and Sinclair, 1970; Borek and Fenoglio, 1976; Gonda et al, 1976). It is possible that the surface topography of cells is correlated with levels of mitotic activity. Studies by Gonda et al (1976), with A549 have shown that this cell line does have extensive surface activity in the form of dense populations of stubby microvilli, agreeing with the observations made here with the control cells.

To summarise, the induction of pulmonary surfactant production in A549 was enhanced following culture on a filter well compared to a conventional plastic substrate. The glucocorticoid response was enhanced by incorporating fibroblasts into the culture. No direct cell-cell contact was necessary, suggesting the intervention of a diffusible factor(s), as confirmed by the activity demonstrated in conditioned medium. It appears that a transformed cell such as A549 can still respond to signals from normal lung mesenchymal cells.

CHAPTER FIVE

PARTIAL PURIFICATION OF FACTOR(S) FROM FIBROBLAST CONDITIONED MEDIUM

Conditioned medium was shown to be active in Chapter four, and it seemed likely that a diffusible factor was responsible. This chapter will describe a series of purification stages in an attempt to isolate and characterise the active component(s), and to reduce the possibility of steroid carryover.

Most of the experiments described in this Chapter were carried out at Glaxo Group Reseach, Greenford, Middx.

INTRODUCTION

Proteins can be separated from each other and other types of molecules on the basis of such characteristics as solubility, charge, specific affinity to chromatographic media, and size. These techniques were used either alone or in combination in an attempt to purify a factor or factor(s) from the conditioned medium shown to be active in the previous chapter.

Cell culture medium represents a complex mixture, containing salts, vitamins, glucose and various essential and non-essential amino acids, and conditioned medium is no exception, and is perhaps even more complex in that it contains metabolic cell products. To attempt to isolate the active component, it was necessary to reduce the complexity of the mixture, using a series of well known protein purification stages. The efficacy was evaluated at each stage by assaying for the ability to stimulate pulmonary surfactant secretion. Also the total amount of protein harvested was measured so that the degree of purification at each stage could be determined.

The first stage selected was ammonium sulphate precipitation. This represents perhaps the simplest form of protein purification and permits localisation of the active fraction by differential solubility. $(NH_2)_4SO_4$ readily precipitates proteins from solution, an effect called "salting out", and it is believed to do so by removing the water of hydration from the protein molecules.

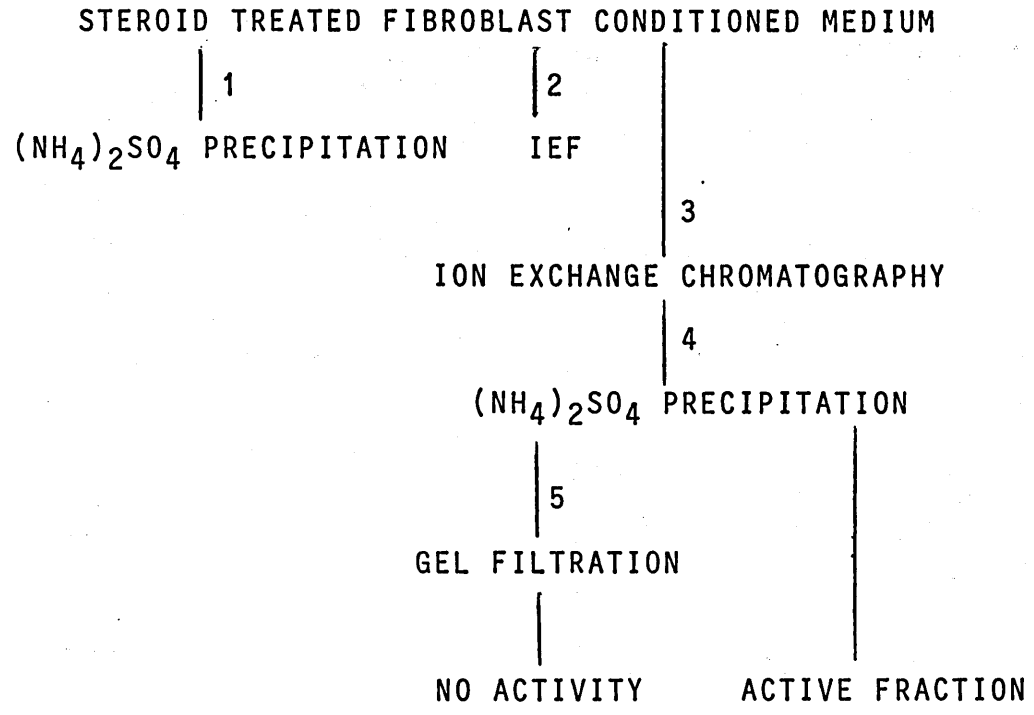
Proteins precipitated by salting out generally retain their native configuration, usually without denaturation. Moreover, $(NH_4)_2SO_4$ is preferred for salting out because it is so soluble in water that very high ionic strengths can be attained.

The isoelectric point of the active fraction, was determined using isoelectric focusing (IEF). IEF is based on the fact that the pH at which a protein is least soluble is it's isoelectric point (pI). This is defined as the pH at which the molecule has no net charge, and at this pH, it fails to move in an electric field. At pH values above or below the pI point, all protein molecules bear a net charge of the same sign, causing them to repel each other. Some proteins are virtually insoluble at their isoelectric pH, therefore this represents a good way of separating them.

Having established the pI value, this allowed the correct choice of an ion exchanger for the main purification step. Ion exchange chromatography separates biomolecules on the basis of their charge characteristics, and is related to, but functionally different from IEF. Ion exchange can very easily be scaled up (for large scale industrial preparations), or down (to pilot scale FPLC), depending on the volume of the mixture to be separated. This step was followed by $(NH_4)_2SO_4$. The sequence of purification steps is shown in Figure 23.

None of these techniques has considered the molecular size. Separations on the basis of size can be achieved using gel

FIGURE 23: SEQUENCE OF PROTEIN PURIFICATION STEPS



filtration chromatography whereby proteins pass through an insoluble but highly hydrated carbohydrate polymer in the form of beads. Small molecules can enter the beads whilst larger ones cannot. Thus the movement of small molecules through the gel bed is retarded. This technique was attempted in the latter stages of the purification process to try to determine the molecular weight.

Another way of determining molecular weights is to use polyacrylamide gel electrophoresis (SDS-PAGE). As well as being useful for molecular weight estimations, this technique proved invaluable at each stage of the purification, since, after staining, it permitted visualisation of individual bands, thereby determining the purity of the mixture at each stage. Also, by using molecular weight markers, and constructing a standard curve, the approximate weights of each band could be determined.

Having purified the factor(s), it was useful to know some of it's physicochemical properties, e.g. was it stable to heat, what was it's reaction to proteases and how did it react after treatment with acid or base; this can be correlated to the IEF experiment. These points are important for characterisation and help in identification.

Finally, an attempt was made to identify the factor(s) using two approaches. Firstly, the activities of the factor(s) were compared with those of known growth factors and secondly, as the factor(s) could be one of the better known growth factors, western blotting

was carried out to see if there was any recognition between the factor(s) and antibodies to known growth factors.

5.1 AMMONIUM SULPHATE PRECIPITATION

50ml aliquots of conditioned medium at 4^0C were precipitated by 4 different salt concentrations, 20,40,60 and 80% w/v, as described in the Materials and Methods chapter. The precipitated samples were resuspended in 2ml of PBS and dialysed extensively overnight. By reducing the volume to 2ml, the medium had been effectively concentrated 25-fold. The total protein collected, as measured by the Bradford assay was 154ug, from a starting volume of 50ml of conditioned medium.

100ul aliquots of each sample, and the final supernatant were incubated with A549 cells in filter wells for 48 hours, as this was the time required to bring about maximum response with conditioned medium, and the cells were then assayed for pulmonary surfactant production as described in the Materials and Methods section.

Figure 24 shows that maximum stimulation was achieved when the cells were incubated with aliquots of the 60% fraction (from now on referred to as MOG-FDF/I) resulting in a 2.6-fold stimulation in surfactant secretion. The total protein present in the MOG-FDF/I was 52.0ug, so the actual concentration of MOG-FDF/I added to the cells was 1.48ug/ml.

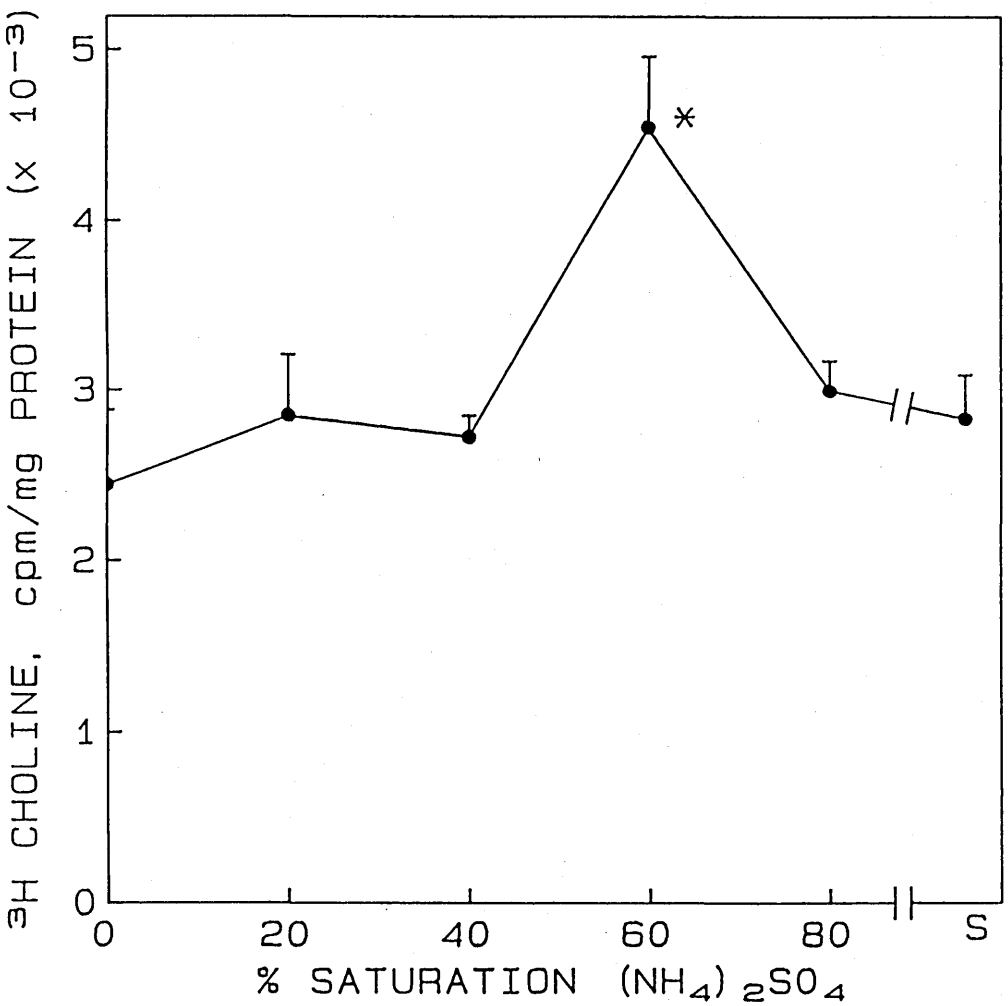

^{3}H CHOLINE, cpm/mg PROTEIN (x 10^{-3})
0
1
2
3
4
5
*
0
20
40
60
80
S
% SATURATION $(NH_4)_2SO_4$

FIGURE 24

Effect of $(NH_4)_2SO_4$ precipitated conditioned medium on pulmonary surfactant production in A549.

A549 cells growing on filter wells were incubated with protein precipitates from conditioned medium and assayed for pulmonary surfactant production as described in the text. S = final supernatant remaining after the precipitation steps.

* $p < 0.02$

Each data point represents the mean of four separate experiments $\pm$ SE

TABLE 7: EFFECT OF $(NH_4)_2SO_4$ PRECIPITATES ON PULMONARY SURFACTANT PRODUCTION IN A549.

% SATURATION $(NH_4)_2SO_4$	% STIMULATION (relative to control)
0	0
20	15
40	6
60	87
80	23
FINAL SUPERNATANT	19

A summary of activities relative to untreated controls is shown in Table 7.

An SDS-PAGE gel of the 4 fractions was performed under reducing conditions (see Materials and Methods chapter), and this is shown in Plate 5. The samples were by no means pure - there was still quite a lot of high molecular weight proteins remaining (>150kDa), which had barely entered the resolving gel, as well as evidence of BSA carryover, even although the samples were prepared from serum free medium. At this stage, it was impossible to determine exactly where the activity lay, as there was no band unique to MOG-FDF/I.

5.2 ISOELECTRIC FOCUSING OF CONDITIONED MEDIUM

Isoelectric focusing was carried out on conditioned medium, dialysed overnight against Tris-HCl, pH 7.4, according to the protocol described in the Materials and Methods section. This yielded 20 separate fractions of approximately 2ml. The pH of each fraction was measured.

The total protein harvested, using the Bradford assay, was 125ug from a starting volume of 50ml of dialysed conditioned medium. Each fraction was tested for stimulatory activity in the surfactant assay, as described in section 5.1. The results are shown in Figure 25. Greatest stimulation was seen in fraction 12 (MOG-FDF/II), at an approximate concentration of 0.15ug/ml, which had a pI corresponding to pH 8.77. As the graph shows, there was also a peak

PLATE 5: SDS-PAGE OF AMMONIUM SULPHATE PRECIPITATED SAMPLES.

Samples were run under reducing conditions as described in the Materials and Methods, and the bands visualised by silver staining.

STD = molecular weight standards, with approximate molecular weights shown beside the final lane.

1 = 20% $(NH_4)_2SO_4$ precipitate.

2 = 40% " " "

3 = 60% " " "

4 = 80% " " "

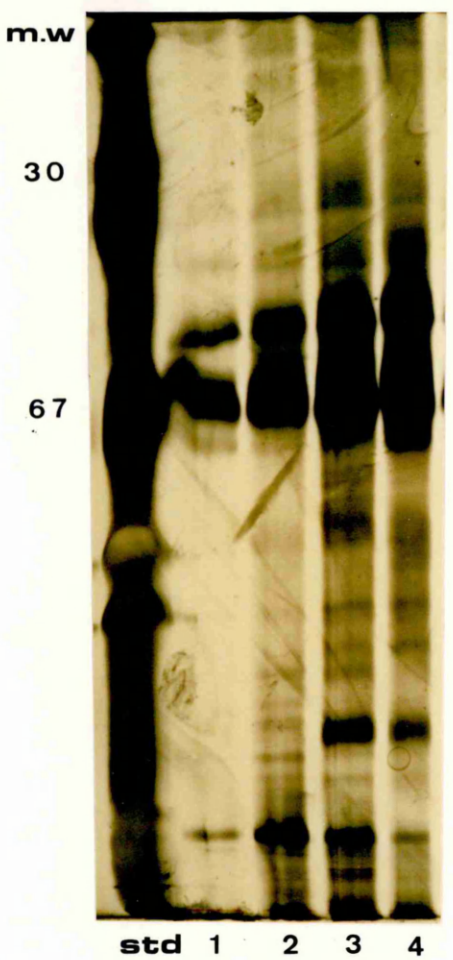
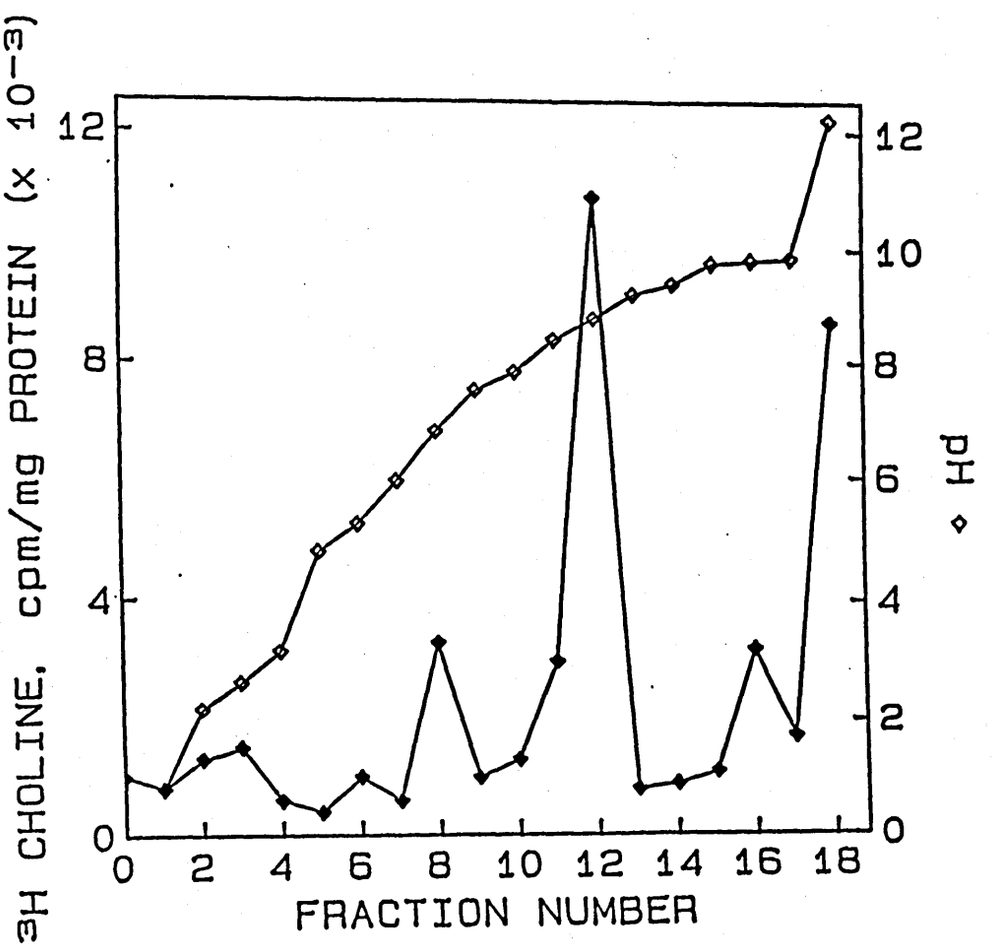

m.w
30
67
std 1 2 3 4

FIGURE 25

Isoelectric focusing activity profile.

IEF was performed as described in the Materials and Methods and the activity and pH of each fraction determined.

Each data point represents the mean of three observations ± SE


3H CHOLINE, cpm/mg PROTEIN (x 10-3)
pH
FRACTION NUMBER
0
2
4
6
8
10
12
14
16
18

of activity at fraction 18. However, this peak corresponds to a sharp pH change often observed towards the end of a run, due to localised high salt concentrations created during the run, and is generally regarded as an artefact of the system, rather than indicating a second pI value.

The active portion was contained in the fractions with a basic pI point, therefore the the protein(s) of interest was likely to be basic, with a pI value of around 8.77, the value for MOG-FDF/II.

Aliquots of each fraction were separated on an SDS-PAGE gel (Plate 6), but as with the previous purification step, there was no unique band in any one fraction, making it difficult to determine exactly where the activity lay. Molecular weight determination of all 20 fractions harvested was carried out using a standard curve, and the molecular weights ranged from 16-220kDa, encompassing an extremely broad spectrum. The fraction showing greatest activity in the assay, fraction 12, showed only 2 bands on the gel, with approximate molecular weights of 46 and 38kDa, but these were found throughout the 20 fractions.

5.3 LARGE SCALE PURIFICATION OF CONDITIONED MEDIUM BY ION EXCHANGE CHROMATOGRAPHY

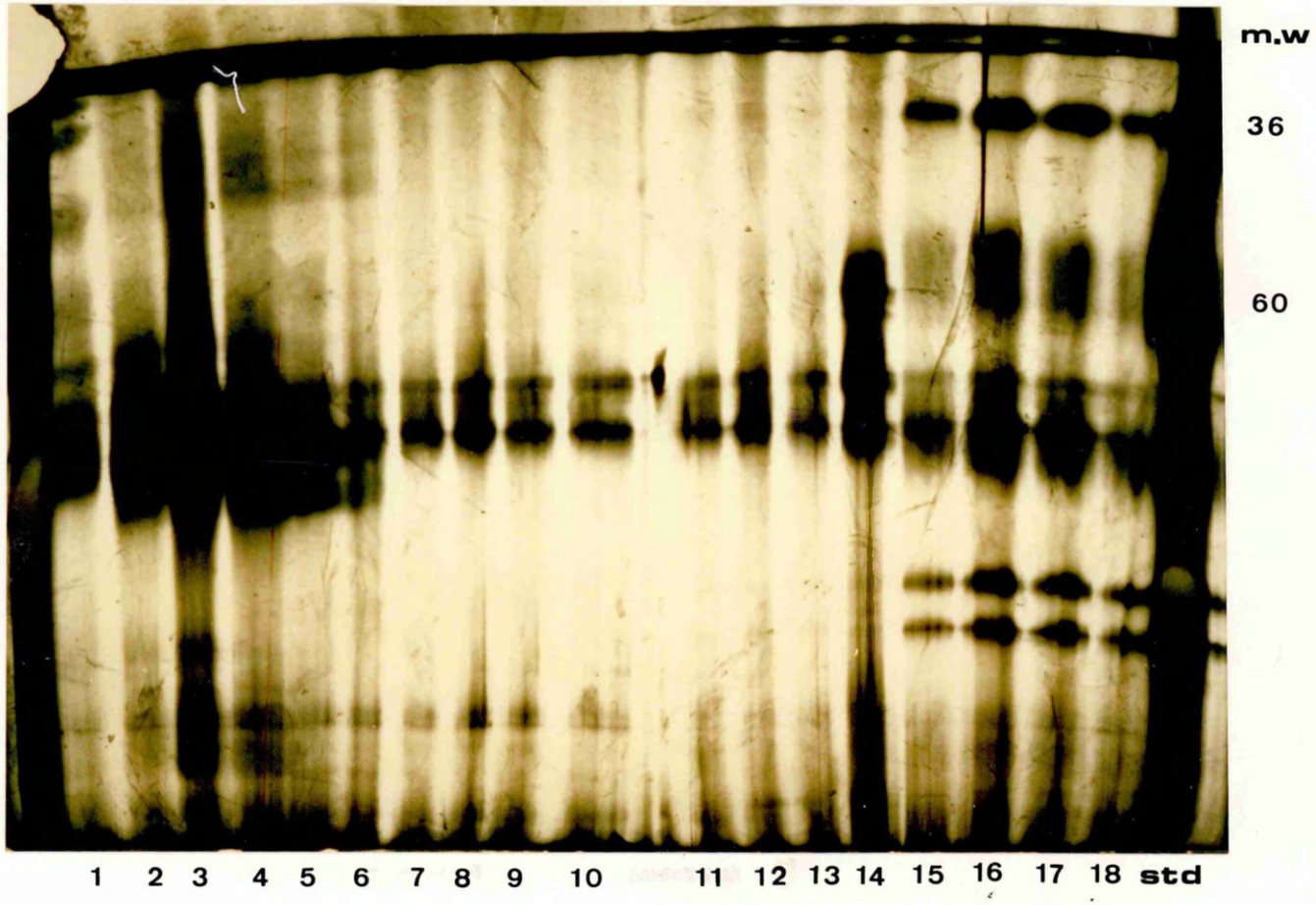
IEF analysis (section 5.2) of the conditioned medium showed the active fraction(s) to have a basic isoelectric point. For this

PLATE 6: SDS-PAGE OF IEF SAMPLES.

Samples harvested from the IEF experiment were run on SDS-PAGE under reducing conditions as described in the Materials and Methods.

Numbers 1-18 refer to the individual samples harvested. Maximum activity was observed in fraction 12.

STD = molecular weight standards, with approximate molecular weights shown next to the final lane.


m.w
36
60
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 std

reason, S-sepharose was chosen as the ion exchanger as it is a cation exchanger.

Eight fractions were harvested from the S-sepharose column, each of approximately 10ml. The total protein yield from these 8 fractions was 3.27mg. Each fraction was assayed for activity as in section 5.1. The activity profile is shown in Figure 26. Activity seemed to be contained in the first few fractions, particularily fraction 2, (MOG-FDF/III; approximate concentration of 1.64ug/ml , resulting in a maximum stimulation of 12-fold over the control values. This was the highest stimulation seen to date. However, this material from fraction 12 was still heterogeneous on SDS-PAGE analysis (Plate 7).

In an attempt to purify the S-sepharose eluate, fractions 1-8 were pooled, to prevent loss of any activity, then precipated in steps at 20,40,60 and 80% w/v $(NH_4)_2SO_4$, and resuspended in a small volume of PBS. Each fraction was assayed for activity and greatest activity was observed in the 60% fraction, MOG-FDF/IV, as previously observed with the unfractionated conditioned medium. A dose response to MOG-FDF/IV is shown in Figure 27.

The gel profile of MOG-FDF/IV was much purer than previously, as shown in Plate 8. Of the 12 bands present in this fraction, there was a unique band which had an estimated molecular weight of 30kDa. This may correspond to the active fraction, but this point will be discussed later.

To try to further purify the active fraction, an attempt was made at gel filtration, by FPLC. The four ammonium sulphate precipated

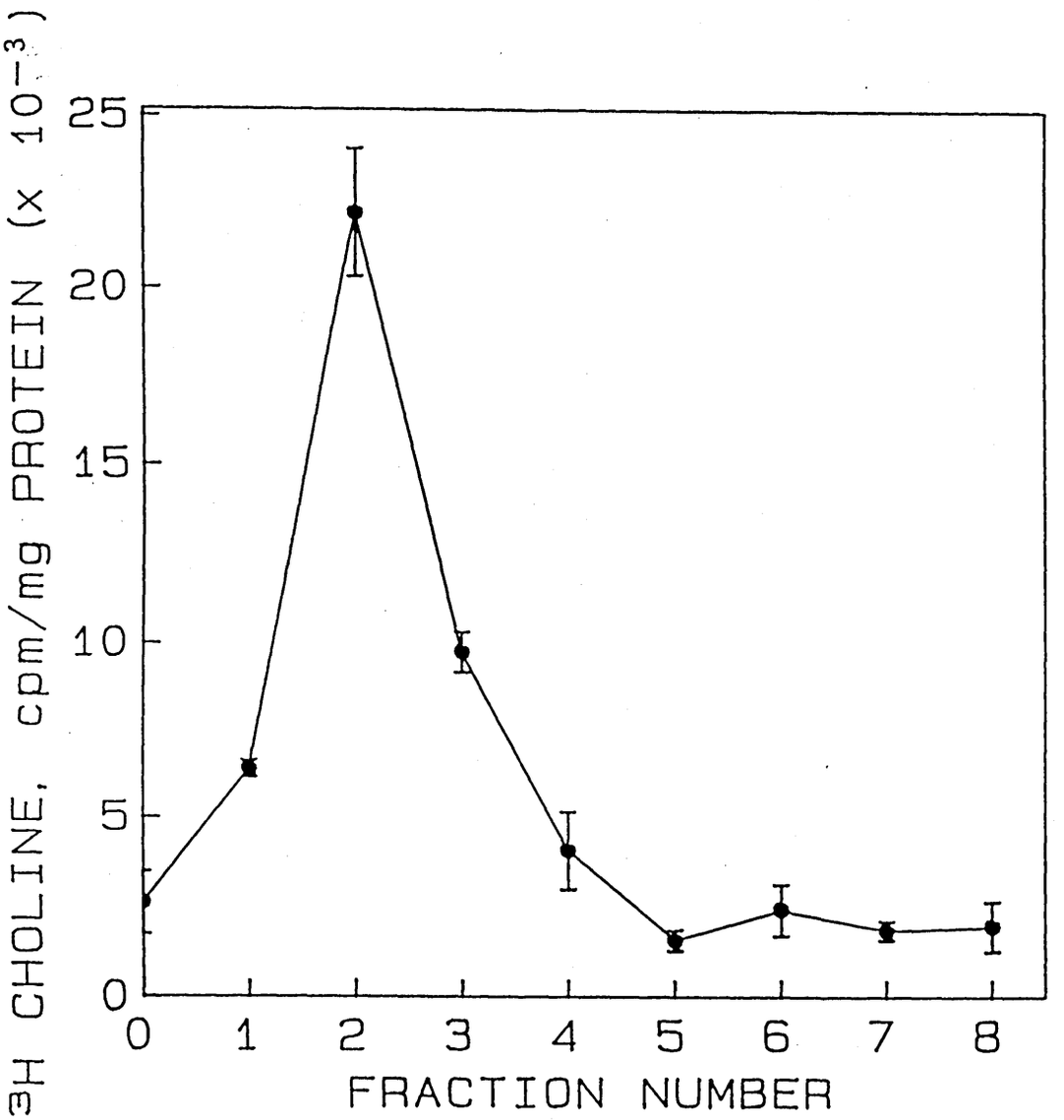

3H CHOLINE, cpm/mg PROTEIN (x 10⁻³)
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
FRACTION NUMBER

FIGURE 26

Activity of fractions eluted from S-sepharose ion exchange chromatography.

Conditioned medium was fractionated as described in the text and each fraction harvested assayed for stimulatory activity.

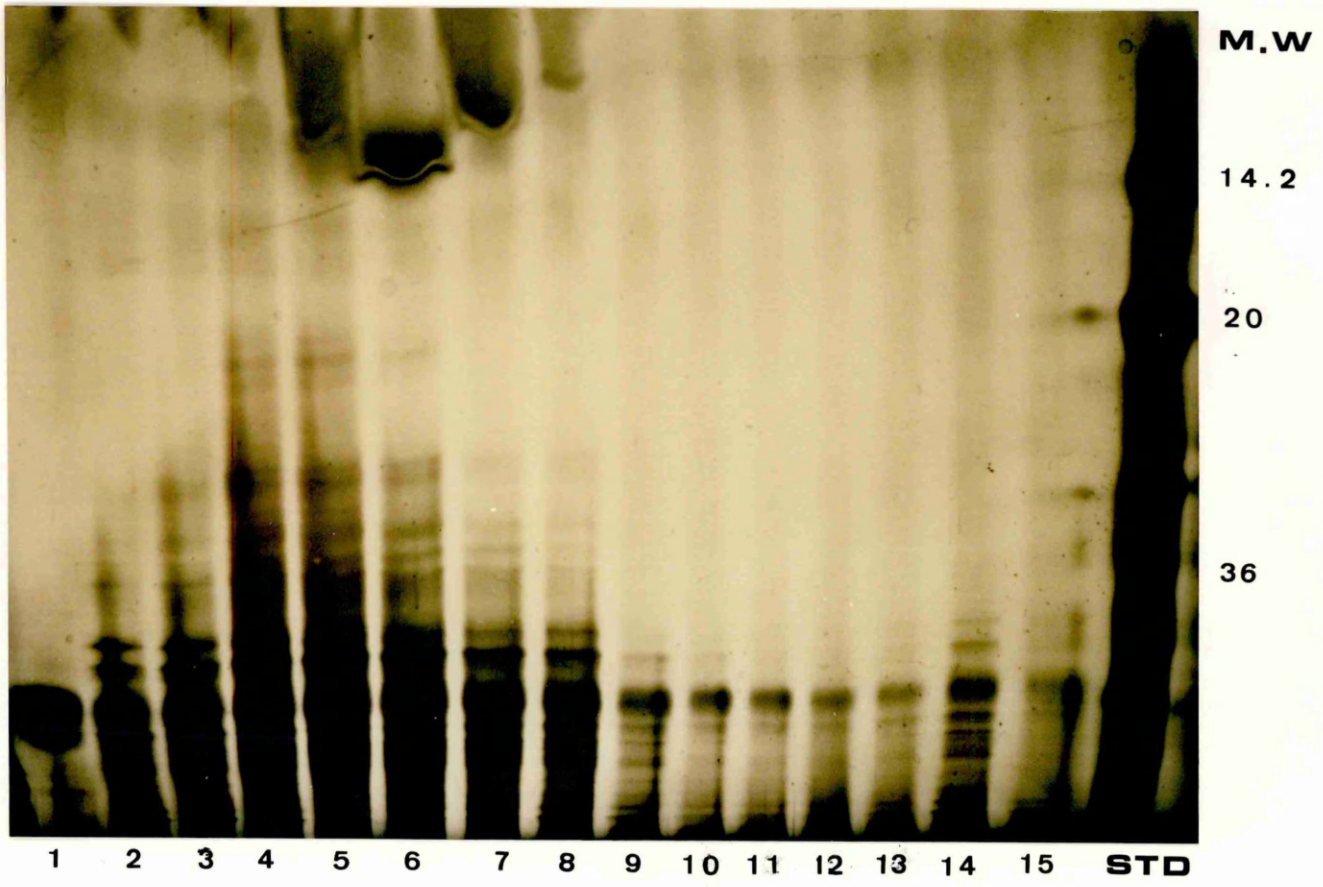
Each data point represents the mean of three observations ± SE of one representative experiment out of a series of three.

PLATE 7: SDS-PAGE OF SAMPLES ISOLATED BY S-SEPHAROSE ION EXCHANGE CHROMATOGRAPHY.

Fractions isolated from the S-sepharose column were electrophoresed under reducing conditions as described in the Methods.

Numbers 1-15 refer to individual samples harvested.

STD = molecular weight standards, with approximate molecular weights shown beside the final lane.


M.W
14.2
20
36
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
STD

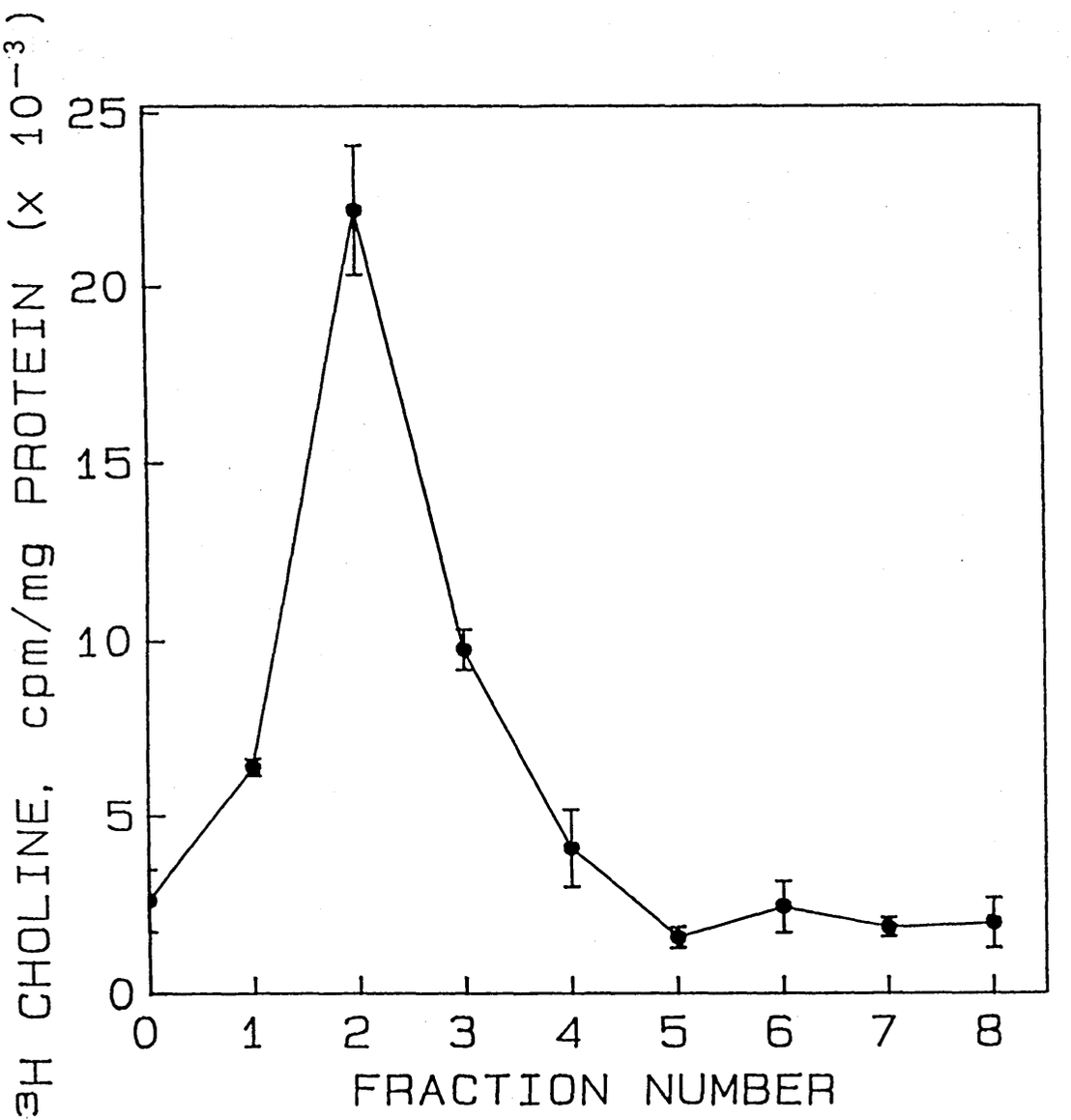

^{3}H CHOLINE, cpm/mg PROTEIN (x 10^{-3})
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
FRACTION NUMBER

FIGURE 27

A549 dose response to MOG-FDF/IV.

A549 cells growing in filter wells were treated with various concentrations of MOG-FDF/IV and assayed for pulmonary surfactant production as indicated in the text.

Each data point represents the mean of three separate experiments $\pm$ SE

PLATE 8: SDS-PAGE OF MOG-FDF/IV.

$(NH_4)_2SO_4$ precipitated S-sepharose samples were electrophoresed under reducing conditions as described in the Methods.

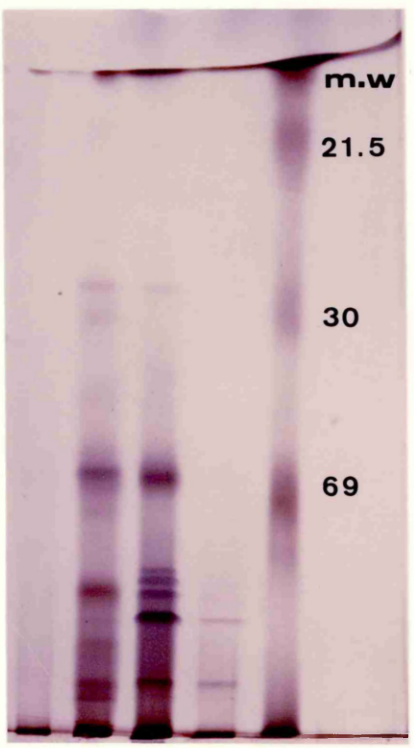
1 = 20% fraction

2 = 40% fraction

3 = 60% fraction (MOG-FDF/IV)

4 = 80% fraction

STD refers to the molecular weight standards, with approximate molecular weights shown next to the final lane.


m.w
21.5
30
69
4
3
2
1
std

samples, including MOG-FDF/IV, were loaded onto a Superose 12 column (Pharmacia) linked to the FPLC system. Superose 12 consists of cross linked agarose beads with a particle size of 30um, and is capable of fractionating samples with a molecular weight range of 1.0-300kDa. Before loading the sample, protein standards (kindly provided by Dr Roberto Solari, Glaxo Group Research, Greenford, Middx.) were loaded first. 200ul aliquots of each sample were injected onto the column and the fractions collected every 2 minutes in PBS. The trace obtained can be seen in Figure 28a. A calibration curve was constructed using the protein standards, and from this the approximate molecular weights of the samples could be determined (Table 8).

TABLE 8 Molecular weight estimation after gel filtration using a Superose 12 column

FRACTION NOS.	ELUTION DISTANCE (cm) (on chart paper	MOLECULAR WEIGHT (kDa)
10-13	3.2-4.1	>290
14-15	4.1-4.8	290-170
16-18	5.1	135
19-20	6.8	36
21-23	7.7-8.6	18-12.5
24-26	9.0-9.7	<10

However, on assaying the fractions, no activity could be detected

FIGURE 28a

Superose 12 FPLC trace.

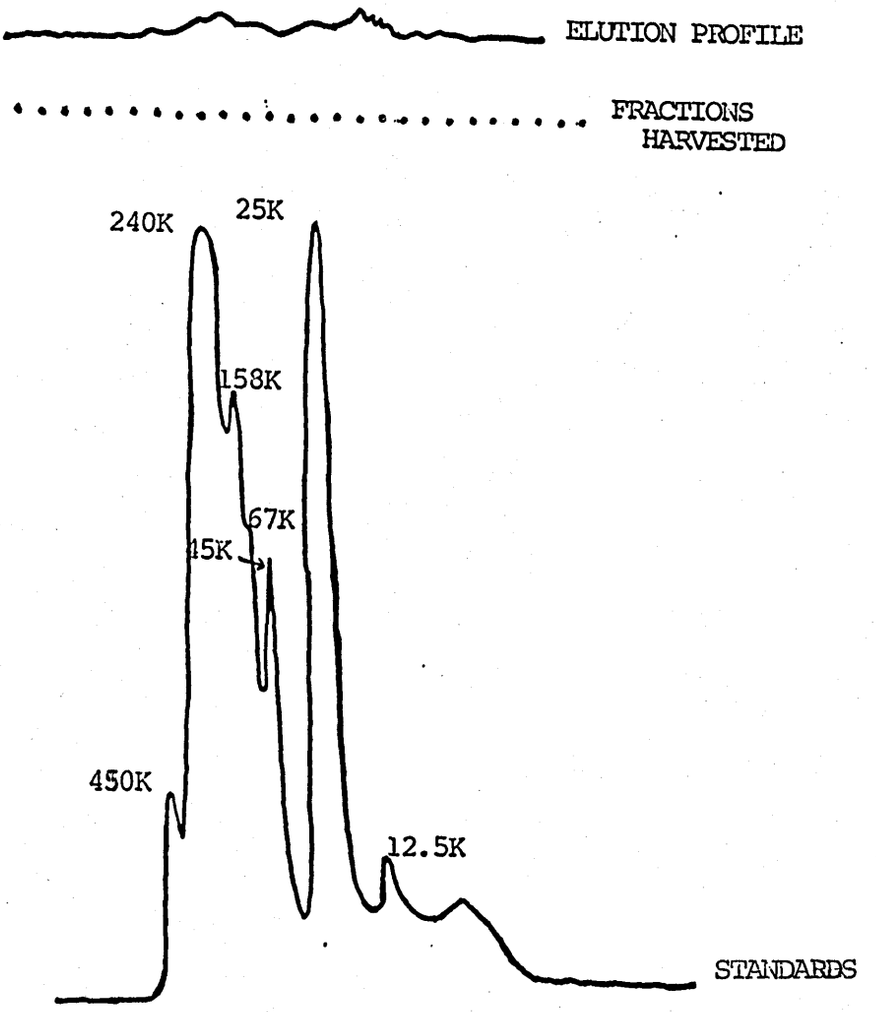
Chromatographic trace obtained after FPLC of MOG-FDF/IV

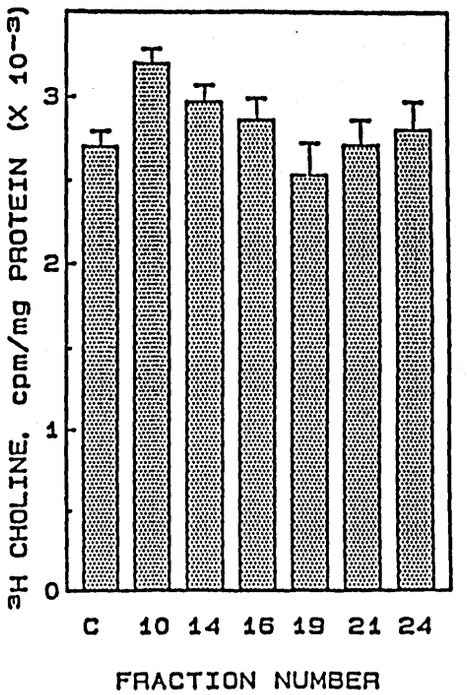
FIGURE 28b

Superose 12 activity profile

Fractions collected from the FPLC run above were assayed for stimulation of pulmonary surfactant production in A549 cells.

Each data point represents the mean of three observations $\pm$ SE of one representative experiment out of a series of three.


ELUTION PROFILE
FRACTIONS HARVESTED
240K
25K
158K
67K
45K
450K
12.5K
STANDARDS


^{3}H CHOLINE, cpm/mg PROTEIN (X 10^{-3})
0
1
2
3
C
10
14
16
19
21
24
FRACTION NUMBER

(Figure 28b). This could be because the loading capacity of the column was only 200ul, and the concentration of the protein solution was 97ug/ml, so each fraction collected contained extremely low amounts of activity, not sufficient to induce surfactant synthesis.

Although MOG-FDF/IV was still clearly heterogeneous, it was decided to proceed with further physicochemical and physiological characterisation, as the time available for further purification was limited, and determination of the phenotypic effects of MOG-FDF/IV was the prime objective. For this reason, MOG-FDF/IV was freeze dried and reconstituted in PBS and used for all subsequent assays. An outline of the purification stages used to isolate MOG-FDF/IV is shown in Figure 29.

The main purification stages, yield and enrichments are summarised in Figure 30 and Table 9.

5.4 PHYSICOCHEMICAL PROPERTIES

Aliquots of freeze dried MOG-FDF/IV, reconstituted in PBS were subjected to a variety of treatments and then assayed for their ability to stimulate pulmonary surfactant production.

FIGURE 29: OUTLINE OF THE MAIN PURIFICATION STAGES REQUIRED TO ISOLATE MOG-FDF/IV.

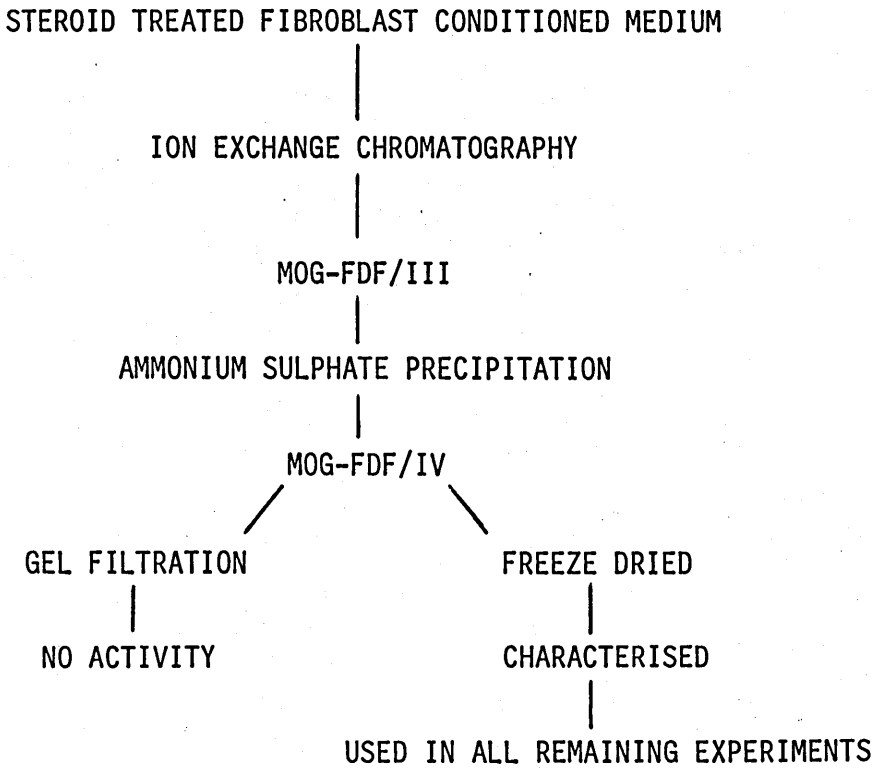


FIGURE 30: SUMMARY OF PURIFICATION STEPS.

AMMONIUM SULPHATE PRECIPITATION (MOG-FDF/I)

Starting volume = 50ml

Total protein harvested (after dialysis) = 153ug

Total protein in 60% fraction = 52ug

% recovery of protein in 60% fraction = 34%

ISOELECTRIC FOCUSING (MOG-FDF/II)

Starting volume = 50ml of dialysed conditoned medium

Total protein harvested = 125ug

Total protein in fraction 12 (most active) = 5ug

% recovery of protein in fraction 12 = 4.2%

ION EXCHANGE CHROMATOGRAPHY (MOG-FDF/III)

Starting volume = 20 litres (diluted 1:4 with 50mM MES buffer)

Total protein harvested = 411ug

Total protein in fraction 2 (most active) = 57ug

% recovery of protein in fraction 2 = 14%

AMMONIUM SULPHATE PRECIPITATION OF POOLED ION EXCHANGE FRACTIONS (MOG-FDF/IV)

Starting volume = 100ml

Total protein harvested = 343ug

Total protein in 60% fraction (most active) = 97ug

% recovery of protein in 60% fraction = 28%

TABLE 9: SUMMARY OF CONDITIONED MEDIUM PURIFICATION STEPS

PURIFICATION STEP	PS/mg PROTEIN	x-FOLD ENRICHMENT[a]
CONTROL	2050	1.0
MOG-FDF/I (60% ammonium sulphate)	4550	2.0
MOG-FDF/II (IEF fraction 12)	11235	5.0
MOG-FDF/III (ion exchange fraction 2)	22172	10.0
MOG-FDF/IV (60% ammonium sulphate ppt of pooled ion exchange fractions 1-8)	34939	17.0

PS = pulmonary surfactant, expressed as incorporation of ^{3}H-choline/mg cell protein.

[a]Related to serum free conditions

5.4.1 Heat treatment

Aliquots were either heated to 100^{0}C in a boiling water bath for 15 minutes then cooled rapidly on ice, or maintained at 56^{0}C for 30 minutes, then cooled on ice.

MOG-FDF/IV was stable to both regimes of heat treatment (Table 10).

5.4.2 Effects of proteases

Samples were incubated with either trypsin (100ug/ml; Worthington) for 2 hours at 37^{0}C or with pronase (Streptomyces griseus; Sigma) at 100ug/ml under the same conditions. The action of trypsin was stopped by the addition of soybean trypsin inhibitor (100ug/ml; Sigma). Pronase activity was terminated by boiling for 5 minutes to denature the enzyme.

The activity of MOG-FDF/IV was reduced by approximately 50% following protease digestion (Table 10).

5.4.3 Stability to acid/alkali

To test for acid stability, aliquots were acidified with an equal volume of 1.0M acetic acid at 4^{0}C for 1 hour, followed by neutralisation with 1.0M NaOH. To measure stability to alkali, aliquots were alkalified to pH 9.0 with 0.5M Tris-HCl for 1 hour at 4^{0}C, and then returned to pH 7.4 with 1.0M HCl.

MOG-FDF/IV was alkali stable but acid labile (Table 10).

TABLE 10: EFFECT OF PHYSICAL AND CHEMICAL TREATMENTS ON THE ABILITY OF MOG-FDF/IV TO STIMULATE PULMONARY SURFACTANT PRODUCTION.

TREATMENT	ACTIVITY (% UNTREATED CONTROL)
CONTROL	100
TRYPSIN (100ug/ml), 2hr 37^{0}C[a]	50
TRYPSIN (100ug/ml) + soybean inhibitor, 2hr 37^{0}C	90
PRONASE (100ug/ml), 2hr 37^{0}C[b]	56
HEAT 56^{0}C, 30 minutes	77
HEAT 100^{0}C, 5 minutes	81
ACID, 1M acetic[c]	50
BASE, pH 9.0[d]	95

[a] The treatment was terminated by the addition of soybean trypsin inhibitor, (100ug/ml).

[b] Pronase was obtained from Streptomyces griseus. Treatment was terminated by boiling for 5 minutes.

[c] The samples were acidified with 1M acetic acid for 1 hour at 4^{0}C, followed by neutralisation with 1M NaOH.

[d] The samples were alkalified to pH 9.0 with 0.5M Tris-HCl for 1 hour at 4^{0}C and then returned to pH 7.4 with 1M HCl.

MOG-FDF/IV appeared to be heat and alkali stable but acid labile and partially protease labile.

5.5 EFFECT OF KNOWN GROWTH FACTORS ON PULMONARY SURFACTANT SECRETION

Induction of pulmonary surfactant production by the activity of MOG-FDF/IV was compared with the activity of known growth factors to look for clues as to its possible identity. All the growth factors (kindly supplied by various people in the Dept. Cellular Sciences, Glaxo Group Research, Greenford, Middx.) were diluted with serum free medium to various predetermined concentrations (as used in assays currently in use at Glaxo). The results of the experiment were expressed as % of untreated control and are shown in Table 11 along with the concentrations of growth factors used.

Of the 8 growth factors assayed, 3 were capable of stimulating surfactant release, namely, PDGF, bFGF and IGF-1. Although insulin was apparently stimulatory, this was not statistically significant. However, none gave as large a stimulation as MOG-FDF/IV. The implications of these observations will be discussed later.

TABLE 11: EFFECTS OF KNOWN GROWTH FACTORS ON PULMONARY SURFACTANT PRODUCTION IN A549

GROWTH FACTOR	CONCENTRATION	% STIMULATION	MOLECULAR WEIGHT (kDa)
CONTROL	---	100	---
TGF-alpha	0.1uM	125	5.6
TGF-beta	1.0ng/ml	104	25
PDGF	3.0 units/ml	311*	32
bFGF	10.0ng/ml	183**	14-18
IGF-1	10.0ng/ml	175**	7
INSULIN	1.0ug/ml	238	5.7
EGF	0.1uM	131	6
BOMBESIN	0.1uM	115	1.6
MOG-FDF/IV	1.0ug/ml	1278***	?

* $p < 0.01$

** $p < 0.02$

*** $p < 0.002$

[NOTE: PDGF specific activity - 3.0 units/ml, 70 000U/mg]

Each observation represents the mean of three separate experiments. Statistical analysis was carried out using the Student's t-test (paired sample test).

5.6 WESTERN BLOTTING

In a further attempt to identify MOG-FDF/IV, a series of western blots were performed using 3 growth factor antibodies. These were selected on the basis of their biological properties and also on the results of section 5.5

5.6.1 TGF-beta blotting

Proteins were transferred onto nitrocellulose paper exactly as described in the Materials and Methods. To identify TGF-beta, a polyclonal anti-TGF-beta kit was used (British Biotechnology). The protocol used was identical to the manufacturers instructions but with the following exceptions:

1. The antibody (stock 2ug/ml in tris buffered saline (TBS) + 0.25% EIA grade gelatin) was diluted 1:500 with TBS + 1% EIA grade gelatin (BioRad).

2. The blot was incubated with the second antibody, biotin-conjugated anti-rabbit IgG, for 1 hour at room temperature, and then overnight at $4^{0}C$.

Detection was by the streptavidin-biotin method, according to the manufacturers instructions.

5.6.2 bFGF and PDGF blotting

Anti-bFGF (AMS Biotechnology) stock 10mg/ml in TBS + 0.25% EIA grade gelatin was diluted 1:500, and anti-PDGF (British Biotechnology) stock 10mg/ml in the same buffer was diluted 1:1000 in TBS + 1% EIA grade gelatin, and incubated with the blots for 1 hour at room temperature. This was followed by 3x10 minutes washes in TBS containing 1% Tween 20 (BioRad), incubation with the second antibody (goat anti-rabbit IgG; BioRad) at a dilution of 1:3000 for 1 hour at room temperature, then overnight at 4^{0}C. The blots were then washed extensively in TBS + 1% Tween 20.

Detection was by the diaminobenzidine (DAB) method. 10mg of DAB and 14ul of 30% hydrogen peroxide were added to a freshly prepared solution of 50mM Tris-HCl (pH 7.6) containing 100mM imidazole (all from Sigma). Once a sufficient level of staining had been attained, this solution was poured off and the blots stored in distilled water.

Photographs of representative blots are shown in Plates 9a,b,c.

It can be concluded from the blots that anti-TGF-beta does not seem to react with the factor. Similarily, purified TGF-beta when incubated with A549 cells did not seem to stimulate pulmonary surfactant secretion, so the possibility of the factor being TGF-beta can be effectively ruled out. The PDGF and bFGF blots showed

PLATE 9: REPRESENTATIVE WESTERN BLOTS.

Plate 9a: TGF-beta blot.

Arrow refers to the position of TGF-beta.

1 = MOG-FDF/IV

2 = TGF-bets standard

Plate 9b: bFGF blot.

1 = MOG-FDF/IV

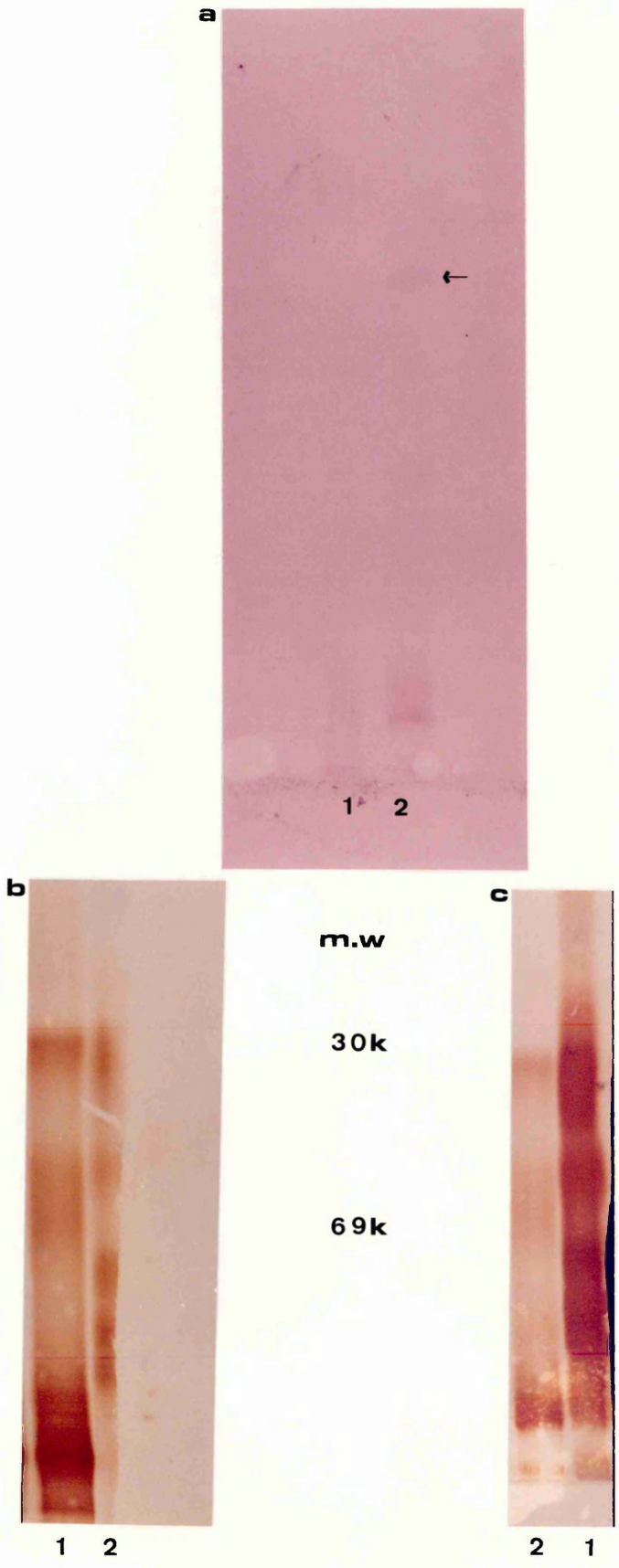
2 = FGF standard

Plate 9c: PDGF blot.

1 = MOG-FDF/IV

2 = PDGF standard

Approximate molecular weights were derived from a separate gel run under identical conditions.


a
1 2
b
m.w
30k
69k
1 2
c
2 1

high levels of nonspecific staining, making it difficult to ascertain, in the time available, if there was any specific binding between the factor and the antibodies. These points will be discussed later.

DISCUSSION

From the results presented in this Chapter, it was possible to purify partially and characterise a factor, or possibly a mixture of factors, from steroid treated fibroblast conditioned medium, using a number of different purification procedures. This factor(s), called MOG-FDF/IV, derived from fractions 1-8 from S-sepharose ion exchange, and then "salted out" by 60% $(NH_4)_2SO_4$, was capable of stimulating pulmonary surfactant production, 12-fold over control values, the highest level of stimulation of all the material tested.

Attempts were made to characterise MOG-FDF/IV using 3 methods; physicochemical properties, comparison of activities with other growth factors and western blotting using growth factor antibodies. The physicochemical properties will be discussed first.

Three different measurements were recorded, namely, the effects of proteases, heat and changing the pH. Incubation of MOG-FDF/IV with the proteolytic enzymes trypsin and pronase could only reduce the activity by one half. This finding was reasonable as far as trypsin

went, since it is a highly specific protease, cleaving polypeptides only on the carboxyl side of lysine and arginine residues. However, after treatment with pronase, which is a particularily agressive protease, since it is rather non specific, one might have expected the activity to be abolished completely. As it was not, this raised doubts as to whether all the stimulatory activity observed with regard to pulmonary surfactant synthesis was actually protein induced. Certainly there was a component of the stimulation which was protein induced, but it may well be that there was a non-protein component as well e.g. perhaps a carbohydrate or a glycolipid bound to a protein.

The possibility of steroid carryover cannot be ruled out, but it is unlikely, however, that steroid carryover was responsible. Firstly, HPLC analysis of conditioned medium (see Chapter 4) showed that < 0.7nM free steroid was detectable in the medium 6 hours after dexamethasone removal, the time at which the medium was collected, and from the dexamethasone dose response curves, > 0.1nM dexamethasone is required to stimulate pulmonary surfactant production. Secondly, treatment of the cells with dexamethasone alone gave an approximately 10-fold smaller stimulation than MOG-FDF/IV. The amount of pulmonary surfactant produced after treatment with dexamethasone was 3.27×10^3 cpm ^{3}H-choline incorporation/mg protein, and 3.79×10^4 cpm/mg protein after treatment with MOG-FDF/IV. Protease resistant activity was still 5-fold higher than the maximum obtained with dexamethasone at 10^{-7}M, and infinitely

higher than any stimulation with 10^{-10}M, the probable maximum concentration in conditioned medium.

MOG-FDF/IV was also found to be acid labile, but, as with the protease experiments, the activity could not be reduced by more than 50%. Perhaps the simple addition of MOG-FDF/IV, with some residual activity remaining after treatment, may still be enough to stimulate pulmonary surfactant, with the cleaved peptide still active but with reduced activity. Or, the treated factor(s) may still have shown a degree of recognition at the receptor site (maybe a reduced affinity), enough perhaps to trigger surfactant secretion.

Treatment with base had no effect on MOG-FDF/IV's ability to stimulate surfactant secretion in A549. This is reasonable, since the IEF experiments determined that the active fraction eluted with a basic pI point. This being the case, it is not surprising that acid treatment reduces the activity.

The molecular weight of MOG-FDF/IV was difficult to determine accurately, since gel filtration by FPLC failed to isolate an active fraction. However, in the 60% $(NH_4)_2SO_4$ precipated MOG-FDF/IV fraction, there was a unique band on SDS-PAGE, with an estimated molecular weight of 30kDa. It is possible that this band might correspond to the activity, but it may be that the activity could be attributed to a band undetectable, even with silver stain which supposedly can detect nanogram quantities of protein. Another

possibility is that MOG-FDF/IV may be glycosylated and this would give a false estimation of the molecular weight. Attempts were made to elute specific bands using native PAGE run under non-reducing conditions (data not shown), but it proved impossible to demonstrate any activity.

The effects of other growth factors on pulmonary surfactant secretion were also determined. Growth factors are defined as polypeptides which stimulate cell proliferation through binding to specific high affinity cell membrane receptors. They are thought not to act in an endocrine fashion, but rather to diffuse over a short range through intracellular spaces and act locally (in a paracrine manner). As well as being found in many adult and embryonic tissues, they are known to be released by a variety of cells in culture (Shields, 1978).

Of the 8 growth factors assayed, 3 showed some significant stimulatory activity, namely PDGF, bFGF and IGF-1. PDGF showed the greatest stimulation, although not as great as MOG-FDF/IV. Although insulin showed stimulation as well, it was not statistically significant.

PDGF is a major mitogen in serum and was originally isolated from expired human platelets where it is stored as a component of the alpha-granules (Ross and Vogel, 1978). On SDS polyacrylamide gels, two bands have been reported, in the molecular weight range 30-32kDa (Goustin et al, 1986). Each of the 2 bands are apparently

composed of dimers of 2 polypeptide chains designated A and B, and the different molecular weights of the 2 bands are thought to be the result of differential processing or degradation of the A chain ends as well as different degrees of glycosylation (Goustin et al, 1986).

Expression of PDGF or a PDGF-like molecule has been shown in a broad spectrum of cells, both transformed and untransformed (Bowen-Pope et al, 1984; Nimian et al, 1984) and whilst PDGF receptors have been found on a variety of mesenchymal cells (Heldin et al, 1981; Bowen-Pope et al, 1984), other than trophoblast cells, most epithelial cells tend to lack PDGF receptors (Heldin et al, 1981). A549, although transformed, is still an epithelial cell and could respond to PDGF, in apparent contradiction to the observations of Heldin et al, 1981. Fibroblasts do produce PDGF, in an autocrine stimulation loop in wound repair, so MOG-FDF/IV may be related to PDGF.

IGF-1 showed a slight stimulation in pulmonary surfactant secretion. Formerly known as somatomedin C, IGF-1 is the best known member of a family of insulin-like peptides, ancestrally related to proinsulin (Blundell and Humbel, 1980). Produced in response to circulating growth hormone, IGF-1 is one of the important growth factors found in serum and plasma (Svoboda et al, 1980), active in stimulating a number of cultured cells (Van Wyk et al, 1981). Moreover, IGF-1 appears to circulate in the plasma noncovalently bound to a specific carrier protein (Furlanetto, 1980). The

molecular weight of IGF-1 is 7kDa. However, nothing smaller than 30kDa was observed on SDS-PAGE gels, although it has already been proposed that there may be smaller molecules present, in such small quantities that they failed to show up after silver staining. Also. after $(NH_4)_2SO_4$ precipitation, there was a dialysis step. The molecular weight cut-off of the the dialysis tubing was 10kDa, so unless IGF-1 was bound to a carrier protein (and this is not an unreasonable supposition, since it circulates in the plasma in such a way), it was presumably lost in the dialysis stage. If bound and retained, IGF-1 may yet be a possible candidate.

bFGF also showed some stimulatory activity. FGF's are members of a family of polypeptide growth factors which show potent mitogenic activity for a variety of cells of mesodermal origin, including primarily fibroblasts, but also vascular and corneal endothelial cells, chondrocytes, osteoblasts, myeloblasts, smooth muscle and glial cells (Thomas and Gimenez-Gallego, 1985). Two forms of FGF exist, acidic and basic FGF. aFGF has a pI of 5.7 with a corresponding molecular weight of 14kDa. bFGF is a larger molecule with a molecular weight of 18kDa. The acidic form shares a 55% sequence homology with the basic form (Thomas and Gimenez-Gallego, 1985). However, there have been reports that pure preparations of both acidic and basic FGF can actually repress terminal differentiation in mouse myeloblasts (Clegg et al, 1987); in the A549 system, cells became more differentiated when treated with MOG-FDF/IV, (although it cannot be discounted that the activities of FGF may be different on different lineages of target cells).

Also, FGF's tend to be associated with healing responses, where they serve a stimulatory function. Furthermore, bFGF has been shown to cause a profound increase in PA levels in bovine capillary endothelial cells (Saksela et al, 1987), which was certainly not the case with A549 (see later).

TGF-beta showed no apparent stimulation of pulmonary surfactant production in A549. TGF-beta has been purified to homogeneity from 4 sources, namely, bovine kidney (Roberts et al, 1983), human placenta (Frolik et al, 1983), expired human platelets (Assoian et al, 1983) and feline sarcoma virus-transformed rat cells (Massague, 1984). The molecular weight of TGF-beta is 25kDa, consisting of 2 identical subunits linked together by disulphide bonds. TGF-beta has been found to be responsible for inducing normal human bronchial epithelium to undergo squamous differentiation in vitro (Massui et al, 1986), characterised by an increase in cell surface area, the formation of cross linked envelopes and the cessation of cell division. It's differentiation-inducing capabilities have also been reported by McMahon and colleagues (1986), who noticed both structural and functional changes in a rat liver epithelial cell line following treatment with TGF-beta. A549 has abundant TGF-beta receptors and indeed secretes latent TGF-beta (Wakefield et al, 1987), but like many cultured cells, cannot repsond to it's own latent TGF-beta. It appears to have lost the ability to activate latent TGF-beta and hence continues to proliferate in the presence of high concentrations of latent autocrine TGF-beta (Sporn et al, 1987; Wakefield et al, 1987).

Anti-TGF-beta did not react with MOG-FDF/IV, as demonstrated in the western blots. Similarily, there was no stimulation of pulmonary surfactant when the cells were incubated with pure TGF-beta suggesting that TGF-beta does not have any differentiating effects on this particular cell line. Indeed, TGF-beta has recently been shown to actually inhibit surfactant apoprotein synthesis in pulmonary type II epithelial cells (Whitsett et al, 1987), which is in direct contrast to the action of MOG-FDF/IV. Therefore it is probably true to say that TGF-beta was not the factor isolated from steroid treated fibroblast conditioned medium.

Western blots of PDGF and bFGF proved difficult to interpret, due to high levels of non-specific background staining. This may be attributed to the fact that a total IgG fraction was used, so that complete monospecificity could not be guaranteed. Another possibility is that MOG-FDF/IV could share some sequence homology with one or both of the growth factor antibodies. However, the blots were far from convincing, making it impossible, in the time available, to draw too many conclusions from them.

In Chapter 4, it was speculated that bombesin might be the factor isolated from conditioned medium, since bombesin-like peptides are abundant in foetal lung (Wharton et al, 1978; Yamaguchi et al, 1983), although after birth levels decline, such that in adults, these peptides are found only sparsely in bronchial neuroendocrine cells. Also, there has been speculation that bombesin may be involved in surfactant production, since bombesin is not expressed

in the immature lungs of infants with respiratory distress syndrome (Ghatei et al, 1983). As previously mentioned, respiratory distress syndrome is associated with a malfunction in pulmonary surfactant synthesis, so it may be that bombesin may have a role in this. Moreover, a structurally related form, gastrin releasing peptide (GRP) is found in the gut where it induces the secretion of gastrin (Lezoche et al, 1981). There is little doubt that the isolated factor has secretory properties regarding A549, so in this respect, and the fact that bombesin can stimulate lung maturity, it could be related to bombesin. However, cultures of A549 when treated with purified bombesin showed no stimulation. Also, the size of bombesin falls far short of the smallest band observed on SDS-PAGE, being a tetradecapeptide of molecular weight 1.6kDa, and would have been excluded by dialysis. For these reasons, bombesin is an unlikely candidate.

It was possible that the MOG-FDF/IV was a mixture of factor(s) rather than one specific factor (indeed the gel profile showed more than one band). In this respect, an interesting avenue to pursue would be to use different combinations of growth factors and see if there was an additive or synergistic effect. For example, bombesin had no effect on pulmonary surfactant production, but it may well be that could have an effect when combined with other growth factors. The growth factors which gave highest stimulation in this work were PDGF (3 fold stimulation), bFGF (1.8 fold stimulation) and IGF-1 (2 fold stimulation). However, combining the total activities of these growth factors arithmetically, gave only a

potential stimulation of less than half of the the value attained for the purified factor(s). Either other growth factors are involved, or the interaction would need to be synergistic.

The rationale behind this work was based on observations by Smith (1979), who were able to partially purify a factor from cortisol treated fibroblast conditioned medium. The factor could accelerate lung maturation in foetal rats. Smith's factor, which he christened fibroblast pneumocyte factor (FPF) was prepared by acid hydrolysis of size fractionated conditioned media. However, MOG-FDF/IV appeared to be acid labile, which would suggest that MOG-FDF/IV is different from Smith's FPF.

Like Smith's, MOG-FDF/IV isolated here was by no means pure. Indeed, Smith reported 4 bands on SDS-PAGE gels, 2 major bands of molecular weights 3.14kDa and 8.9kDa, plus 2 intermediate bands (molecular weights not specified). Later, in 1983, Smith and Sabry again purified FPF, calling it FPF-II. Again there were 4 bands, this time of slightly higher molecular weights, 12, 10, 8 and 5kDa, but these could be attributed to different degrees of glycosylation.

Clearly, there were major differences between the molecular weights of FPF/FPF-II and MOG-FDF/IV, further suggesting the involvement of a different (and possibly unique?) factor.

CHAPTER SIX

EFFECT OF MOG-FDF/IV ON OTHER PHENOTYPIC MARKERS AND CHEMOSENSITIVITY IN VITRO

This chapter will be divided into 2 parts. The first part will concentrate on the effects of MOG-FDF/IV on other phenotypic markers e.g. plasminogen activator activity, clonogenicity in soft agar, DNA and GAG synthesis. The second part will deal with some effects on the chemosensitivity of A549 cells pretreated with MOG-FDF/IV.

The implications of the results of both sections will be brought together in a general discussion at the end.

PART ONE: PHENOTYPIC ALTERATIONS

Up until this point, the only phenotypic modulation to be studied has been the ability of the MOG-FDF/IV to stimulate pulmonary surfactant secretion in A549. It is possible that the effect of MOG-FDF/IV was to act solely on the induction on surfactant synthesis, rather than having a more general differentiating effect, so it was necessary to look at other parameters to see whether or not this was the case.

Three markers were selected, namely, glycosaminoglycan (GAG) synthesis (a marker associated with differentiation; Shirasuna et al, 1988), plasminogen activator activity (a marker associated with malignant cells; Duffy and O' Grady, 1984) and the effect on cloning in suspension (a further malignancy-associated marker; Freedman and Shin, 1974) relative to monolayer cloning.

Before considering these parameters, it is first necessary to define exactly what is meant by differentiation markers and malignancy-associated markers. The term "differentiation marker" will be used to define the expression of the specific characteristics which are expressed in a functionally mature cell, and which are generally lacking in undifferentiated or transformed cells. Malignancy, as such, cannot be demonstrated in vitro, but in vivo a tumour is classified as being malignant once it has crossed the basement membrane and invaded normal tissues. It has been reported by Barsky et al (1983), by immunofluorescence and

immunoperoxidase techniques, that invasive tumours lack the basement membrane components laminin and type IV collagen, whilst their benign counterparts showed intact basement membranes with linear staining of laminin and type IV collagen. Malignancy-associated properties are by no means unique to malignant cells, since many normal cells express them as well, particularily during development and wound repair.

The three phenotypic markers chosen often show a correlation, since it is frequently observed that with increased progression of a tumour, the histology usually indicates a much poorer degree of differentiation, coupled to an increased invasive capacity and hence an increased malignancy. On the basis of these observations, it is often assumed that there is an inverse relationship between the expression of differentiation and malignancy-associated properties, such that the induction of differentiation has been proposed as a potential mode of therapy (Spremulli and Dexter, 1984; Freshney, 1985). If a cell population becomes more differentiated, this will presumably change the status of the tumour, causing a reduction in the proliferative pool, whilst increasing the pool of differentiated cells.

Changes in GAG synthesis have been correlated with discrete stages of development in embryonic systems (Toole, 1981) and the commitment to a particular pathway of cellular differentiation may be mediated by, or intrinsically associated with, changes in the cell surface molecules (Lyman et al, 1976; Fukada et al, 1980).

Normal maturation of granulocytes and monocytes also appears to be accompanied by specific changes in the GAG profiles (Kolsted et al, 1983). GAGS have been shown to be important as structural and functional elements in tissues (Hascall and Hascall, 1981) and as potent regulators of blood coagulation (Bjork and Lindahl, 1981; Marcum and Rosenberg, 1985). For these reasons, GAGS represent a useful way of measuring an alteration in the differentiation of cells.

GAGS are polyanionic carbohydrates of the cell surface, composed of linear high molecular weight polysaccharide chains, most often covalently bound to a core protein in the matrix or on the cell surface. They form repeating disaccharide units which bear a strong negative charge, due largely to varying degrees of sulphation and carboxylation. Common GAGS include hyaluronic acid, chondroitin 4- and 6-sulphate, keratan sulphate, dermatan sulphate, heparin and heparan sulphate. The biological function of GAGS is partly related to their physicochemical properties, but they also play an important role in cell-cell and cell-substrate interactions (Rollins and Culp, 1979).

GAGS therefore have a wide variety of specialised functions, but the most interesting with respect to this study is their ability to alter when a cell becomes committed to a particular pathway of cellular differentiation. By measuring GAG synthesis, this should provide further evidence as to whether treatment with MOG-FDF/IV really does alter the phenotype of the cells.

Plasminogen activators (PA's) are serine proteolytic enzymes which catalyse the conversion of biologically inactive plasminogen to plasmin, the enzyme responsible for dissolving fibrin (Dano et al, 1985). In addition to its role in fibrinolysis, increased PA activity has been linked to the expression of the malignant phenotype, including tumorigenesis (Laung et al, 1975; Duffy and O'Grady, 1984), and an increased metastatic potential (Dano et al, 1985). This correlation is further supported by observations with fresh lung biopsies and lung cell lines, that most (although not all) transformed cells show an increased secretion of PA activity when compared with their corresponding non-transformed counterparts (Markus et al, 1980). The increased release of PA occurs whether the transformation is accomplished by chemicals, oncogenic DNA or RNA viruses (Duffy and O'Grady, 1984; Camiolo and Greco, 1986), and Ossowski and Reich (1983) showed that an antibody raised against a PA from a human epidermoid carcinoma, HEp3, whilst unable to inhibit tumour growth at the site of the primary inoculation, could nevertheless prevent or strongly inhibit, metastasis to the embryonic lung. However, PA activity is by no means unique to transformed cells, since it is found in nearly all animal tissues, particularily where wound repair is going on, and in many body fluids.

I have shown in an earlier chapter that MOG-FDF/IV is capable of stimulating pulmonary surfactant production in A549 cells, so if differentiation and malignancy are interrelated, it may be capable of reducing PA levels. Indeed, some of the classical

differentiating agents have been shown to reduce PA activity e.g. sodium butyrate has been reported to reduce PA activity in glioma cell lines (Gross et al, 1988), DMSO has similar effects on the PA levels in human epidermoid carcinoma cells (Ossowski and Belin, 1985) and dexamethasone and glial maturation factor, which enhance differentiation in glioma, repress PA (McLean et al, 1986). Hence,if the cells are becoming more differentiated, and therefore possibly less malignant, we might expect to see a reduction in PA levels.

A reduction in suspension cloning is, like PA, a further indication of a reduction in malignancy-associated properties. Transformed cells readily form colonies in suspension (MacPherson and Montaigner, 1964), unlike their normal counterparts. Hence, if a cell is becoming more differentiated, and presumably less malignant, one might expect to see a reduction in clonogenicity in soft agar.

Another parameter to be considered is the effect of MOG-FDF/IV on cell growth. In Chapter 4, the effect of conditioned medium on A549 cell growth was examined, and although their was no apparent effect on cell growth, it is important to study the effect of purified MOG-FDF/IV, since it has already been shown that it has a differentiating effect.

6.1 EFFECTS OF MOG-FDF/IV ON A549 CELL GROWTH

6.1.1 Effect on terminal cell density

Mid-log phase cultures of A549 growing in Nunclon $25cm^2$ flasks were exposed to various concentrations of MOG-FDF/IV in serum free medium for 3 days. At the end of the incubation period, the cells were trypsinised and counted electronically. As can be seen in Figure 31, treatment of A549 with MOG-FDF/IV during log phase had no effect on terminal cell density.

6.1.2 Effects on ^{3}H-thymidine incorporation

A549 cells were plated out at a concentration of 10^5 cells/ml in 24 well plates. After overnight incubation, the medium was removed and replaced with fresh medium containing MOG-FDF/IV (1.0ng-10ug/ml). DNA synthesis was assayed 24 hours later with a pulse of ^{3}H-thymidine (0.5uCi/ml, 2uCi/mmol; Amersham) for 2 hours at 37^0C. The cells were then rinsed twice with ice cold PBS and lysed with 1N NaOH. The lysate was neutralised with 4N HCl and the acid insoluble materials precipitated with 10% TCA (BDH) on ice. The precipitate was collected on a Millipore filter (0.45uM) and the radioactivity determined in a liquid scintillation counter (Packard). The results, expressed as percentage of untreated control, are shown in Table 12 and although there was an apparent difference between DNA

FIGURE 31

Effect of MOG-FDF/IV on the terminal cell density of A549 in monolayer culture.

Log phase cultures of A549 growing in 24 well plates were incubated with various concentrations MOG-FDF/IV as indicated in the text.

Each data point represents the mean of three separate experiments $\pm$ SE.

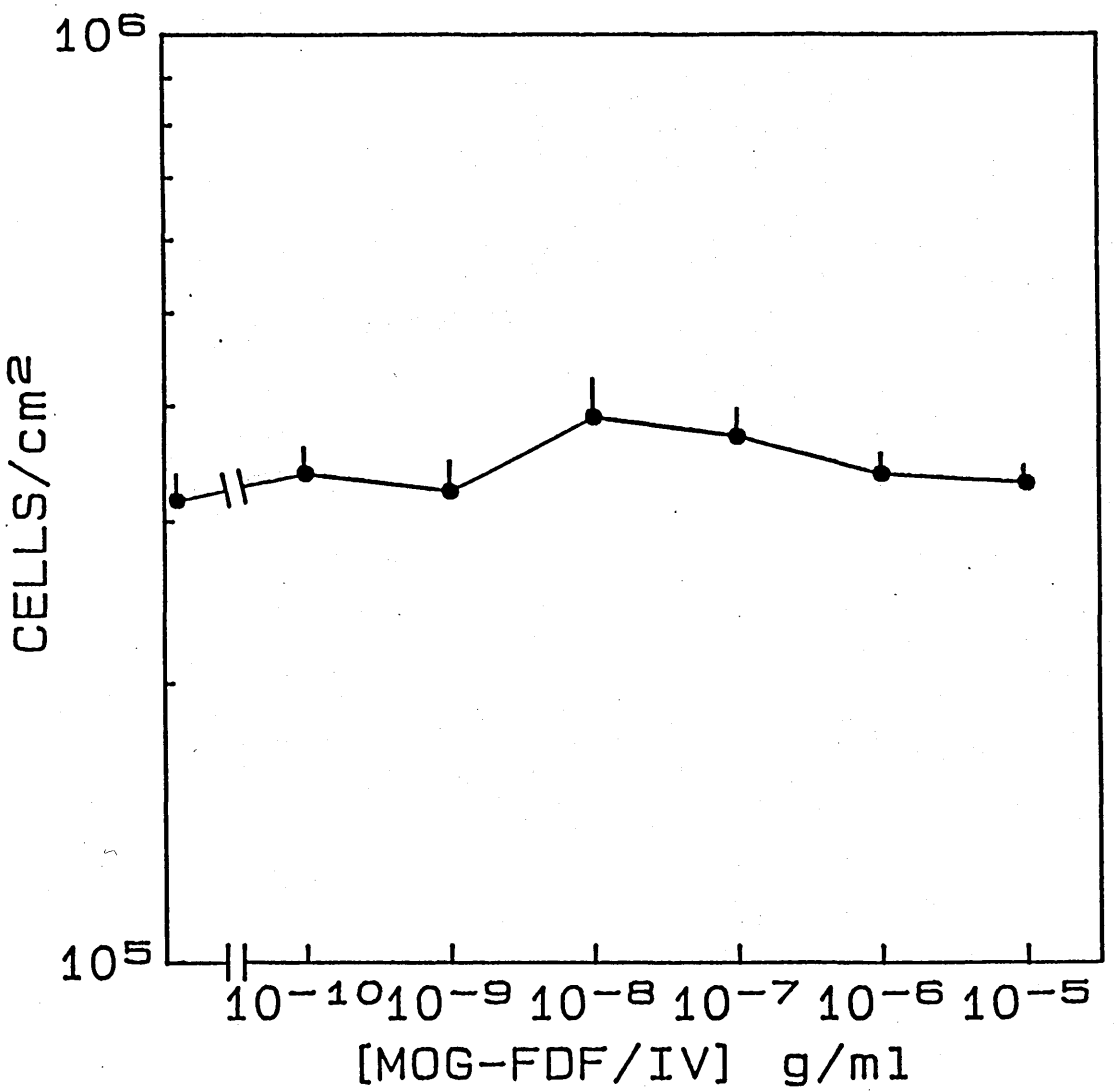

10^6
10^5
CELLS/cm^2
10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}
[MOG-FDF/IV] g/ml

TABLE 12: EFFECT OF MOG-FDF/IV ON ^{3}H-THYMIDINE INCORPORATION.

AMOUNT ADDED (ug/ml)	cpm ± SE ($x10^{-4}$)		% UNTREATED CONTROL
0	2.32	0.31	100
0.001	2.07	0.19	89
0.01	1.87	0.09	80
0.1	1.68	0.26	72
1.0	1.96	0.29	84
10	1.82	0.35	78

Each observation represents the mean of four separate experiments ± SE.

synthesis in treated and untreated cells, this was not statistically significant.

6.2 EFFECTS OF MOG-FDF/IV ON GAG SYNTHESIS

GAG synthesis was measured as follows. A549 cells were seeded at a concentration of 10^5 cell/ml into Nunclon 24 well plates and allowed to plate down overnight. Cells were then incubated with various concentrations of factor (0.001ug/ml-10ug/ml), made up in serum free medium, for 48 hours. At the end of the incubation period, the medium was aspirated and replaced with fresh culture medium containing 5uCi/ml, (100mCi/mmol) ^{3}H-acetate (NEN) and incubated for a further 24 hours. The medium and cell lysate were digested together with pronase E (Streptomyces griseus; Sigma) made up in 0.2M Tris-HCl, pH 7.8 (Sigma) at 55^0C overnight containing 12.5ug/ml of hyaluronic acid and chondroitin sulphate (both Sigma) as cold carriers. The total digest was precipated with 2% cetylpyridinium chloride (CPC;Sigma) for 1 hour at 37^0C and the resultant GAGS collected on a membrane filter (Millipore; 0.45um) under vacuum. The filters were transferred to scintillation vials for counting (Packard Liquid Scintillation counter) and GAG synthesis expressed as percentage of the untreated control. The results are shown in Table 13 and there was an increase in total cellular and secreted GAGS after treatment with MOG-FDF/IV.

TABLE 13: EFFECT OF MOG-FDF/IV ON PRODUCTION OF GAGS IN A549.

AMOUNT ADDED (ug/ml)	TOTAL GAGS (cpm ± SE x 10^{-5})		% UNTREATED CONTROL
0	1.47	0.11	100
0.001	2.03*	0.07	137
0.01	2.04**	0.08	139
0.1	1.99*	0.13	135
1.0	1.60	0.15	108
10	1.45	0.12	98

* $p < 0.05$

** $p < 0.02$

Each measurement represents the mean of four observations ± the SE.

Data from sections 6.1.2 and 6.2 were plotted on the same graphs to allow direct comparison between DNA and GAG synthesis to be made. This is shown in Figure 32. It appears that GAG synthesis but not DNA synthesis can be altered by pretreatment of the cells with factor, causing a rise in GAG synthesis with a non-significant reduction in DNA synthesis. An increase in GAG synthesis may be related to cellular differentiation.

6.3 EFFECT OF MOG-FDF/IV ON PLASMINOGEN ACTIVATOR ACTIVITY IN A549 AND WIL

PA was measured according to a modification of the chromogenic assay developed by Whur et al (1980). The assay is a two step process resulting in an amido-lytic reaction which releases para-nitroaniline from the synthetic chromogenic substrate, S-2251 (H-D-valyl-L-leucyl-L-lysine-para-nitroaniline) employed in the assay. This is cleaved by plasmin and PA is measured as the plasminogen-dependent release of para-nitroaniline. A schematic representation of this is shown in Figure 33.

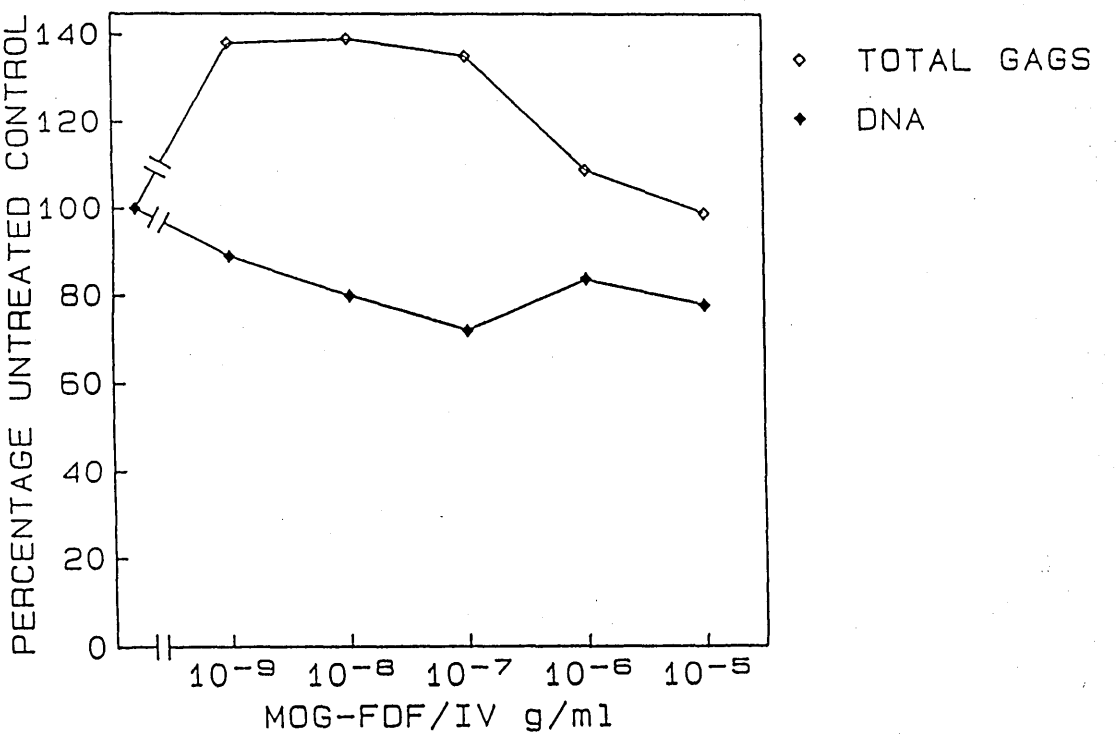
Cells were seeded at a density of $5x10^4$ cells/ml into Costar 6.5mm filter wells and exposed to 1ug/ml of MOG-FDF/IV in serum free medium for 48 hours. The medium was then changed to serum free, phenol red free (see section 6.3.1) overnight (the assay is colorimetric, so phenol red present in normal culture medium may interfere). This was aspirated, the cell monolayers were

FIGURE 32

Combined effect of MOG-FDF/IV on ^{3}H-thymidine incorporation and GAG synthesis in A549 expressed as percentage of untreated control.

DNA synthesis was measured by ^{3}H-thymidine incorporation and total GAGS by ^{3}H-acetate incorporation into CPC precipitates of cell digests and medium and both observations converted to percentage untreated control as described in the text.

Each data point represents the mean of 4 separate experiments $\pm$ SE


PERCENTAGE UNTREATED CONTROL
140
120
100
80
60
40
20
0
10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶ 10⁻⁵
MOG-FDF/IV g/ml
TOTAL GAGS
DNA

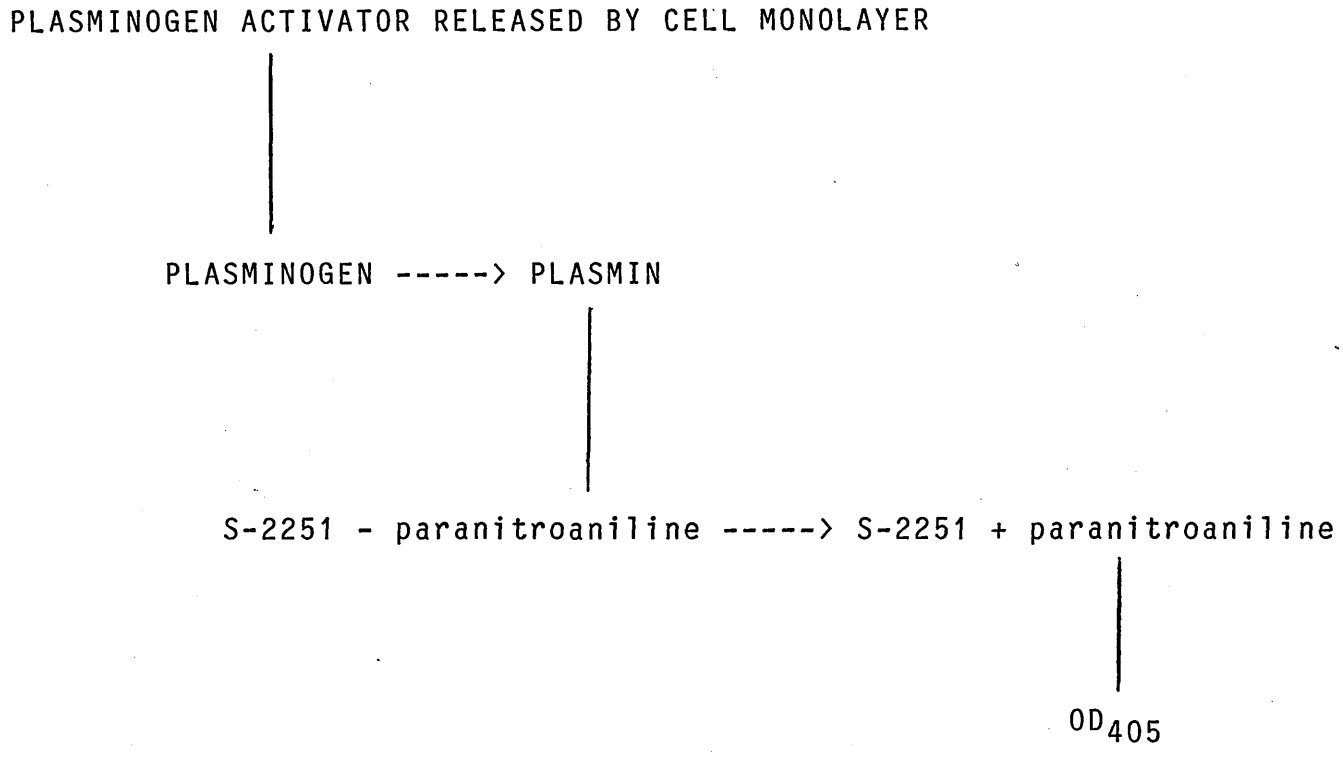


FIGURE 33 SCHEMATIC REPRESENTATION OF THE PA ASSAY

washed twice with HBSS (phenol red free), and then incubated with 0.5ml aliquots of reaction mixture (see 6.3.1) for 2 hours at 37^{0}C. The reaction was terminated by the addition of 0.5ml of 5% acetic acid (BDH), and the absorbance at 405nm was measured on a Gilford spectrophotometer. PA in plough units/ml was determined by means of a standard curve, using urokinase as the standard,. Protein determination of the cell monolayers was carried out using the Bradford protein assay (see Materials and Methods), and the final results expressed as plough units/mg of protein.

The results of the PA assay are tabulated in Table 14, and clearly show that treatment of both A549 and WIL caused a significant reduction on PA activity in both these cell lines.

6.3.1 Appendix to PA assay

(i) phenol red free medium

HBSS (phenol red free; Flow)	95ml
Vitamins (Gibco)	1.0ml
MEM essential amino acids (Gibco)	2.0ml
Non-essential amino acids (Gibco)	1.0ml
Glutamine, 200mM (Gibco)	1.0ml
10% glucose (BDH) in HBSS	1.0ml
Sodium bicarbonate (Gibco)	1.1ml

1N NaOH - enough to bring the pH to 7.4

TABLE 14: PLASMINOGEN ACTIVATOR ACTIVITY OF A549 AND WIL AFTER PRETREATMENT WITH MOG-FDF/IV.

CELL LINE	PA ACTIVITY (PU/mg PROTEIN)				% REDUCTION BY MOG-FDF/IV
	CONTROL	± SE	TREATED	± SE	
A549	4.73	0.49	1.53*	0.13	32
WIL	13.16	0.09	3.83**	0.21	29

* $p < 0.02$

** $p < 0.002$

PU = Plough units.

Each observation represents the mean of three separate experiments ± SE.

The solution was filter sterilised using a 0.22um Millex GV filter (low protein binding)

(ii) reaction mixture (per 16mm well)

Plasminogen (5cu/ml in HBSS; KabiVitrum)	100ul
S-2251 (5mM in HBSS; KabiVitrum)	100ul
Poly-D-lysine (1.5mg/ml; Sigma)	50ul
HBSS (phenol red free; Flow)	250ul

6.4 EFFECT OF MOG-FDF/IV ON CLONING OF A549 CELLS

The clonogenicity of A549 both in monolayer and in soft agar was compared. Inhibition of cloning in monolayer would suggest a cytotoxic or a cytostatic effect (or both). While agar cloning would also measure this, it is supposedly transformation-dependent. Hence a disproportionate reduction in agar cloning compared to monolayer cloning would imply reduced transformation.

6.4.1 Cloning in monolayer

A549 cells were pretreated with various concentrations of MOG-FDF/IV (0.1ng-10ug/ml) for 48 hours and then cloned according to the protocol in the Materials and Methods section.

The results are shown in Table 15 and it can be seen that there was

TABLE 15: EFFECT OF MOG-FDF/IV ON CLONING IN MONOLAYER.

[FACTOR] (ug/ml)	COLONY NUMBER ± SE		PLATING EFFICIENCY (%)
0.01	188.83	1.90	37.37
0.1	196.67	1.96	39.33
1.0	202.72	2.48	40.54
10.0	204.72	3.06	40.94
0	207.33	2.22	41.47

Each observation represents the mean of three separate experiments ± SE.

no significant effect of the factor on the clonongenic potential in monolayer, with an average plating efficiency of 40% and a typical colony number of approximately 200 colonies/petri dish in both control and treated cells.

6.4.2 Cloning in soft agar

Clonogenicity in soft agar was performed on A459 cells which had been pretreated with either MOG-FDF/IV (1ug/ml), fibroblast conditioned medium or steroid treated fibroblast conditioned medium for 48 hours. The results are shown in Table 16 and a photograph of some typical MTT stained clones is shown in Plate 10. A549 was able to grow well in semisolid agar, with a plating efficiency of around 33%, as measured by the number of colonies containing more than 10 cells. Treatment with conditioned medium (both with or without steroid pretreatment) had no effect on clonogenicity. However, pretreatment with MOG-FDF/IV caused a significant reduction in colony number by some 30%.

TABLE 16: EFFECT OF MOG-FDF/IV ON SUSPENSION CLONING IN SOFT AGAR.

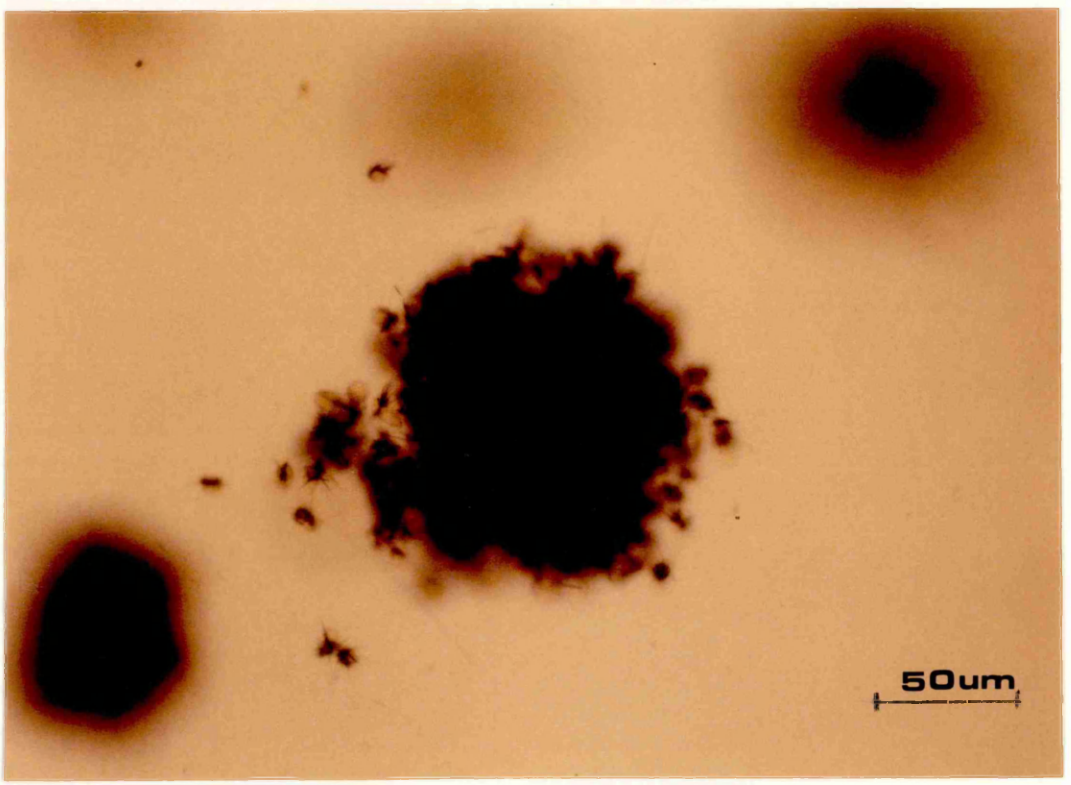
	COLONY NUMBER (Mean ± SE)		PLATING EFFICIENCY (%)
CONTROL	333	26	33
CONDITIONED MEDIUM	294	18	29
DX TREATED CM	313	16	31
MOG-FDF/IV (1ug/ml)	221*	16	22

* $p < 0.02$

Abbreviations: DX dexamethasone
CM conditioned medium

Each value represents the mean of three separate experiments ± SE.

PLATE 10: TYPICAL SOFT AGAR CLONE AFTER STAINING WITH MTT.


50um

PART TWO: ASPECTS OF CHEMOSENSITIVITY

Tumour cell resistance to cytotoxic drugs is considered to be one of the major causes of failure of clinical chemotherapy. This emergence of resistance is considered to be the major cause of death in lung, breast and ovarian cancers. Clearly, strategies to circumvent resistance have to be developed. It has recently been proposed by Lotan and Nicholson (1988), that anticancer therapy may be improved by the sequential use of cytotoxic and differentiating agents in an effort to suppress tumour cell diversification.

In the first part of this Chapter, it was shown that pretreatment with MOG-FDF/IV could induce differentiation and suppress malignancy-associated properties in A549. It was therefore of interest to see if pretreatment of the cells with MOG-FDF/IV could in any way alter the sensitivity of A549 to a commonly used cytotoxic drug, Adriamycin, since the combination of differentiation inducing agents and low doses of conventional cytoxic agents represents a possibility for clinical application of differentiation therapy. The efficacy of this has been demonstrated by Kasukabe *et al* (1987), who showed that continuous treatment of myeloid leukaemia cells with both differentiation inducers such as 1 alpha, 25-dihydroxyvitamin D_3 and anti-leukaemic drugs such as 1-beta-D-arabinofuranosylcytosine was more effective therapeutically than treatment with a differentiation inducer alone both *in vitro* and *in vivo*. The advantages of such combinations may be maximal induction of terminally differentiated cells and suppression of

drug resistant populations whilst limiting the development of acquired resistance.

Adriamycin belongs to the anthracycline group of compounds and is an antibiotic isolated from cultures of the fungus, Streptomyces peucetius. Adriamycin is believed to intercalate with DNA, inserting itself between the double helical strands of the DNA molecule, where it effects DNA damage through interaction with type II DNA topoisomerases, inducing double strand breakages. However, there is evidence to suggest that the drug can also be actively cytotoxic without entering the cells, by interaction at the cell surface (Tritton and Yee, 1982). The cytotoxic effects of Adriamycin are maximal during S phase of the cell cycle, but there is some activity during other phases of the cycle as well.

Treatment of A549 with MOG-FDF/IV has been shown to alter the phenotype and repress malignancy-asociated properties in this cell line. A more differentiated cell may show greater sensitivity to cytotoxic drugs. Experiments were devised to examine alterations in chemosensitivity associated with phenotypic change.

6.5 EFFECTS OF PRETREATMENT WITH MOG-FDF/IV ON CHEMOSENSITIVITY OF A549 TO ADRIAMYCIN.

The chemosensitivity assay was performed using an adaptatation of the MTT assay (Mossman, 1983), as modified by Plumb et al (1989).

Cells were seeded into 96 well microtitre plates (Flow Laboratories) at a concentration of $5x10^3$ cells/ml and left in a 37^0C incubator for 48 hours. The medium was removed and the cells treated with 1ug/ml of MOG-FDF/IV in serum free medium for 24 hours. Parallel cultures as controls were set up and they received serum free medium only. Following exposure to MOG-FDF/IV, the cells were refed with fresh culture medium containing 10% FCS and Adriamycin ($10^{-10}M$-$10^{-4}M$) for a further 24 hours. For the next 3 days the cells were fed daily with fresh, drug free, medium and on the fourth day after drug treatment, the MTT assay was performed. Cells were refed with 200ul of culture medium containing 10mM HEPES buffer (Gibco). 50ul of a stock solution of MTT (5mg/ml; Sigma) was added to each well, and the plates incubated in the dark at 37^0C for 4 hours. The medium was then removed from the wells and the water insoluble purple formazan crystals dissolved in 200ul of DMSO (BDH) per well. 25ul of Sorenson's glycine buffer (see mitogenesis assay) was added to each well and the plates read in an ELISA plate reader (BioRad, model 2550) at an absorbance of 570nm.

A graph of the results is shown in Figure 34a,b, and shows that pretreatment of the cells with MOG-FDF/IV had no effect on the ID_{50} of A549 to Adriamycin. Control cultures had an ID_{50} of $1.0 \times 10^{-8}M$ and pretreated cells had the same ID_{50}.

FIGURE 34a

Chemosensitivity of A549 to Adriamycin in vitro.

The chemosensitivity of A549 to Adriamycin was determined using the MTT assay as outlined in the text.

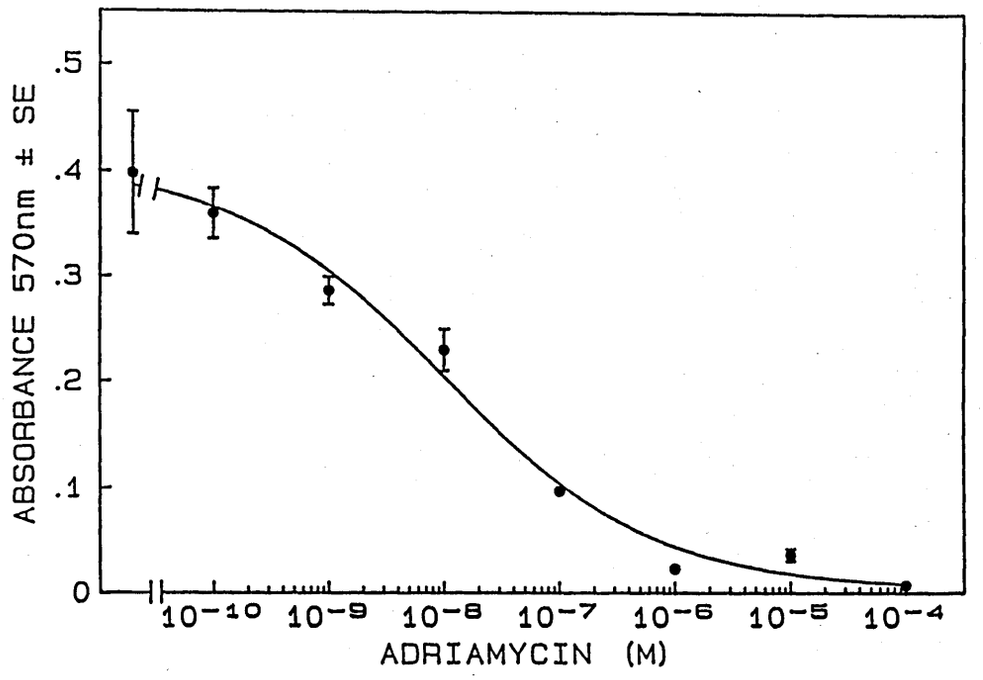
n = 3 ± SE

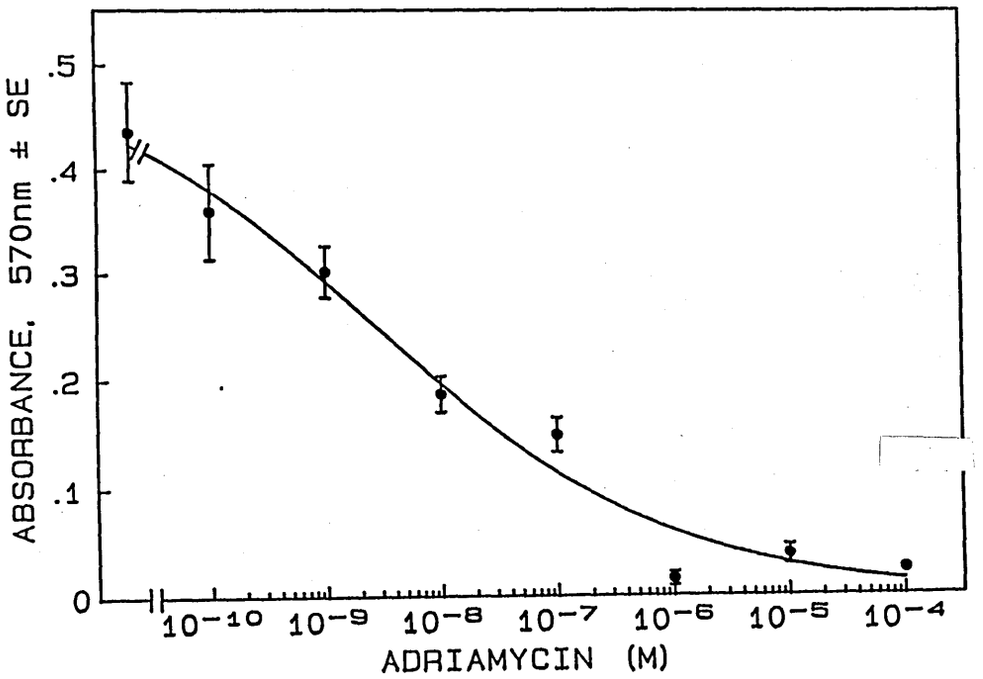
FIGURE 34b

Effect of pre-treating A549 with MOG-FDF/IV on the chemosensitivity of A549 to Adriamycin in vitro.

A549 cells growing in 96-well plates were pre-treated with MOG-FDF/IV and their chemosensitivity to Adriamycin measured using the MTT assay as described in the text.

n = 3 ± SE


ABSORBANCE 570nm ± SE
.5
.4
.3
.2
.1
0
10⁻¹⁰ 10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶ 10⁻⁵ 10⁻⁴
ADRIAMYCIN (M)


ABSORBANCE, 570nm ± SE
.5
.4
.3
.2
.1
0
10⁻¹⁰ 10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶ 10⁻⁵ 10⁻⁴
ADRIAMYCIN (M)

6.6 CHEMOSENSITIVITY OF A549 CELLS AFTER CULTURE UNDER MAXIMUM INDUCING CONDITIONS

The optimal conditions for culture of A549 cells for induction of pulmonary surfactant synthesis has been shown to be a filter well, where increased synthesis of pulmonary surfactant was observed when the cells were treated with a soluble mediator(s) purified from dexamethasone-treated fibroblast-conditioned medium. If phenotypic change is related to chemosensitivity, then the optimal conditions for differentiation, may cause an alteration in the chemosensitivity of the cells to Adriamycin. Under these optimal conditions, the chemosensitivity of A549 was investigated.

A549 cells were grown to log phase in Costar 24.5mm filter wells (see Materials and Methods) in the presence or absence of a confluent monolayer of fibroblasts growing on the bottom of the dish which housed the filter well. The cells were either exposed to adriamycin (doxorubicin; 0.01-10uM) for 24 hours, or 0.25uM dexamethasone for 48 hours, or a combination of both, with adriamycin added in the second 24 hour period. After Adriamycin exposure, the cells were trypsinised and cloned in monolayer at a concentration of 100 cells/ml, according to the protocol described in the Materials and Methods section. The results are shown in Tables 17a,b and Figure 35.

The effect of Adriamycin, with and without dexamethasone, on the clonogenicity of A549 exposed in filter wells was measured as a

TABLE 17a: EFFECT OF TREATMENT WITH ADRIAMYCIN ± DEXAMETHASONE IN FILTER WELLS ON THE CLONOGENICITY OF A549 IN MONOLAYER.

[ADR] (uM)	COLONY NUMBER ± SE				PLATING EFFICIENCY	
	+DX		-DX		+DX	-DX
0	208	2	250	3	41	50
0.001	278	4	283	1	55	56
0.01	291	4	273	3	58	54
0.1	316	3	245	1	63	49
1.0	147	6	160	2	29	32
10	0	0	0	0	0	0

TABLE 17b: EFFECT OF TREATMENT WITH ADRIAMYCIN ± DEXAMETHSONE IN FILTER WELLS IN THE PRESENCE OF LF113 ON THE CLONOGENICITY OF A549 IN MONOLAYER.

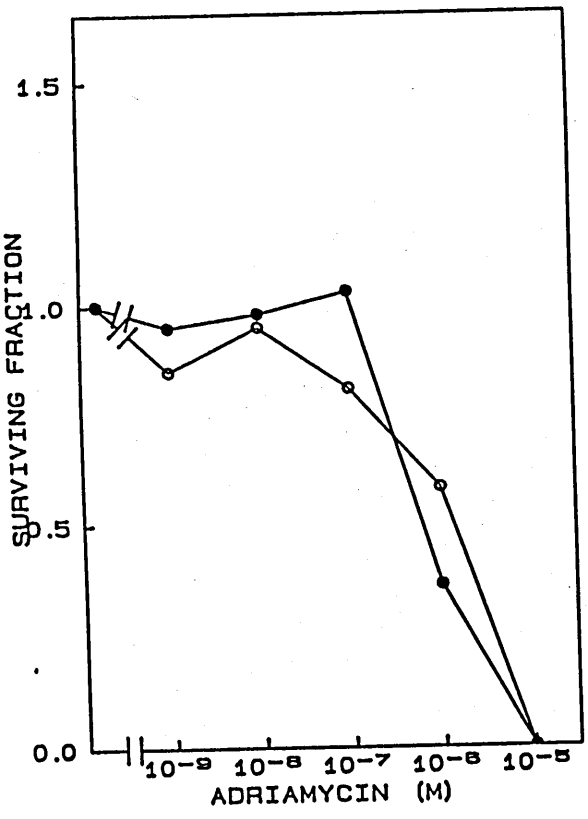
[ADR] (uM)	COLONY NUMBER ± SE				PLATING EFFICIENCY	
	+DX		-DX		+DX	-DX
0	333	3	298	1	66	59
0.001	316	3	252	1	63	50
0.01	325	2	284	2	65	57
0.1	344	4	242	4	68	48
1.0	123	7	169	6	24	34
10	2	0.5	0	0	0.004	0

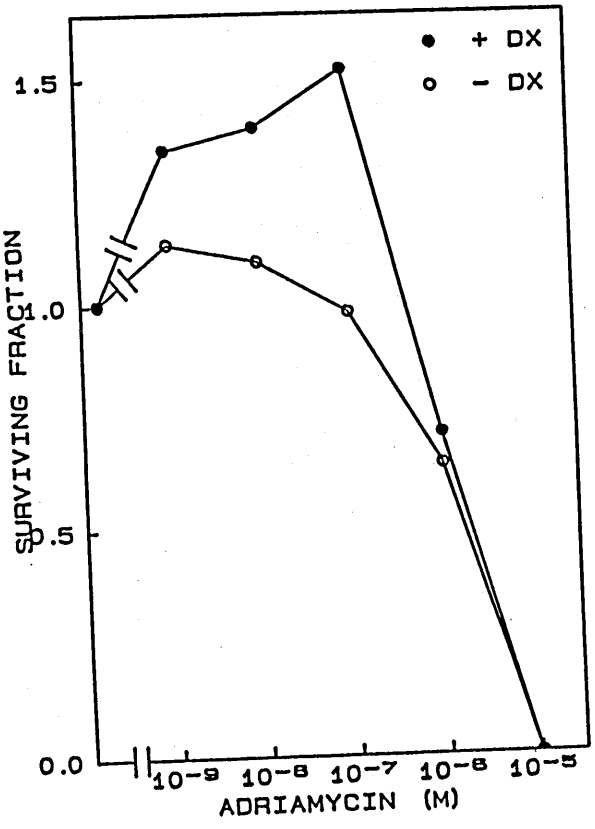
Each observation represents the mean of three separate experiments ± SE.

FIGURE 35: A549 SURVIVING FRACTION AFTER EXPOSURE TO DEXAMETHASONE AND ADRIAMYCIN.

Figure 35a: Control surviving fraction

Figure 35b: Surviving fraction after drug exposure in the presence of fibroblasts


1.5
1.0
0.5
0.0
SURVIVING FRACTION
10-9 10-8 10-7 10-6 10-5
ADRIAMYCIN (M)


+ DX
- DX
1.5
1.0
0.5
0.0
SURVIVING FRACTION
10-9 10-8 10-7 10-6 10-5
ADRIAMYCIN (M)

simple assay for cytotoxicity. Low concentrations of Adriamycin (<0.1uM) caused an increase in the plating efficiency of A549, while concentrations exceeding 0.1uM were cytotoxic. In the presence of dexamethasone, the effect was similar, although the overall stimulation in plating efficiency was even greater.

The ID_{50}'s were shifted slightly; in the absence of steroid, the ID_{50} was 1.55uM, while in the presence of steroid, it was 1.75uM. On the addition of fibroblasts, the mitogenic effect of Adriamycin was abolished, both in the presence and absence of steroid, although overall, the plating efficiencies were higher, particularily after steroid treatment. This was reflected in a corresponding shift in ID_{50} from 1.35uM to 1.0uM with the drug combinations. These results are summarised in Table 18.

TABLE 18 SUMMARY OF ID_{50}'S AFTER DIFFERENT DRUG TREATMENTS

CULTURE CONDITIONS	ID_{50} (uM) ADRIAMYCIN	
DEXAMETHASONE(uM)	0	0.25
A549 ALONE	1.55	1.75
A549 + FIBROBLASTS	1.35	1.0

In the presence of fibroblasts, the ID_{50} value was reduced after

each treatment regime, although this was not statistically significant.

DISCUSSION

The main aim of this Chapter was to examine the effects of MOG-FDF/IV on other phenotypic markers, namely, DNA and GAG synthesis, PA activity and clonogenicity in suspension. As well as the phenotypic markers, the chemosensitivity of A549 to a cytotoxic drug, Adriamycin, was investigated, to see if there was any alteration in the chemosensitivity asociated with phenotypic change.

It was of interest that MOG-FDF/IV increased the total GAG synthesis in A549 cells. This observation concurs with the findings of Shirasuna et al (1988), who showed that incubation of a salivary adenocarcinoma cell line with fibroblast conditioned medium could increase GAG synthesis. Moreover, they presented evidence which suggested that a soluble factor from the conditioned medium was responsible. However, like Shirasuna's data (1988), the experiments here looked at gross changes in total GAGS synthesis, and without knowing exactly which GAGS were involved, the GAGS data are difficult to interpret.

Although there was an apparent reduction in ^{3}H-thymidine incorporation after incubation with MOG-FDF/IV, this observation

was not statistically significant. Shirasuna et al (1988) have reported an inverse correlation between DNA and GAG synthesis following treatment of a human salivary adenocarcinoma cell line with fibroblast conditioned medium, with a reduction in DNA synthesis and an increase in GAG synthesis. With certain reservations, the data presented here could agree with that of Shirasuna et al. However, by direct cell counting, the terminal cell density of A549 was unaffected by treatment with MOG-FDF/IV suggesting there was no effect on cell growth.

Treatment of A549 and a lung adenocarcinoma, WIL, with MOG-FDF/IV resulted in a significant reduction in PA levels in both cell lines. This suggests that the MOG-FDF/IV was capable of reducing PA activity in both these cell lines and agrees with observations made by several groups that hormones or hormone-like substances can alter the secretion of PA's (Mira-y-Lopez et al, 1983; Ossowski and Belin, 1985; Gross et al, 1988). Increased PA levels have been correlated with the malignant phenotype in a number of experimental systems, although the expression of PA is by no means unique to tumour cells (Duffy and O'Grady. 1984; Camiolo and Greco, 1986). It is interesting to speculate that there may be an inverse relationship between maliganancy-associated properties i.e. PA expression and the induction of a more differentiated phenotype, since treatment of A549 cells with MOG-FDF/IV not only reduced absolute PA levels, but also increased the synthesis of pulmonary surfactant and total GAGS. The possibility of such a relationship agrees with the findings of Frame et al (1984), who showed that in

a panel of early passage glioma cell lines, less well differentiated cell lines expressed high PA levels, with a decrease in PA expression in cell lines displaying a more differentiated phenotype.

Of the three members of the PA group of enzymes (circulating PA found in the blood, tissue PA (tPA) and urokinase-like PA (uPA), the PA most commonly associated with tumour cells is uPA. This has been proven using studies with monoclonal antibodies. 90% of the PA expressed in human lung tumours is uPA, compared to only 50% in normal tissues (Markus et al, 1980). The same is true of breast, although the difference is less, where 80% of the PA is uPA in tumours, and only 62% in normal breast (Evers et al, 1982). In retrospect, measurement of uPA may have given a better indication of exactly which PA was involved.

Clonogenicity in soft agar was reduced by about 30% without any effects in monolayer, suggesting a specific growth inhibition in suspension. This is in line with the observations of Shirasuna et al (1988), who noticed a marked reduction, not only in colony number, but also in colony size when a salivary adenocarcinoma cell line was treated with WI-38 conditioned medium.

These phenotypic measurements imply that the effect of MOG-FDF/IV was not solely on pulmonary surfactant synthesis, but that a coordinated phenotypic effect was occurring. As surfactant synthesis is a significant marker of differentiation in this cell

line, and GAG synthesis a further differentiation marker and reduced suspension cloning, coupled with a concomitant reduction in PA are indicative of a reduction in malignancy associated properties, it would appear that MOG-FDF/IV may be altering the phenotype of A549 to a less malignant and more differentiated form (See also Chapter 7).

In addition to the phenotypic markers just described, the effects of MOG-FDF/IV on A549 chemosensitivity were also examined. There was no significant difference between the ID_{50} of A549 to Adriamycin, as measured by MTT reduction, with or without pretreatment with MOG-FDF/IV. As is the case for most non-small cell lung carcinomas, A549 is fairly resistant to cytotoxic drugs, so it may not have been the best cell line to have chosen if an increase in resistance was expected. It should, however, have shown any increased sensitivity. The slight increase shown in the presence of fibroblasts and dexamethasone, though not significant, is in agreement with previous unpublished data from this laboratory, linking increased pulmonary surfactant synthesis with Adriamycin sensitivity (McCormick and Freshney, unpublished observations).

The effect of combined treatment with Adriamycin and dexamethasone on the clonogenicity of A549 pre-exposed to the drugs on filter wells, was also studied, since culture of A549 on filter wells appeared to cause maximal stimulation of the cells to produce pulmonary surfactant. With Adriamycin alone, there was an increase

in plating efficiency and a growth stimulation (a larger colony size) at subtoxic drug concentrations (> 0.1uM). The ability of adriamycin to stimulate growth of a variety of cell types at low titres is not an uncommon event, and has been reported sporadically in the literature over the years, most recently by Vichi and Tritton (1989).

Relative to control values, dexamethasone alone reduced the surviving fraction by some 20%, with a corresponding reduction in plating efficiency. This observation is not surprising, bearing in mind that in a previous chapter of this thesis, dexamethasone was shown to have a cytostatic effect on A549.

When dexamethasone was combined with various concentrations of Adriamycin, overall there was an enhanced clonal growth at subtoxic concentrations (>0.1uM), suggesting that differences in chemosensitivity were not due to altered cell proliferation. This is in agreement with observations by McCormick and Freshney (1987), who showed that A549 cloned in the presence and absence of dexamethasone showed increased sensitivity to Adriamycin at high steroid concentrations, but greater resistance at high low steroid concentrations. Both concentrations could, however, enhance clonal growth.

On the addition of fibroblasts transfilter, low concentrations of Adriamycin again had a growth stimulatory effect, and in general the stimulation was of a similar order of magnitude as observed

with A549 cultured under the same conditions but in the absence of fibroblasts. Dexamethasone, however, was stimulatory, increasing the surviving fraction by about 10% in the presence of LF113. Similarily, Adriamycin plus dexamethasone increased the surviving fraction by 20-30%, in the subtoxic range. In previous Chapters, dexamethasone was shown to be stimulatory for the growth of LF113 cells, as well as causing the production of MOG-FDF/IV. So, while MOG-FDF/IV had a differentiating function on A549, it appears to be making the cells more resistant by increasing the clonal cell growth. However, without knowing exactly what effect the Adriamycin was having on the fibroblasts, the results of the coculture experiments are difficult to interpret. It appears, though, that Adriamycin chemosensitivity was not altered significantly, either by the presence of MOG-FDF/IV or by altering the culture conditions.

From the results presented here, it appears that MOG-FDF/IV can alter phenotypic markers other than pulmonary surfactant, confirming that the cells were becoming more differentiated and less malignant. However, there did not appear to be any major alteration in A549 chemosensitivity to Adriamycin.

CHAPTER SEVEN

STUDIES *IN VIVO*

If MOG-FDF/IV was causing a coordinated shift from malignancy to differentiation, a decrease in tumour growth *in vivo* might be expected. This Chapter will describe experiments on tumours generated as xenografts in nude mice to examine this question.

The design of all the experiments in this Chapter and the analysis of results were carried out by myself. However due to my allergic response to the animals, the experimental techniques were carried out by Mr Tom Hamilton and Dr Jim Cassidy. Processing and staining of xenograft sections was carried out by Mr Tony Savage, Glaxo Group Research, Greenford, Middx.

INTRODUCTION

Another aspect concerning the biological effects of MOG-FDF/IV was its effects *in vivo*. It has already been shown that it could act as a differentiating inducer for A549 *in vitro*. Does it fulfil a similar role *in vivo*?

With few exceptions, most animal tumours only grow in the syngeneic host. Heterotransplantation only works in a privileged site or in an immune-deficient host. Human tumours can be grown *in vivo* using immunodeficient "nude mice". These mice are genetically athymic and lack T lymphocytes although B lymphocytes are still present. Because they are T cell deficient, nude mice are usually maintained under sterile conditions to minimise the risk of infection from external sources.

One of the problems associated with growing tumours in a non-syngeneic host is knowing whether or not the xenograft still retains the characteristics of the tumour from which it was derived. This has been investigated by Shorthouse *et al* (1980) who showed that histology and chromosome analysis of the xenografts indicated that the characteristics of the original tumour were maintained in the xenografts. A further problem is correlating the *in vivo* and *in vitro* response, and this is particularily important when dealing with chemotherapeutic compounds. In many cases, the chemotherapeutic response of xenografts shows a positive

correlation with the response of the patient (Shorthouse *et al* 1980).

A correlation between *in vivo* and *in vitro* responses has been shown with a number of putative differentiating agents. Steroid hormones, which have been demonstrated to have antiproliferative effects against several different tumour cell lines *in vitro*, have similar such effects *in vivo*. Dexamethasone was cytostatic towards a human NSCLC cell line *in vitro*, and when grown as a xenograft, treatment of the hosts with the steroid caused total cessation of xenograft growth (McLean *et al*, 1986). Polar solvents such as NMF and DMF can alter the growth characterstics of human colon cancer cell lines *in vitro*, with an increase in population doubling times, a decrease in saturation density, and a loss of clonogenicity in soft agar (Dexter *et al*, 1979). This correlates well with the *in vivo* response where both DMF and its metabolite, NMF inhibited the growth of two colon carcinomas xenografted in nude mice (Dexter *et al*, 1982).

In vitro, treatment of A549 cells with MOG-FDF/IV has been shown to cause a shift to a more differentiated phenotype, and a resultant loss of malignancy-associated properties. This Chapter will examine whether or not this applies *in vivo*.

Another parameter investigated was target cell specificity, since most of the previous experiments had dealt almost exclusively with

A549. To determine whether MOG-FDF/IV was specific to A549, three other xenografts, of lung and non-lung origin were also examined.

7.1 EFFECT ON A549

A549 xenografts were prepared as described in the Materials and Methods section. Four groups of mice were used, and each contained at least five animals. Group one (controls) received daily intraperitoneal (i.p) injections of 5ul/g body weight of PBS. Group two received i.p. injections of dexamethasone (100ng/g), since it has been shown previously that dexamethasone could induce, directly, pulmonary surfactant production in A549. Group three received MOG-FDF/IV (50ng/g) i.p., and group four received i.p. injections of both dexamethasone and MOG-FDF/IV. Each group were treated daily for 12 out of 14 days (excluding the weekend). Tumour volumes were estimated using double caliper measurements three times weekly and converted into mm^3 (on the assumption that the tumour was elliptical in section) using the following formula (Fergusson et al, 1986):

$$\text{TUMOUR VOLUME } (mm^3) = D\ d^2\ pi/6$$

Where D = larger diameter

d = smaller diameter

At the end of the experiment, the tumours were excised and processed for light microscopy as described in the Materials and Methods.

There was a striking difference in both the relative sizes (in vivo and freshly excised), and the overall appearance of the freshly excised tumours (Plates 11-12), with the controls showing evidence of vascularisation, while the MOG-FDF/IV treated xenografts had a whitish appearance with no evidence of surface blood vessels.

Histologically, the control group contained many viable tumour cells, with little evidence of structure. There were some stromal elements, and signs of vascularisation (Plate 13a). The dexamethasone treated group showed areas of necrosis. (Plate 13b). The most interesting observation was seen in group three which received MOG-FDF/IV (Plate 13c). There was a striking difference in the tumour histology, with evidence of extensive structural reorganisation, with stromal infiltration and signs of glandular-like or duct-like formation, and the appearance of columnar epithelium. This is shown in greater detail in Plate 13e. There were very few (if any) blood vessels and small pockets of necrosis. In group 4 (both MOG-FDF/IV and dexamethasone), the histology was intermediate to groups two and three (Plate 13d).

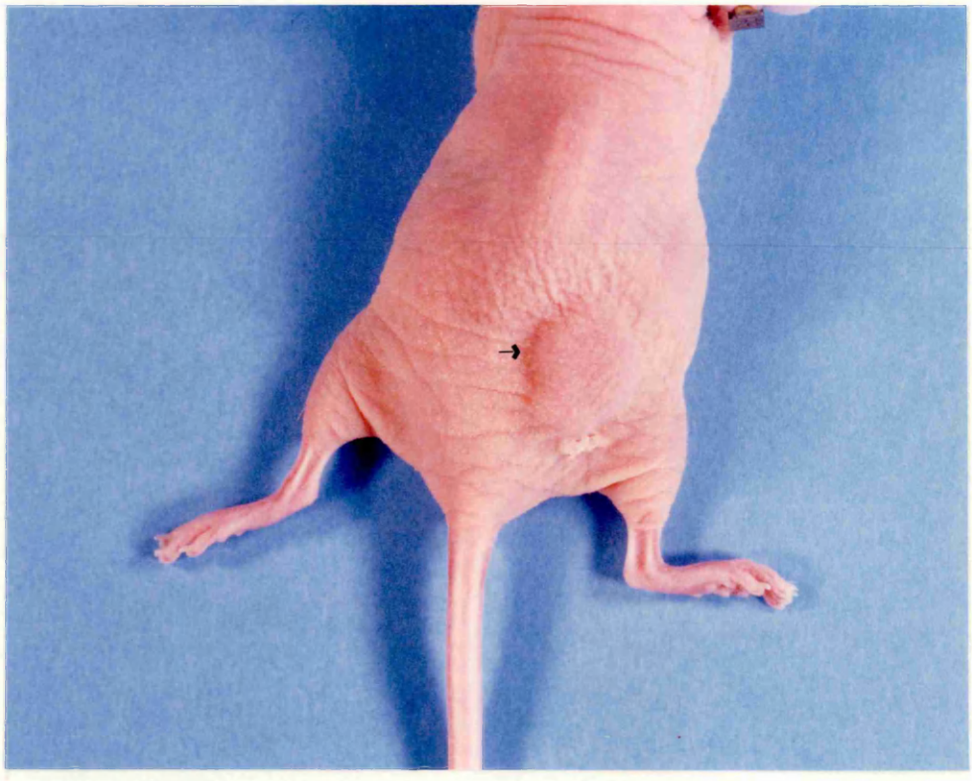
While the control xenografts continued to increase in volume throughout the course of the experiment, the tumour volumes of all

PLATE 11: NUDE MICE BEARING A549 XENOGRAFTS.

Arrow represents site of implantation.

Plate 11a: Control mouse approximately one month after first implantation.

Plate 11b: Mouse pretreated with MOG-FDF/IV.
One month post implantation, 12 injections of MOG-FDF/IV during the last 14 days.



1cm

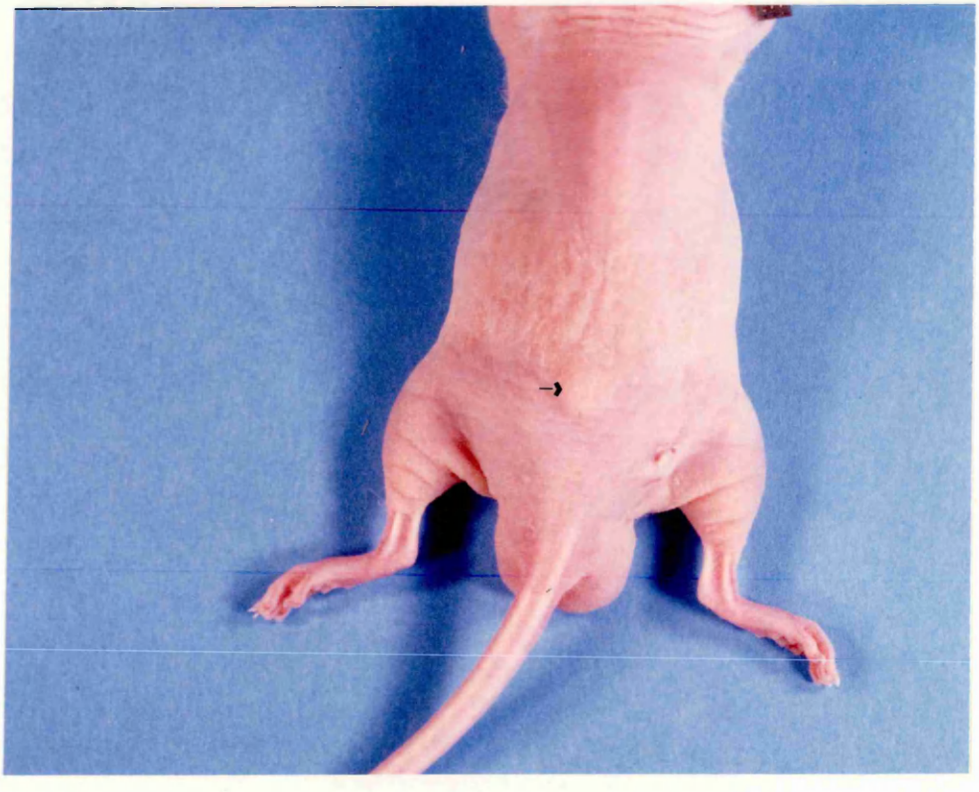


PLATE 12: GROSS MORPHOLOGICAL APPEARANCE OF EXCISED A549 XENOGRAFTS.

C = control

D = dexamethasone

B = dexamethasone and MOG-FDF/IV

F = MOG-FDF/IV

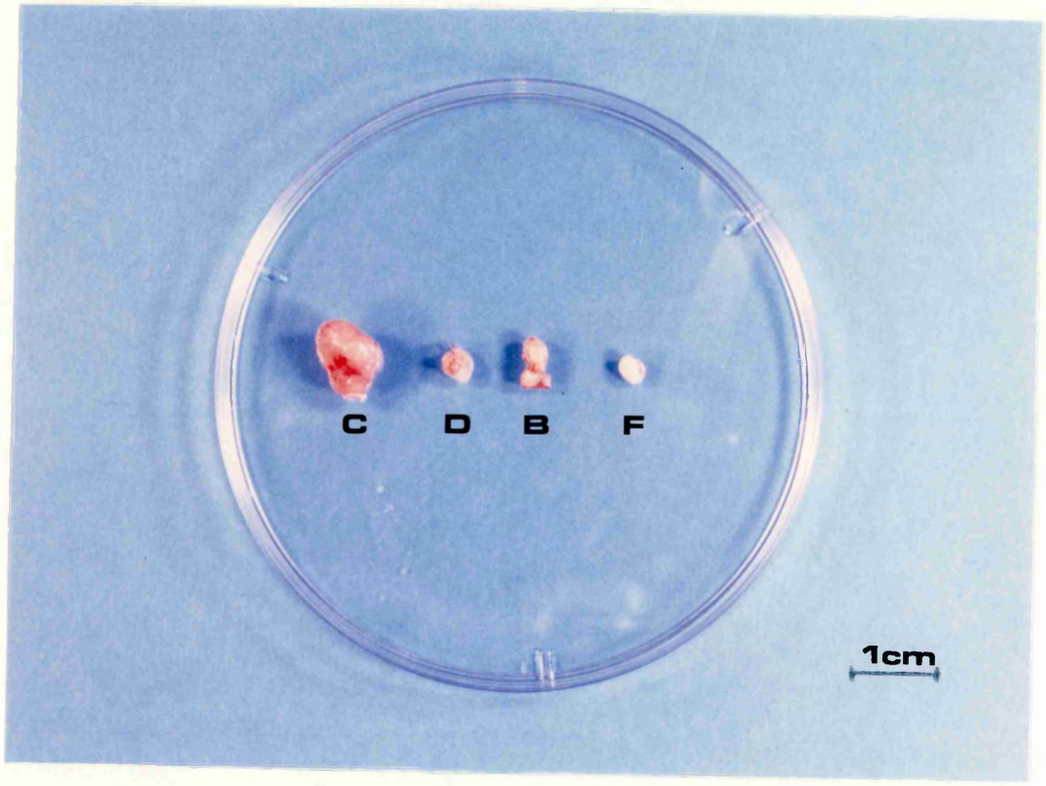

C
D
B
F
1cm

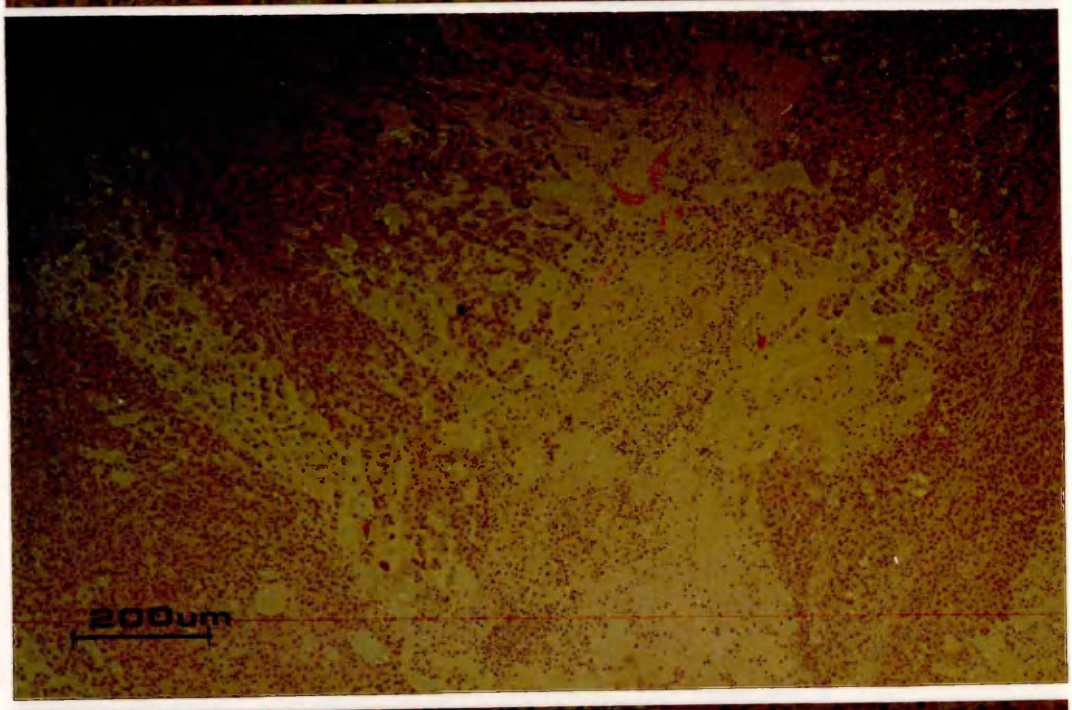
PLATE 13: HISTOLOGICAL APPEARANCE OF A549 XENOGRAFTS

H & E stain

Plate 13a Control xenograft, one month after first implantation.

Plate 13b Dexamethasone treated xenograft, one month post-implantation, 12 days out of the last 14 with 100ng/g MOG-FDF/IV i.p.

Plate 13c MOG-FDF/IV treated xenograft, one month post-implantation, 12 days out of the last 14 with 50ng/g MOG-FDF/IV.


200um

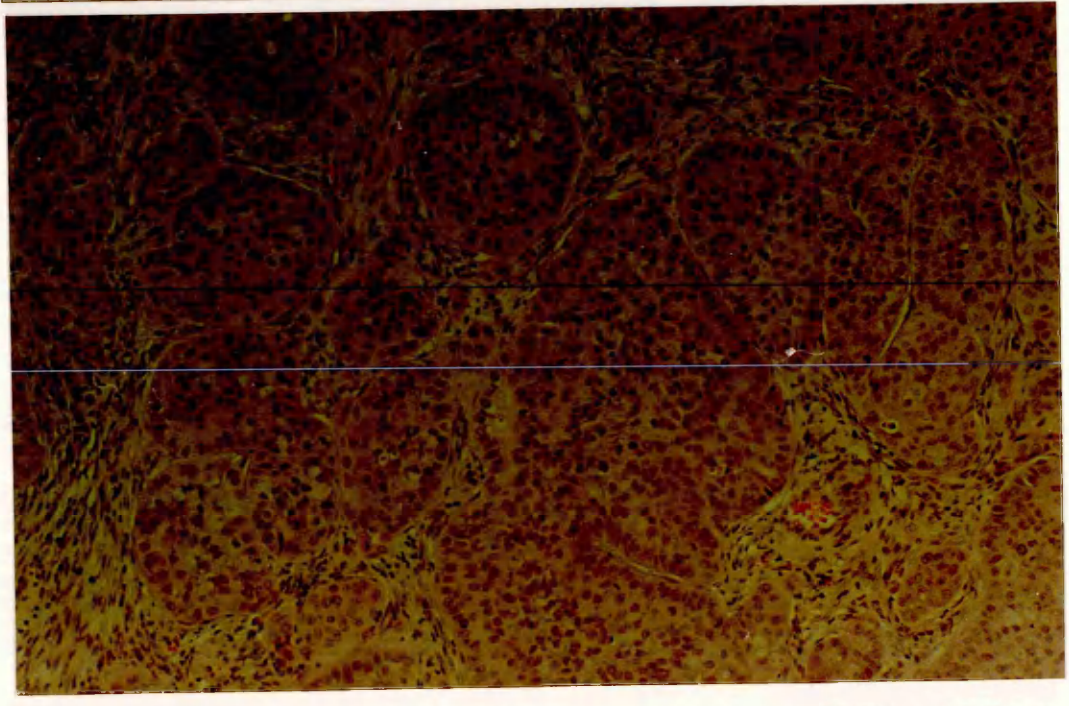
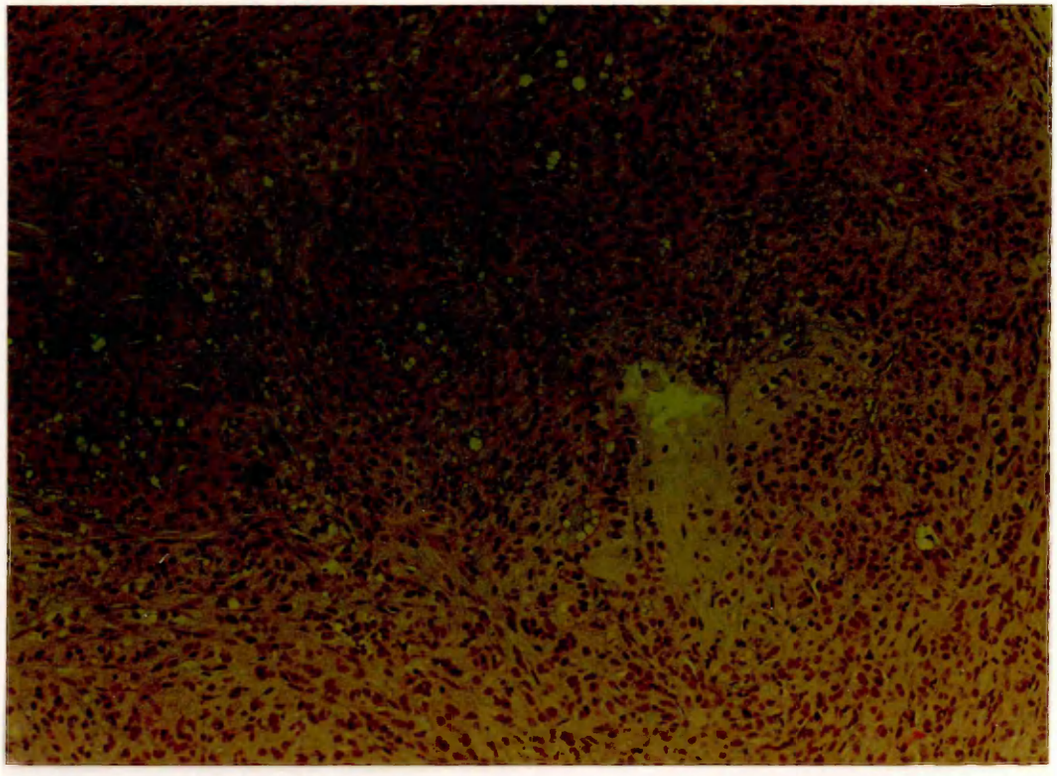


Plate 13d: Histological appearance of dexamethasone and MOG-FDF/IV treated A549 xenografts (treatment as 13b and c combined).

Plate 13e: Higher power photomicrograph of 13c showing columnar epithelium surrounding a "duct-like" structure.



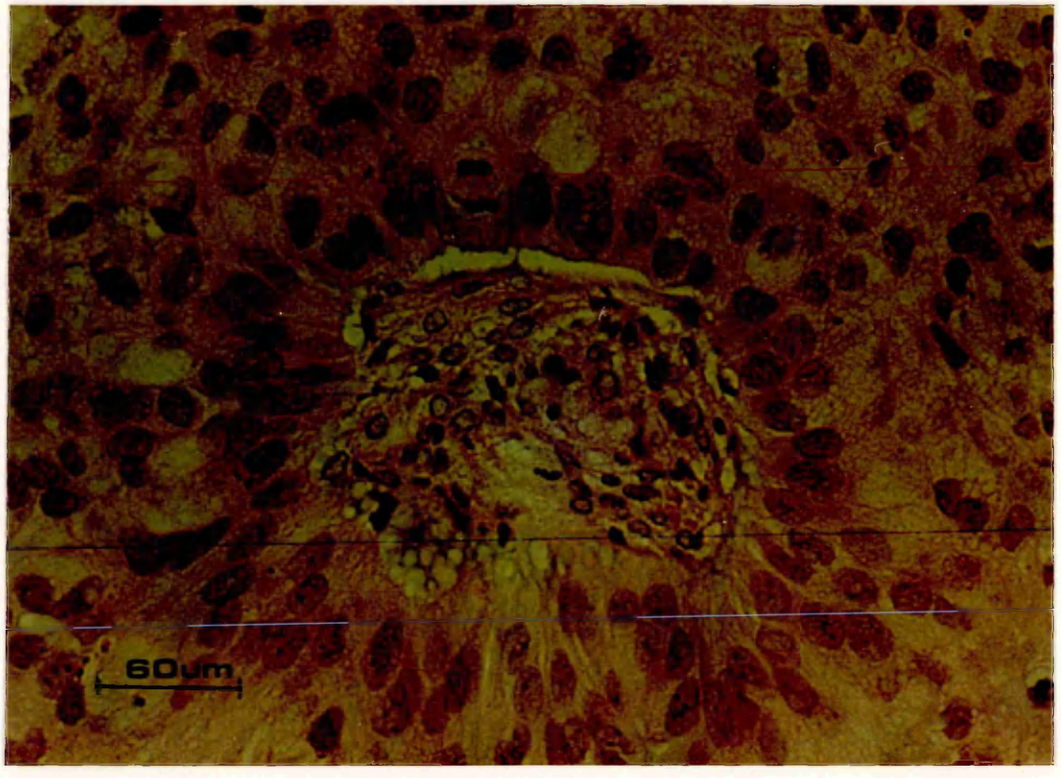

60um

FIGURE 36

Effect of i.p. administration of dexamethasone on the growth of A549 xenografts in nude mice.

Mice were treated with dexamethasone, i.p. three times weekly and the tumour volumes estimated as described in the Materials and Methods. Tumour status as Plate 13.

n = 5 $\pm$ SE

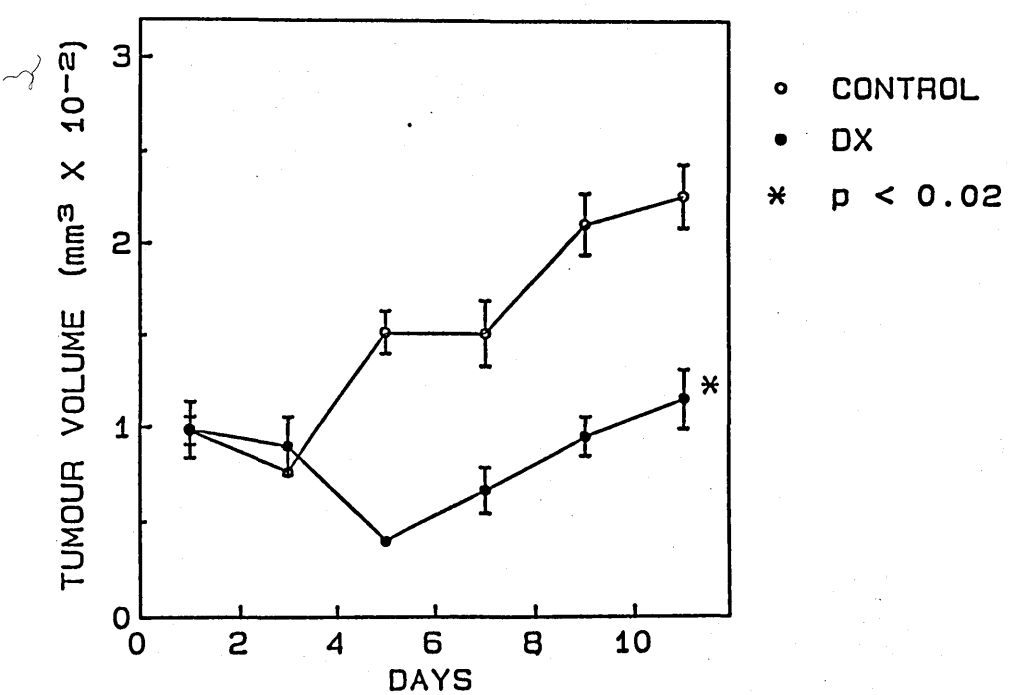

TUMOUR VOLUME (mm³ X 10⁻²)
0
1
2
3
0
2
4
6
8
10
DAYS
*
CONTROL
DX
* p < 0.02

FIGURE 37

Effect of i.p. administration of MOG-FDF/IV on the growth of A549 xenografts in nude mice.

Mice received i.p. injections of MOG-FDF/IV three times weekly and the tumour volumes were determined using double caliper measurements, as outlined in the text. The tumour status was as described in Plate 13.

$n = 5 \pm SE$

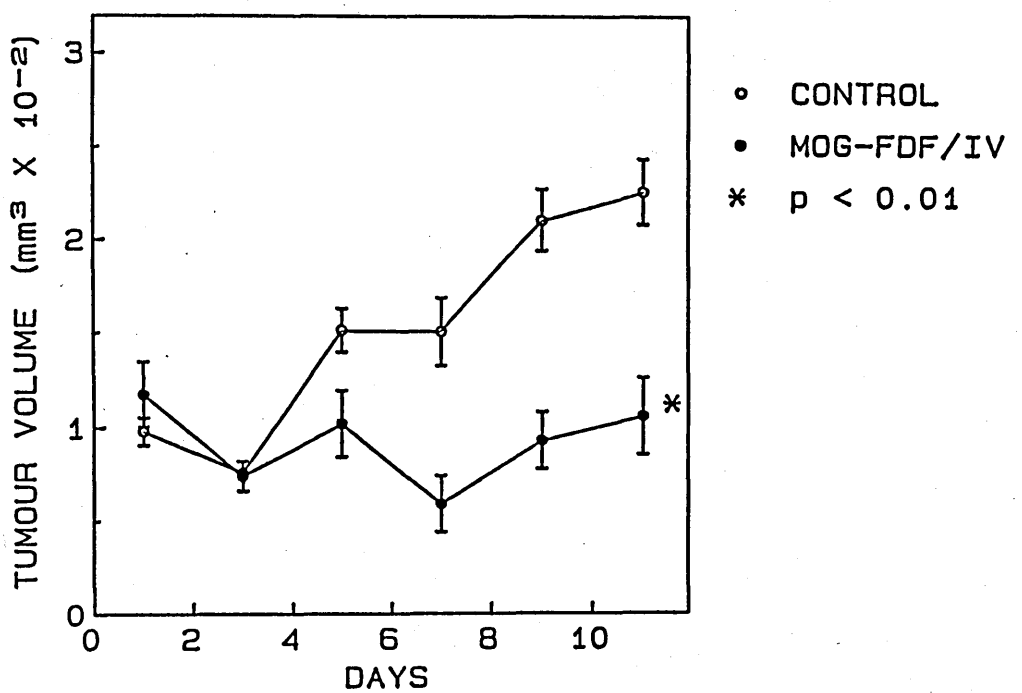
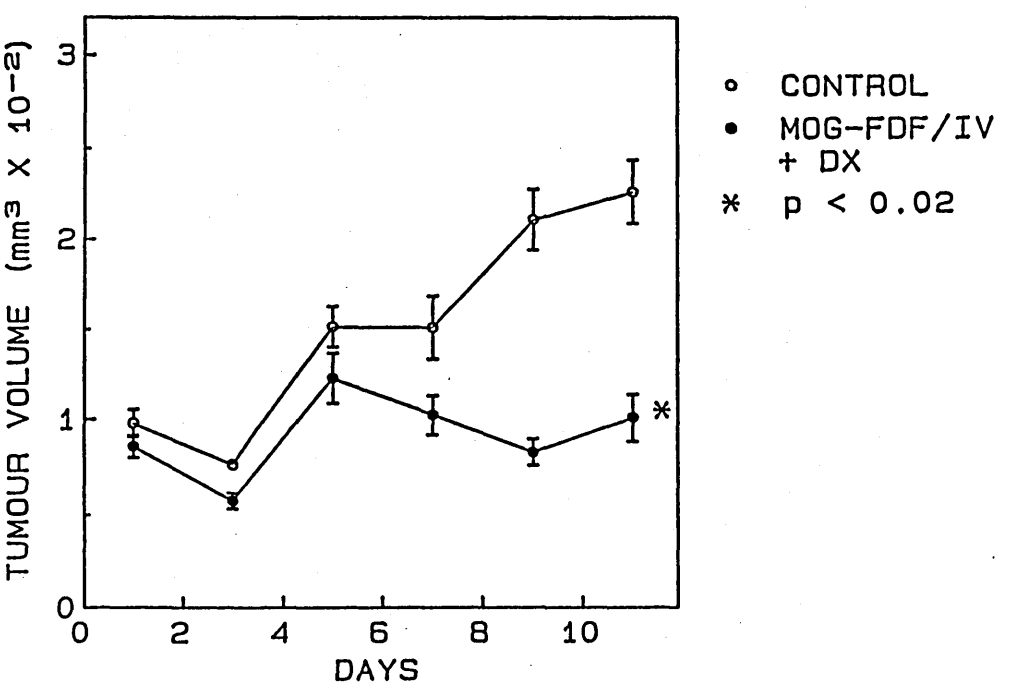

TUMOUR VOLUME (mm³ X 10⁻²)
0
1
2
3
0
2
4
6
8
10
DAYS
*
CONTROL
MOG-FDF/IV
* p < 0.01

FIGURE 38

Effect of simultaneous i.p. administration of MOG-FDF/IV and dexamethasone on the in vivo growth of A549 xenografts.

Nude mice bearing A549 xenografts (status as Plate 13) received i.p. injections of both MOG-FDF/IV and dexamethasone 3 times weekly as described in the text. Tumour volumes were measured by double caliper measurements.

n = 5 ± SE


TUMOUR VOLUME (mm³ X 10⁻²)
0
1
2
3
0
2
4
6
8
10
DAYS
*
CONTROL
MOG-FDF/IV + DX
* p < 0.02

three treated groups indicated significant inhibition of growth. This is shown in Figures 36-38.

7.2 EFFECT ON LT-34

LT-34 is an early passage NSCLC derived from a lymph node metastasis grown as a xenograft in nude mice and then cultured in vitro. Xenografts were established as previously described in the Materials and Methods, but only two experimental groups were used and they received either PBS or MOG-FDF/IV (dosage and treatment regimes as 7.1). No histological examination was carried out.

As can be seen in Figure 39, treatment with MOG-FDF/IV appeared to reduce the tumour volume during the course of the experiment, although this reduction was not statistically significant.

7.3 EFFECT ON A2780 OVARIAN CARCINOMA

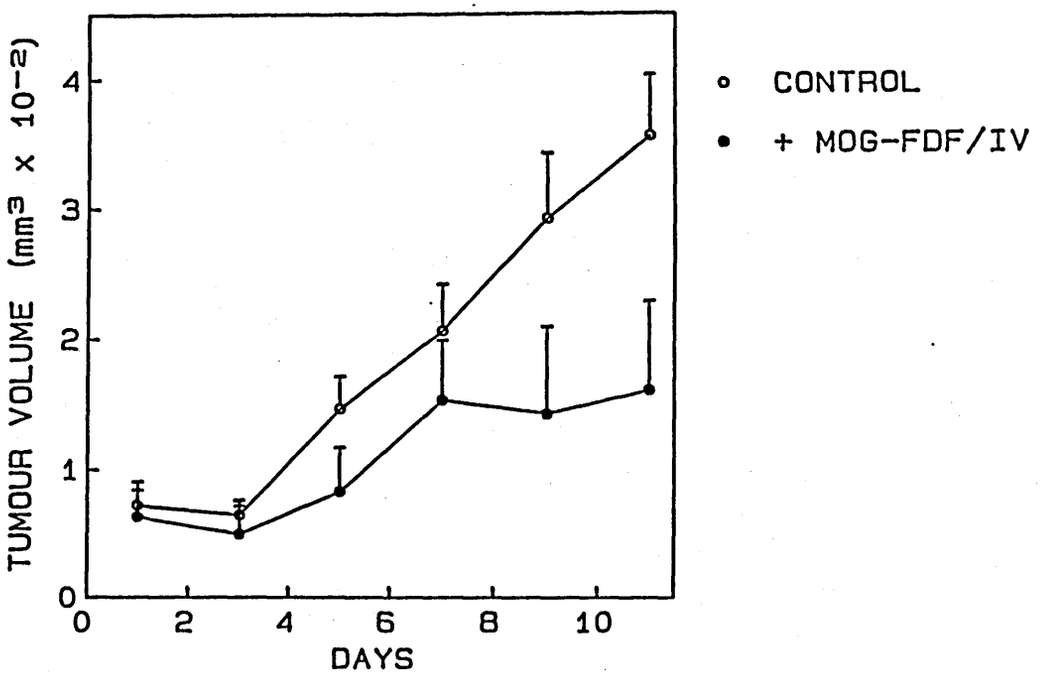
Xenografts were established as previously described, but since the tumour was of ovarian origin, female BALB/c mice were used. There were two experimental groups, a control group and a group which received MOG-FDF/IV (dosage and treatment regimes as 7.1). At the end of the experiment, both sets of tumours were excised and processed for light microscopy.

FIGURE 39

Effect of i.p. administration of MOG-FDF/IV on the growth of LT-34 xenografts in nude mice.

Nude mice bearing LT-34 xenografts (approximately three weeks post-implantation), were treated with MOG-FDF/IV for 12 out of 14 days and the tumour volumes determined by double caliper measurements.

$n = 2 \pm SE$


TUMOUR VOLUME (mm³ x 10⁻²)
0
1
2
3
4
0
2
4
6
8
10
DAYS
CONTROL
+ MOG-FDF/IV

As can be seen from Figure 40, MOG-FDF/IV had no effect on the growth of A2780 xenografts. Similarily, there was no evidence of structural reorganisation at the histological level (Plate 15), with both controls and treated tumours containing densely packed cells with evidence of blood vessels and granulocytes.

7.4 EFFECT ON H69V

H69V is a SCLC which grows as a monolayer, and was derived in this laboratory from the parental line, NCI-H69, which is a classic SCLC. Xenografts were established as described in the Materials and Methods, and the two experimental groups (control and MOG-FDF/IV) received the same dose and treatment regimes as 7.1. The xenografts were examined histologically at the end of the treatment.

MOG-FDF/IV appeared to inhibit growth in H69V xenografts, but sample number was low, and variance too high to make the differences statistically significant (Figure 41). There was some evidence of stromal infiltration in the treated group (Plate 16), and some structural reorganisation, although much less than that observed in the A549 xenografts treated with MOG-FDF/IV.

FIGURE 40

Effect of i.p. administration of MOG-FDF/IV on the growth of A2780 xenografts.

Female BALB/c nude mice bearing A2780 xenografts (status as Plate 13) were treated with MOG-FDF/IV as described in the Materials and Methods for 12 out of 14 days, and the tumour volumes estimated by double caliper measurements.

$n = 3 \pm SE$

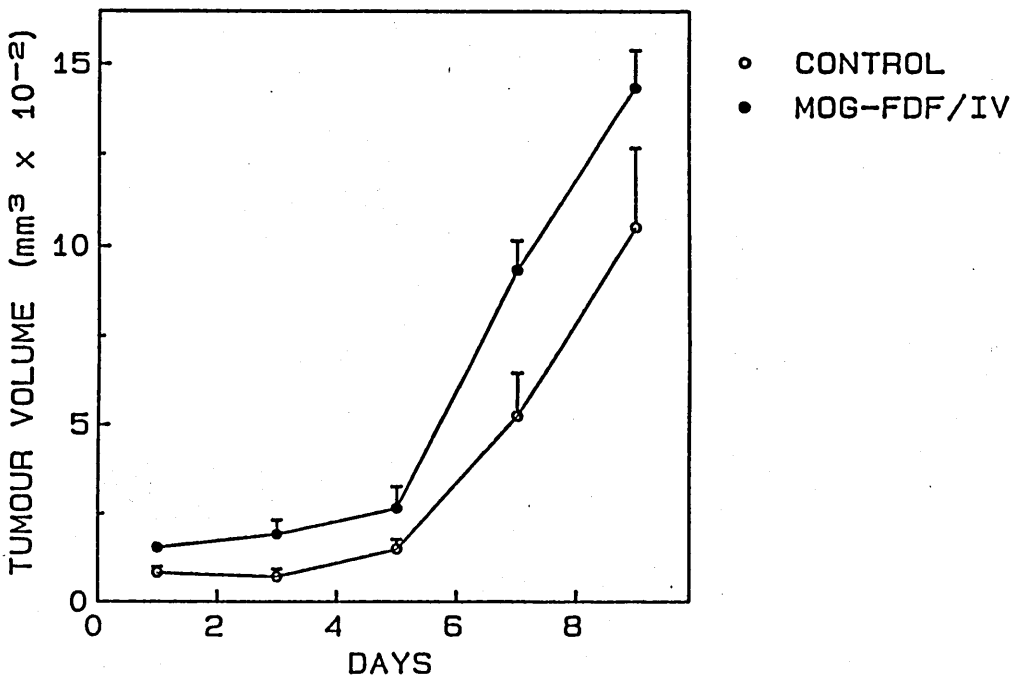
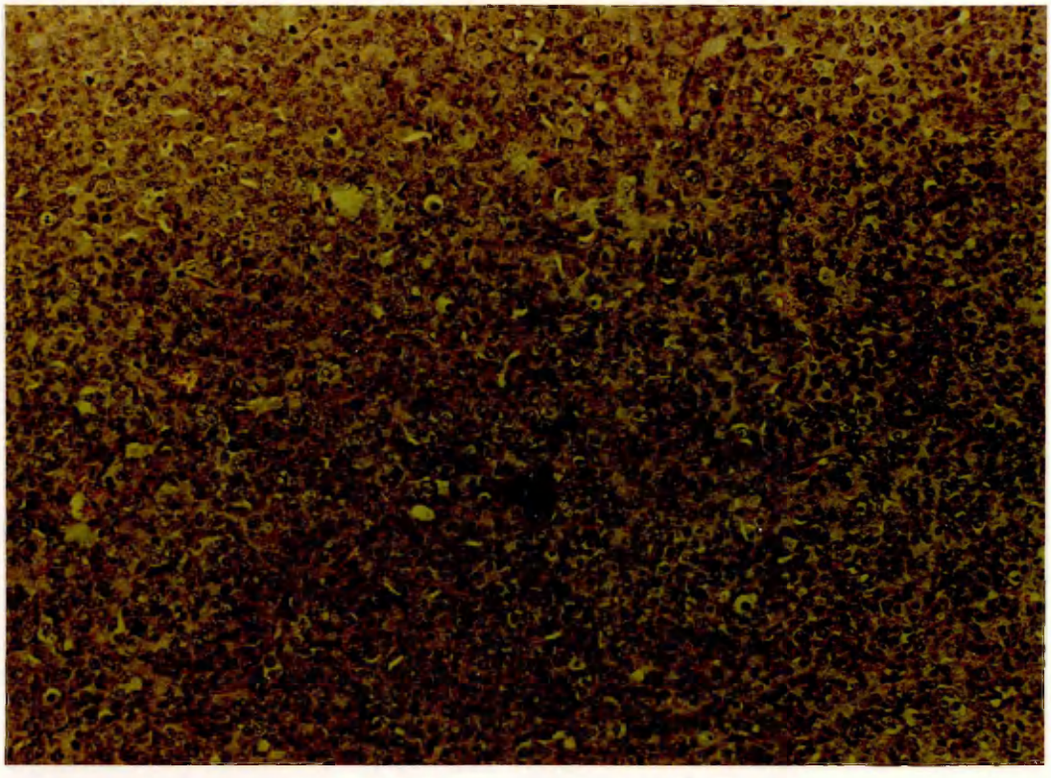

TUMOUR VOLUME (mm³ x 10⁻²)
15
10
5
0
0
2
4
6
8
DAYS
CONTROL
MOG-FDF/IV

PLATE 14: HISTOLOGICAL APPEARANCE OF CONTROL AND TREATED A2780 XENOGRAFTS.

H & E stain.

Plate 14a: Control xenograft, one month post-implantation.

Plate 14b: MOG-FDF/IV treated xenograft (treatment as Plate 13).



150um

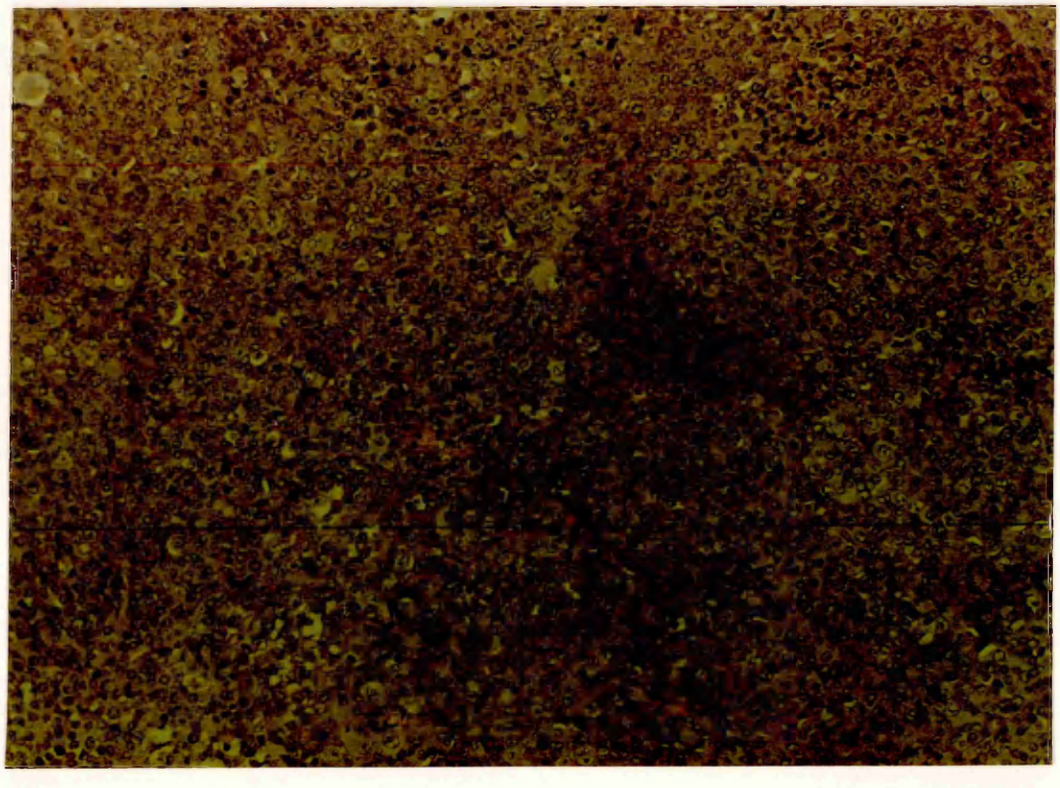


FIGURE 41

Effect of MOG-FDF/IV on the growth of H69V as xenografts.

Mice received a daily i.p. injection of MOG-FDF/IV, and tumour volume was estimated every 2 days as described in the text.

Each data point represents the mean of two observations ± SE.

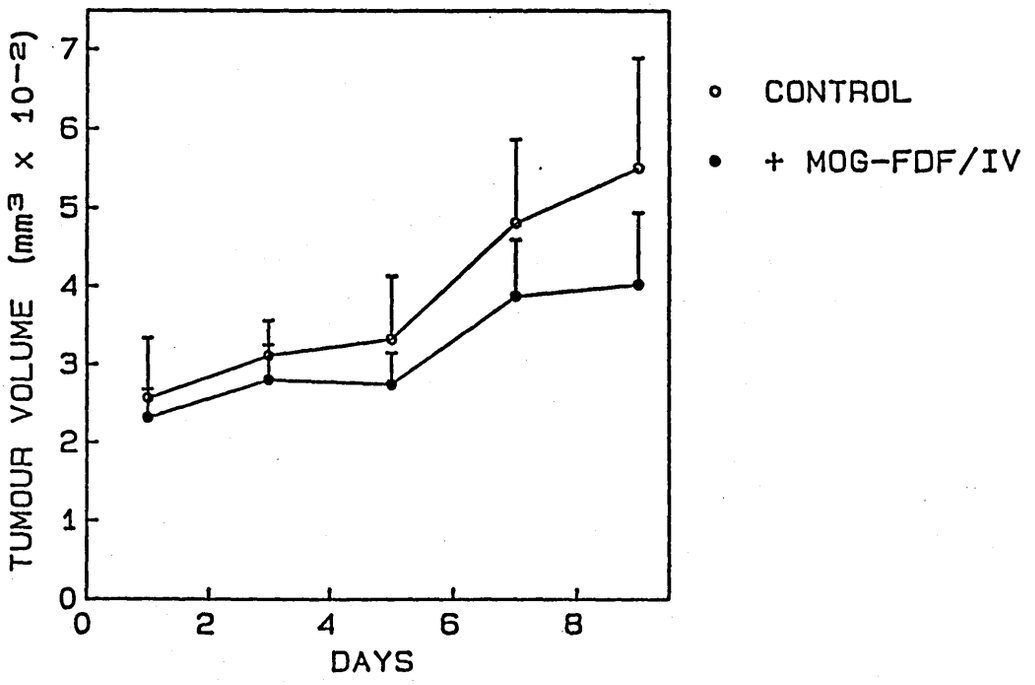
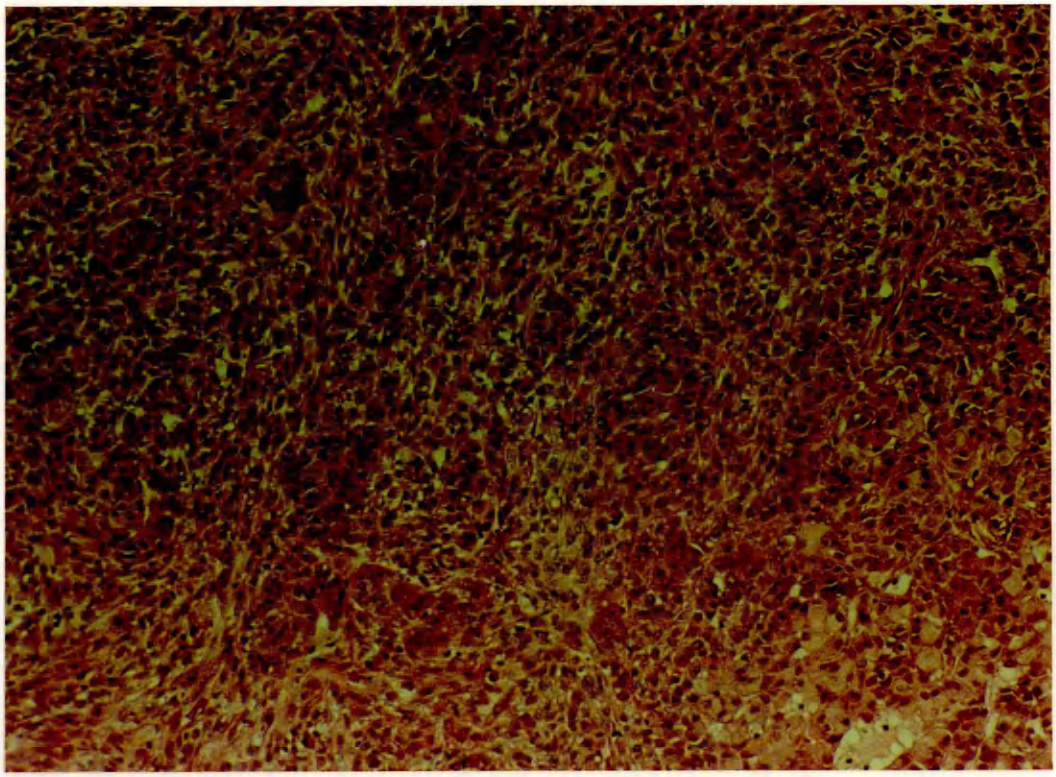

TUMOUR VOLUME (mm³ x 10⁻²)
0
1
2
3
4
5
6
7
0
2
4
6
8
DAYS
CONTROL
+ MOG-FDF/IV

PLATE 15: HISTOLOGICAL APPEARANCE OF H69V XENOGRAFTS.

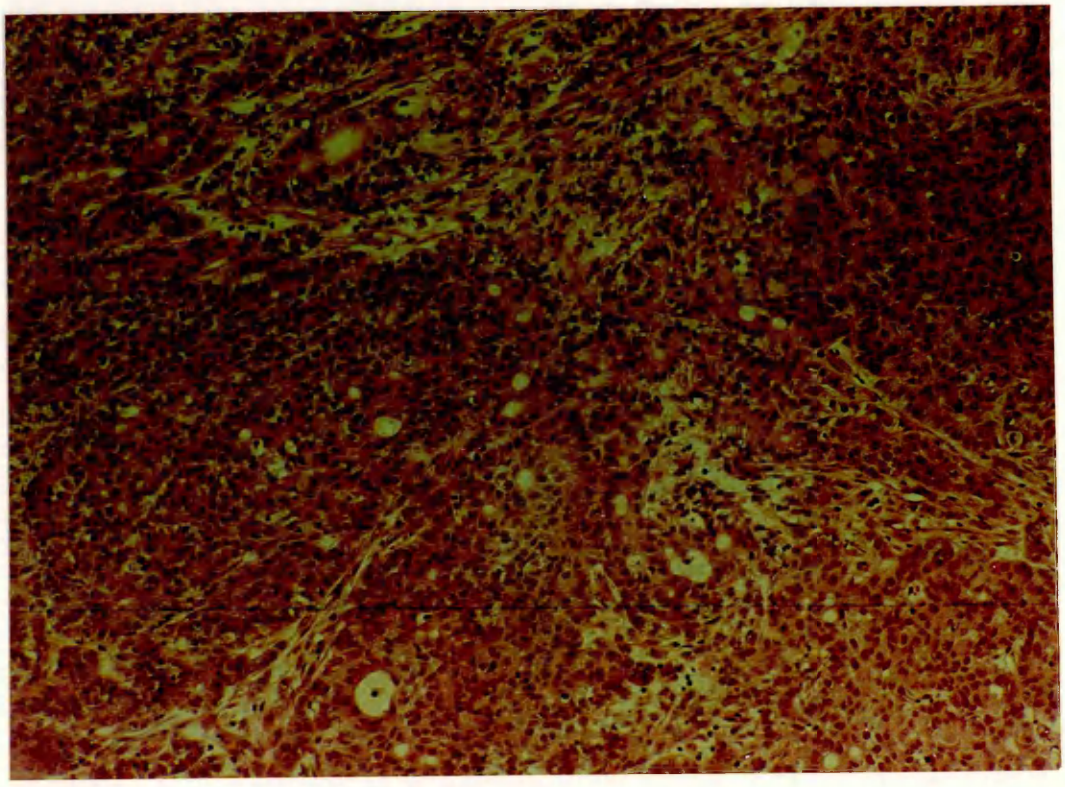
H & E stain.

Plate 15a: Control xenografts, one month post-implantation.

Plate 15b: MOG-FDF/IV treated xenografts (treatment as Plate 13).



200um



DISCUSSION

This Chapter set out to examine the effects of MOG-FDF/IV on the *in vivo* response of A549, to see if there was any correlation with the reponses *in vitro*. To assess target cell specificity, three other xenografts were examined in parallel.

After treatment with MOG-FDF/IV, freshly excised A549 xenografts had quite different gross morphology from the PBS controls. The control group displayed evidence of extensive vascularisation whilst the xenografts treated with MOG-FDF/IV had a distinctly whitish appearance with no surface evidence of vascularisation. The dexamethasone treated group and the group receiving both MOG-FDF/IV and dexamethasone, although having a reduced size compared to controls, still showed surface capillaries. This is somewhat surprising, since it is believed that one *in vivo* action of dexamethasone is to affect vascular functions in diseased tissues and Braunschweiger and Schiffer (1986), have shown this to be precisely the case with xenografts of RIF-1 tumours growing in nude mice. However, the gross difference observed here was in surface vasculature, which is systemic rather than tumour. The data showed that tumour growth was severely curtailed and necrosis was evident, in agreement with vascular collapse reported by Braunschweiger and Schiffer (1986). From these observations, it is possible that MOG-FDF/IV is acting on the tumour vasculature, and, given that the complete identity of MOG-FDF/IV is not yet

known, then it could be acting on the vascular endothelial cells, perhaps by making the tumour less angiogenic.

MOG-FDF/IV could inhibit the growth of A549 xenografts. This was significant with a p value of < 0.01. MOG-FDF/IV was administered as an i.p. injection, so to reach the xenograft, where, presumably it was having its effect, it would have to get into the host circulation. In a previous Chapter, an attempt was made to identify MOG-FDF/IV, and although it was clearly heterogeneous, from SDS-PAGE analysis, it appeared that the molecular weight was probably not smaller than 10kDa, because of dialysis. If this is the case, is difficult to imagine how such large molecules could pass through the peritoneal cavity and into the bloodstream. Certainly there is a lot of proteolytic activity associated with the peritoneal cavity, so it is possible that MOG-FDF/IV is being degraded into a smaller and more manageable form by the peritoneal enzymes whilst still retaining biological activity. In a smaller form, it may be able to pass into the bloodstream more readily, thus becoming more accesible to the tumour. Previous experiments showed that MOG-FDF/IV was only 50% protease labile; the remaining activity may be small enough to enter the vascular system. In this respect, an important experiment to carry out would be to treat the mice with i.v. rather than i.p. injections of MOG-FDF/IV. This should prove whether or not peritoneal enzymatic activity was necessary to allow MOG-FDF/IV to reach the correct site.

As well as reducing xenograft growth, treatment with MOG-FDF/IV resulted in extensive structural reorganisation within the xenograft itself, with evidence of stromal infiltration. It has been reported that untreated NSCLC tumours contain prominent fibrous stroma when grown as xenografts in nude mice (Bergh, 1988). However, the apparent lack of stromal elements in both the control and the dexamethasone treated xenografts implied that MOG-FDF/IV was responsible for stromal infiltration. Given that MOG-FDF/IV originates from stromal cells, it is interesting to speculate that it may be recruiting host stromal cells into the xenograft, perhaps in an attempt to isolate the xenograft and prevent it from undergoing metastasis. In a human basal cell carcinoma, after growing as xenografts in nude mice for between two and five months, 15/17 contained differentiated epithelium with morphologically recognisable stroma (Stamp et al, 1988).

The formation of glandular-like structures and the appearance of ductal epithelium in the MOG-FDF/IV treated xenografts would seem to point to a more differentiated phenotype. Such a claim has been reported recently by Hatekeyama and colleagues (1988), who looked at the effect of subcutaneous administration of dexamethasone four times weekly for eight weeks, to nude mice bearing xenografts of a salivary gland adenocarcinoma. This regime completely inhibited tumour growth and enhanced an apparent luminal structure in the xenograft sections. They claimed that the steroid was inducing cellular differentiation by acting through glucocorticoid receptors. In this study, dexamethasone, whilst reducing the

volume of the xenografts, induced no such structural reorganisation. Moreover, in the study by Hatekeyama et al (1988), the mice were treated with the steroid for eight weeks, so it is highly likely that they would experience some sort of toxic effect after such long term steroid treatment. In one series of experiments in this study (results not shown), the dexamethasone treatment was carried on for a further week, and by the middle of the third week of treatment, almost half of the mice had died, presumably from steroid toxicity.

From the A549 histology, it could be that MOG-FDF/IV is inducing differentiation. The consequences of this may have important implications; by inducing the cells to differentiate, the cells are presumbably becoming more normal, leading to a benign rather than a malignant tumour. The release of angiogenesis factors may be reduced, and the invasive capacity limited. The differentiaited component of the tumour is also likely to be less proliferative.

To determine whether MOG-FDF/IV was specific to A549, its effect on three other xenografts was examined. MOG-FDF/IV had no effect on the in vivo growth of A2780 (ovarian carcinoma). With H69V (derived from SCLC but which shows both NSCLC and SCLC phenotype), and LT-34 (another NSCLC), there was an apparent reduction in tumour growth, but on statistical analysis, this was not significant. A possible reason for this may be that fewer mice were used than with the A549 experiments, due to a shortage of

MOG-FDF/IV. In this respect, it would be worthwhile to repeat these experiments using larger numbers of animals.

In the H69V group, there was some evidence of stromal infiltration in both the control and the treated groups. As mentioned above, observations by Bergh (1988), have shown that in general xenografts of NSCLC contain stromal elements, although in the case of A549, this was only true in the group treated with MOG-FDF/IV. However, H69V is not strictly speaking, a NSCLC. Although derived from a classic SCLC, NCI-H69, work in this laboratory has shown that it displays both NSCLC and SCLC features (Zareen Khan, personal communication).

In a previous chapter (Chapter six), levels of PA were measured in A549 and in another NSCLC, WIL, following treatment with MOG-FDF/IV, and there was a significant reduction in PA in both treated cell lines. This suggested that perhaps MOG-FDF/IV was non-specific. To follow up these observations, attempts were made to grow the cell line WIL as a xenograft. This cell line normally grows very well as a xenograft, but after trying for several weeks, it became apparent that the cell line, for some reason had lost the capacity to form xenografts. On the basis of this observation, and the results with LT-34 and H69V, it is difficult to say whether or not MOG-FDF/IV is specific to A549, or to lung, but it certainly has a more significant effect on A549 both *in vivo* and *in vitro*.

In vitro tissue culture experiments, whilst useful in determining the efficacy of potential anticancer agents often have certain shortcomings, most importantly in correlating the *in vitro* response with the predicted response *in vivo*. One of the questions posed in the Introduction to this Chapter was to ask if there was such a correlation with the response of A549 to MOG-FDF/IV. From the results presented in this Chapter, there does appear to be an *in vitro* and *in vivo* correlation. Therefore MOG-FDF/IV is effective *in vivo* apparently retaining its differentiating properties observed *in vitro*.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

This Chapter will discuss the main findings of this thesis and their implications, with suggestions for future work.

8.1 PREAMBLE

The main aim of this thesis was to examine the effect of glucocorticoids on the role of human foetal lung fibroblasts, MOG-LF113 in the *in vitro* and *in vivo* differentiation of a human alveolar lung carcinoma, A549.

In the general introduction (Chapter One), the developmental failures in neoplasia were outlined and the concept of re-inducing differentiation in these tissues was introduced. It was shown that normal embryonic differentiation requires the support of the mesenchyme for organogenesis and the correct expression of the mature, differentiated phenotype. This raised the possibility that such a system could be applied to human epithelial tumours. After all, tumours often adopt a quasi-embryonic phenotype and may be more responsive to embryonic-like induction.

8.2 STROMAL-EPITHELIAL INTERACTIONS IN NEOPLASIA

As well as being necessary in normal epithelial development, the influence of the stroma was shown to be important for the glucocorticoid induced expression of differentiated functions in A549. This was measured by increased production of pulmonary surfactant, a marker of differentiation in A549 cells. Direct fibroblast-epithelial cell contact was not important, since

conditioned medium collected from steroid treated fibroblasts was effective, and the role of a diffusable factor was implicated.

If such an induction is possible, why is the host stroma ineffective under normal circumstances? Probably the main reason is that the fibroblasts used in this study were derived from foetal lung. Foetal fibroblasts have important inductive properties during embryogenesis, and since most tumours often adopt an embryonic phenotype, they may respond more readily to foetal induction. Also, in a tumour, the host stroma is very often damaged by factors produced by the tumour, in such a way that it can no longer give the correct inductive signals, so the normal differentiation pathway is perturbed.

8.3 IDENTITY OF MOG-FDF/IV

It was possible to partially purify a heterogeneous mixture of proteins from fibroblast conditioned medium using several protein purification techniques. This mixture was designated MOG-FDF/IV. It was shown to be heat and alkali stable, but acid labile and partially (50%) protease labile. From the results presented here, it is difficult to say, and probably unwise to speculate about the possible identity of identity of MOG-FGF/IV.

The main problem was the uncertainty about the molecular weight. On SDS-PAGE gels, there was nothing smaller than 30kDa visible

after silver staining, although the possibility that the activity may have been attributed to a fraction present in such small quantities that it it failed to show up, even after silver staining, has been previously discussed.

However, with the knowledge that is available, it is possible to look at where MOG-FDF/IV fits in with respect to known growth factors. This is shown in Table 19.

The actions of MOG-FDF/IV are, not surprisingly, closest to those of Smith's FPF, since it was on the consequences of these observations that this study was based. However, as discussed previously, Smith's factor was prepared by acid hydrolysis of conditioned medium, and MOG-FDF/IV was acid labile. In one of Smith's reports (Post et al, 1984), the group was able to produce a monoclonal to FPF. An attempt was made to obtain some of this, but this proved unsuccessful. This was unfortunate, since it would have been useful to help further identify MOG-FDF/IV.

The actions of MOG-FDF/IV are quite different from the other three growth factors in the Table. A549 was (with the exception of the animal studies), really the only cell line studied in any great detail, where the principal action of MOG-FDF/IV was to induce pulmonary surfactant production. This occurred with the native form of MOG-FDF/IV, i.e. without prior acidification or alkalisation. Nevertheless, by examining the effects of MOG-

TABLE 19: ACTIONS OF MOG-FDF/IV COMPARED TO OTHER GROWTH FACTORS

GROWTH FACTOR	SIZE (kDa)	CELL SOURCE	TARGET CELL	FUNCTION
PDGF	32kDa	Platelets, endothelial cells, placenta	Mesenchymal cells, smooth muscle placental trophoblast	Paracrine and possible autocrine role in tissue repair. Stimulation of connective tissue cell growth
TGF-beta	25kDa	Platelets, kidney, placenta, cultured cells	Fibroblasts, keratinocytes, mammary epithelium, melanoma, carcinoma	Inhibits cell growth, promotes differentiation. Controls embryonic develpoment
FGF	14-18kDa	Brain, pituitary	Endothelial cells, fibroblasts	Important in wound healing, angiogenesis, nerve regeneration
FPF	5-7kDa (?)	Steroid treated lung fibroblasts	Type II pneumocytes	Stimulates lung maturation
MOG-FDF/IV	?	Steroid treated lung fibroblasts	Alveolar carcinoma, (others?)	Induces pulmonary surfactant, represses PA, reduces tumour volume in vivo

FDF/IV on other cell lines, it may turn out to have different effects on different lineages of target cells.

8.4 ACTIONS OF MOG-FDF/IV

Another question to be considered is the possible mode of action of MOG-FDF/IV. As already discussed, the principal role of MOG-FDF/IV in vitro was to induce pulmonary surfactant production in A549 cells. However, dexamethasone was also capable of stimulating pulmonary surfactant production in A549 cells. Since two apparently different compounds can bring about the same end product, it must be asked if they act in the same way i.e. do they share a final common pathway or do they act through different pathways?

From the results presented here, there are probably two possible scenarios. Firstly, dexamethasone could have a direct effect via glucocorticoid receptors. Indeed, A549 cells have been shown in the past to possess glucocorticoid receptors (McLean, 1986), so it is not surprising that these cells can respond to this steroid.

The other possibility is that dexamethasone, acting via steroid receptors in A549, stimulates the production of MOG-FDF/IV by A549 and the cells respond to this in an autocrine manner, influencing gene expression and resulting in pulmonary surfactant production. In a human breast carcinoma cell line, ZR-75, alpha-interferon

induces production of TGF-beta, regulating their growth in an autocrine fashion (Kerr et al, 1988).

In view of the cell growth data, it is likely that dexamethasone and MOG-FDF/IV act via different routes, but have the same end result. Whilst dexamethasone was cytostatic to A549 at both log and plateau phases of the cell growth cycle, MOG-FDF/IV did not appear to have any significant effect on the growth of A549 cells.

8.5 ROLE OF FDFs IN DEVELOPMENT

FPF has been identified in normal lung. In the liver, fibroblast hepatocyte factor (FHF), which is antigenically different to FPF, has also been partially purified (Dow et al, 1984), and this is important for hepatic development. Such regulatory peptides may be commonplace in embryonic stimulation, with each major organ having its own specific FDF. In neoplasia, the same situation may also arise, whereby mutual interactions between the stroma and the epithelium is altered, and the differentiation inducing, or maintaining effect of the stroma is lost.

8.6 INTER-RELATIONSHIP BETWEEN MALIGNANCY AND DIFFERENTIATION

It has often been proposed that there is an inter-relationship between differentiation and malignancy-associated properties,

whereby an increase in differentiation would be correlated with a decrease in malignancy-associated properties. Marker properties representing the mature differentiated and malignancy-associated phenotype were identified and biochemical and biological assays used to quantify them. Looking at the results as a whole, it appears that such a relationship now applies to A549 cells. MOG-FDF/IV was found to induce differentiation, measured by an increase in pulmonary surfactant production, an increase in the total cellular and secreted GAGS, and the induction of a more differentiated phenotype in xenografts, as well as repressing malignancy-associated properties (a reduction in PA and clonogenicity in soft agar). There appears to be an inverse correlation between between differentiation and malignancy.

8.7 CORRELATION BETWEEN IN VITRO AND IN VIVO RESULTS

There did appear to be a correlation between the in vivo and in vitro results, with differentiation in vitro accompanied by an apparent differentiation in vivo. Such a relationship raises the possibility that restoration of normal differentiation by paracrine factors may represent a novel way of treating epithelial tumours.

8.8 FUTURE PROSPECTS

The results of this study have raised a number of interesting points which would merit future examination.

Greatest pulmonary surfactant production was observed when the A549 cells were cultured on a filter well. As discussed previously, this could be due to one of two possibilities. Since the origin of A549 is reputed to be from an alveolar carcinoma, the *in vitro* conditions on the filter well may mimic more closely the *in vivo* environment. Whether this only applies to alveolar epithelial cells is not known, but it would be beneficial to culture other lung epithelial cells from different areas of the lung under such conditions to see if there was an increase in the expression of differentiated functions.

Perhaps the most important point is the identity and further characterisation of MOG-FDF/IV. As was evident from the results of Chapter 5, MOG-FDF/IV was by no means purified to homogeneity. Obviously for future studies it was be a great advantage to know if MOG-FDF/IV was a unique factor or one of the better known growth or differentiation factors. Having a purified factor would not only make some of the experiments much easier, since there may be the possibility of raising a monoclonal antibody to it, but also raise the possibility of having the factor sequenced and cloned. Also, it would be much easier to radio-label something of

specific identity, rather than an impure mixture. This would be important e.g. for receptor competition assays.

Another point is the relationship between MOG-FDF/IV and other growth factors, to see if there are any additive, synergistic or antagonistic effects. The results presented here have indicated that PDGF, bFGF and IGF-1 all have significant effects in stimulating pulmonary surfactant production. It may well be that MOG-FDF/IV is actually a mixture of growth factors which are working in combination. If this is so, it would be beneficial to unveil their interactive effects. Such experiments are currently underway in this laboratory.

MOG-FDF/IV can induce differentiation and repress malignancy-associated properties in A549, but it is not known if this represents terminal differentiation or not, since differentiation pathways in solid tumours are not very well characterised. This would be an important aspect to be considered at some stage. Connected to this, is the potential effect of MOG-FDF/IV on the invasive properties of A549, and there are several ways of measuring this e.g invasion of embryonic chick heart (Mareel et al, 1979).

Other important aspects to consider are specificity for the both the target epithelium, the fibroblasts from which the factor may be derived, and, to a lesser extent, the phenotypic inducer. The specificity of mesodermal induction may be dependent on the source

of the epithelium. It is possible that the effect of MOG-FDF/IV is specific to alveolar or lung epithelium, but an inductive effect on other epithelial tissues may be found if the correct markers were examined. The specificity of MOG-FDF/IV was carried out to a limited extent here, and it was shown that perhaps it was specific, at least to NSCLC. However these results need further examination using a greater range of tumour models.

In this study, only foetal lung fibroblasts were used. Adult fibroblasts, although they have probably lost their inductive properties, would nevertheless be worthwhile trying. Foetal fibroblasts from a source other than lung would also be a good option, since this would determine stromal specificity. It would also be interesting to examine the prolonged effect of A549 on MOG-LF113, to see if fibroblasts exposed to tumour cells could still respond in the same way to glucocorticoid and release active factor.

It was shown that dexamethasone could act directly on A549 cells, where, as well as inducing pulmonary surfactant, it had a cytostatic effect. From the observations in this study, it has already been discussed that there are two possible modes of action (see above). It would be of value at some stage to purify dexamethasone treated A549 conditioned medium to see if such an effect was found. Of less importance would be to try other steroids, either dexamethasone analogues or different steroids. It would also be of value to look at other phenotypic inducers such

as sodium butyrate, both directly on the A549 cells and indirectly through the fibroblasts to see if production of MOG-FDF/IV could be stimulated by substances other than steroids.

Many studies have implied that the correct substratum is important for the expression of differentiated properties in epithelial cells. In this respect, it may be of use to culture the cells on basement membrane coated dishes or filter wells and monitor the effects.

In conclusion, it appears that stromal-epithelial interactions are important for the induction of differentiation and the reduction of malignancy-associated properties in human alveolar lung carcinoma cells. This is regulated by a factor, possibly unique, which is released from glucocorticoid stimulated foetal lung fibroblasts. The results of this study suggest that neoplastic cells still retain the capacity to respond to paracrine phenotypic inducers. Although still in an early stage, it is possible that such inducers may have a role in cancer therapy in the future.

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