MECHANISMS INVOLVED IN RETINOIC ACID-INDUCED INHIBITION OF METASTATIC MELANOMA CELL LUNG COLONIZATION.

BY

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ABBREVIATIONS.

Arg(R)	Arginine.
Asp(D)	Asparagine.
BSA	Bovine Serum Albumin.
BSS	Balanced Salt Solution.
CAM	Cell Adhesion Molecule.
CPAE	Calf Pulmonary Arterial Endothelial Cells.
CRABP	Cellular Retinoic Acid Binding Protein.
Cys(C)	Cysteine.
DEAE	Diethylaminoethyl Cellulose.
DMEM	Dulbecco's Minimal Essential Medium.
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid.
EHS	Engelbreth-Holm-Swarm Sarcoma.
FCS	Foetal Calf Serum.
FGF	Fibroblast Growth Factor.
GAG	Glycosaminoglycan.
Gly(G)	Glycine.
Ile(I)	Isoleucine.
Leu(L)	Leucine.
MEM	Minimal Essential Medium.
PAI-1	Plasminogen Activator Inhibitor 1.
PAI-2	Plasminogen Activator Inhibitor 2.
PBS	Phosphate Buffered Saline.
PMSF	Phenylmethylsulfonyl Fluoride.
PN-1	Protease Nexin 1.
Pro(P)	Proline.
RA	Retinoic Acid.
RAR	Retinoic Acid Receptor.

RARE	Retinoic Acid Response Element.
RXR	Retinoid X Receptor.
Ser(S)	Serine.
TGF	Transforming Growth Factor .
TIMP	Tissue Inhibitor of Metalloproteinase.
t-PA	Tissue type-Plasminogen Activator.
Tris	2-Amino-2-(hydroxymethyl)-1,3-propandiol.
Try(Y)	Tryptophan.
u-PA	Urokinase type-Plasminogen Activator.
VLD	Very Low Density Cells.

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"Simply the best"

SUMMARY.

This project was initiated to study some of the mechanisms by which retinoic acid inhibits the experimental lung colonization of mice by murine melanoma cells. Using the metastatic cell line B16F1M2, this was investigated in three ways.

When B16F1M2 cells treated with 10^{-6}M retinoic acid are injected via the tail vein into syngeneic mice, the number of lung colonies produced is reduced when compared to those established from control cells. In vitro, retinoic acid has an inhibitory effect on B16F1M2 cell growth and the morphology of the cells is affected with a shifting to a more "normal" form. The first stage of the project was to examine the effect of retinoic acid on the adhesion of these cells to basement membrane components, intact extracellular matrix and to other cells (both homotypic and heterotypic).

It was found that retinoic acid pretreatment inhibited the adhesion of the cells to laminin/nidogen and type IV collagen in a time-dependent and concentration-dependent manner, while adhesion to fibronectin remained unaffected. The adhesion of the cells to intact subendothelial extracellular matrix was also inhibited. The ability of the cells to spread on the substrates was also affected by pretreatment with retinoic acid, with cells remaining rounded on laminin/nidogen and type IV collagen. The density of the cultures also affected cell adhesion, with low-density cultures exhibiting reduced adhesion when compared to almost confluent cultures.

Reports have suggested that some of the effects of retinoic acid are reversible once the molecule is removed from the system. This was found to be the case for adhesion to laminin/nidogen and type IV collagen, although removal of the retinoid did not allow cells to return to control levels within the two days of the experiment. Addition of the retinoid after two days did reduce the degree of adhesion.

Cell-cell adhesion was also investigated. B16F1M2 homotypic adhesion was inhibited by retinoic acid treatment. If both the monolayer and the seeding cell suspension were exposed to retinoic acid, an inhibition in adhesion was also observed. Cell aggregate formation was similarly affected with cells treated with 10^{-6}M retinoic

acid forming aggregates which were more readily dispersed and, indeed, these were also smaller than those formed by control cells. Heterotypic adhesion to endothelial cells was inhibited and this was accompanied by a change in cell morphology with adherent retinoic acid-treated cells remaining rounded on the endothelial monolayer.

The second part of the project was to investigate the ability of the cells to degrade the extracellular matrix. Subendothelial extracellular matrix was prepared and labelled with either [^{3}H] proline which labelled collagen or newly synthesised collagens present or $^{35}SO_4$ which labelled the sulphated glycosaminoglycans. Retinoic acid was found to have an inhibitory effect on the release of products labelled with ^{35}S, but a variable effect on the release of ^{3}H-labelled products. This is in disagreement with many other reports.

One specific enzyme, plasminogen activator, was investigated more closely. B16F1M2 cells grown in the presence of 10^{-6}M retinoic acid were found to exhibit increased activity of this enzyme, both intracellularly and extracellularly.

The third part was to examine the changes, both qualitative and quantitative, in cell surface glycosaminoglycans induced by retinoic acid. In the initial separation the glycosaminoglycans from retinoic acid-treated cells eluted as two peaks when the first separation was carried out as compared to one for the control cells. Enzymatic digestion and further chromatography - both DEAE cellulose and gel filtration - revealed little or no difference in the profiles obtained from both sets of cells. Quantitatively, there was a decrease in sulphated glycosaminoglycan production in cells grown in the presence of retinoic acid. Both the heparan sulphates and the chondroitin sulphates were demonstrated to have a reduced degree of sulphation following exposure to retinoic acid.

INTRODUCTION

1:1. GENERAL INTRODUCTION.

1.The Metastatic Process.

Metastasis from a primary malignant tumour is a complex multistep process. Once the initial transforming event has occurred, the neoplastic cells proliferate forming the primary tumour mass. Cells detach from the tumour and invade the surrounding interstitial connective tissue matrix, infiltrating the intercellular spaces. The cells then cross the basement membrane and enter the circulation via the blood or lymph systems. Evidence suggests that much of the invasive activity of the cells is due to the activation and secretion of a variety of degradative enzymes by both tumour cells and those of the host. These enzymes are capable of degrading the various barriers crossed by the cell before reaching the circulation (Liotta et al, 1983). The tumour cells are also capable of active migration which may be controlled by the secretion of autocrine or paracrine factors, stimulating the second messenger signalling systems (Liotta et al, 1989a). Once invasion has occurred, the cells may grow at the initial site of penetration, or alternatively, they may be released as either single cells or as small aggregates of cells into the circulation.

The survival of tumour cells in the circulation is an important step in the continuation of the metastatic process. However, the presence of viable neoplastic cells in the blood or lymph does not give a positive indication of eventual metastatic development (Weiss, 1985). Circulating tumour cells are subjected to trauma and this is responsible for the death of the majority of them (Weiss, 1987). While circulating, it is possible for the cells to interact with host cells such as platelets, lymphocytes and monocytes and many of these interactions are thought to be cytotoxic to the tumour cells (Fidler et al, 1978). Those cells which survive the trauma of recirculation and any cytotoxic interactions, and remain viable, may be able to arrest in the microvasculature at a distant site forming, potentially, a metastatic colony.

Detachment of cells from the primary tumour, the heterotypic or homotypic clumping of cells, and the cellular adhesion to the microvascular endothelium or exposed basement membrane at the site of metastatic formation, are all dependent upon cell surface moieties on both tumour and host cells. It is probable that variations in the expression and regulation of cell and substrate adhesion molecules play an important role in the success of the metastatic process (Juliano, 1987).

Once the tumour cells have arrested within the target organ, they must extravasate in order to form secondary growths. This requires penetration of the vascular basement membrane, followed by dissolution of the extravascular connective tissue stroma, thus allowing the cell to pass through it. To form a metastatic lesion, the cell must proliferate and grow, establishing a secondary tumour which can, itself, generate the metastatic sequence thus possibly initiating further secondary growths (Weiss, 1985).

The ability of tumour cells to spread is very complex and depends on an interplay between host and tumour cell properties. The forces of selection, when applied to a heterogeneous starting population of cells are thought to allow the emergence of pre-existing populations suggesting that, on the whole, metastasis is a selective process (Hart & Fidler, 1980; Poste & Fidler, 1980).

The first step in the metastatic process is the detachment of cells from the primary tumour, which may be brought about by the loss of intercellular junctions, alterations in the chemical composition and physical properties of the cell coat, and loosening of interactions between the cell and the substrate (Schirrmacher, 1985). The site of release of the tumour cell is an important factor, as if cell detachment occurs prior to contact between the tumour and blood vessels, the tumour cell will probably enter the lymphatic system which, in the instance of melanoma, may occur by active locomotion (Weiss & Ward, 1983). If contact with blood vessels has been made prior to detachment, then venous dissemination will be the preferred initial route (Weiss & Ward, 1983). Entry to the blood system requires invasion and results in exposure of the tumour cells to the vessel lumen.

Cells detach from the primary tumour by a combination of factors, including mechanical agitation generated by body movements and trauma (Weiss, 1980), however, the flow of blood is not considered to be a major mechanism of detachment. Cells can also enter the circulation by shedding into vascular clefts which are lined by tumour cells, as opposed to vascular endothelium, thus this mechanism of entry does not require prior invasion and is a common feature of sarcomas (Willis, 1952).

Necrosis is a common feature of solid tumours and may play a role in cell detachment. Work on tumour necrosis in breast cancer has demonstrated a positive correlation between necrosis and treatment failure (Fisher et al, 1978), confirming earlier work which indicated that there was a higher incidence of tumour cells in the blood where there was necrosis of the primary tumour as compared to those tumours where little or no necrosis was found (Malmgren, 1968).

The heterogeneity of the cells within the tumour must also be a consideration, as a tumour originated from a single transformed cell will have a cell population which is both phenotypically and genotypically heterogeneous (Fialkow, 1979). Tumours can be heterogenous in several different ways as can be seen in tumours of the same histological type but, coming from different individuals and tumour progression within one patient, ie from tumourigenic non-invasive via invasive, non-metastatic to invasive and metastatic subpopulations (Schirrmacher, 1985). Heterogeneity can also occur within a single tumour at any one time as revealed histologically, while malignant tumours have been shown to be heterogenous with respect to metastatic capacity (Fidler, 1973a; Poste et al, 1982). The heterogeneity of the tumour cell population also has an effect on its stability, as has been demonstrated in clonal lines of B16 melanoma of both high and low metastatic potential, with the highly metastatic cells often being less stable (Poste et al, 1981). Cells of the highly metastatic KV-2237 fibrosarcoma have been shown to be composed of subclones exhibiting a wide range of metastatic phenotypes, whereas a clone which had low metastatic potential was found to retain this phenotype. However, several groups have shown that individual subpopulations, and their

subsequent clones are heterogeneous in their stability (Chambers et al, 1981; Neri & Nicolson, 1981; Miller et al, 1983). Changes in the tumour subpopulations can be sudden or gradual and this can result in phenotypes which are either more or less malignant (Heppner & Miller, 1983), also changes can occur after passage either in vivo or in vitro. Clonal analysis of B16 melanoma metastases, produced experimentally by intravenous injection, revealed that they were unicellular in origin (Talmadge et al, 1982; Poste et al, 1982). During the early stages of clonal growth, most lesions contain cells with no distinguishable metastatic phenotypes, however the progressive stage is accompanied by the emergence of cells which have altered metastatic properties (Poste et al, 1982; Talmadge et al, 1984).

Heterogeneity may result from genetic errors arising in either the classical genetic mechanisms, or from the production of cellular variants as in normal tissue differentiation. Genetic errors may include mutations in structural genes, mutations in regulatory genes, or major genomic changes due to numerical or structural alterations to the chromosomes (Schirrmacher, 1985). Oncogenes may affect gene regulation by varying their number and insertion sites, however recent work suggests that many of the modified properties of metastatic populations are not necessarily acquired or regulated independently, but may be controlled through the modification in expression of a small number of key genes (Greenberg et al, 1989). These genes are normally essential for the regulation of diverse cellular functions including proliferation, differentiation, cell-cell communication and motility (Bishop, 1987; Sager, 1989).

In addition to genetic factors, microenvironmental factors may also be involved in tumour heterogeneity, and it is possible that the tumour microenvironment may activate genetic programs within the tumour cell subpopulation causing phenotypic changes similar to those in normal tissue differentiation (Schirrmacher, 1980; Altevogt et al 1982). For this to occur, the influence of the host must be not only selective but also inductive, and if this is the case then multiple phenotypes can

be expressed by one common genotype. Microenvironmental soluble factors as well as cell-matrix contact or cell-cell contact-mediated signals may have a regulatory role on tumour cell phenotypes and expression of heterogeneity (Reid, 1982).

Heterogeneity is also seen in normal tissues, and tumour cells may give rise to variants through a process resembling normal tissue differentiation (Pierce, 1974). Differentiation and dedifferentiation can be illustrated using clones of the B16 mouse melanoma cell line with pigment production being used as a marker of differentiation. Using time-lapse photography, differentiation appeared unrelated to cell division and could be reversed in a proportion of cells, but dedifferentiation was associated with cell proliferation, with pigmented clones being small and most of the nonpigmented clones being large (Bennett, 1983). This suggests that the functions which are associated with differentiation can switch on and off.

An extension of this may be that metastatic capability can be modulated by cell shape in the B16 melanoma cell line (Raz & Ben-Ze'ev, 1983). Normal cells attach and spread on a solid surface until the surface is completely covered by a sheet of cells which then stop proliferating - anchorage dependent growth (Stoker et al, 1968). Proliferation of nontransformed cells will occur when hormonal growth factors are available, but such factors are ineffective if the cells are grown as suspension cultures (Otsuka & Maskowitz, 1975). Anchorage-dependent growth may then be cell-contact and cell-shape dependent (Maroudas, 1975), with the loss of anchorage dependent growth control being one of the major manifestations of tumorigenicity (Vasiliev, 1985).

In an analysis of the rate of DNA synthesis as a function of cell spreading on a substrate, cell variants of B16 melanoma and UV-2237 fibrosarcoma tumour cell lines displaying low, intermediate and high metastatic potentials, displayed no direct correlation between the metastatic phenotype and the changes in DNA synthesis in response to changes in cell shape (Raz & Ben-Ze'ev, 1982). However, if the difference between the control of cell shape is studied, growth of cells in a spherical configuration induces a marked increase in their metastatic capability and these cells will attach and spread more quickly when resuspended and set up in culture. The

alterations in the metastatic capabilities can be reversed by allowing the cells to grow as a monolayer for 24-48h (Raz & Ben-Ze'ev, 1983), and this suggests a central role for changes in cell shape in the modulation of metastatic capability. In vivo, metastases are not necessarily caused by cells with the highest metastatic potential, but by cells which have a metastatic phenotype (Poste, 1983). Additionally, signals from the microenvironment can regulate the expression of various genetic programs, which in turn may activate, or repress, various cellular activities which may affect the metastatic potential (Schirrmacher, 1980), and the reversible modulation of the metastatic capability of changes in cell shape is compatible with this.

The shape of cells in vitro is determined by the interaction of the cell with the extracellular matrix and/or with neighbouring cells (Edelman, 1985). These points of cellular interaction with the environment are characterised by the specific organisation of cytoskeletal elements in characteristic molecularly defined structures. Specific integral membrane proteins mediate these interactions between the cytoskeleton, the extracellular matrix and neighbouring cells. Such interactions may have a role in determining pattern formation and tissue morphogenesis (Obrink, 1986).

Tumour cells have well developed cytoskeletal networks (Raz & Geiger, 1982; Vasiliev, 1985; Ben-Ze'ev, 1985), and the organisation of these may have an effect on the active directional mobility of metastatic cells as well as their ability to deform (Raz & Ben-Ze'ev, 1987). Analysis of the organisation of these networks revealed significant differences between cells of low and high metastatic potentials. The differences were found mainly in the actin-containing networks, with highly metastatic variants having distorted actin bundles (Raz & Geiger, 1982; Zvibel & Raz, 1985). Further work revealed that the organisation of actin is a primary manifestation of a stable cellular phenotype and as such is important in metastasis (Raz & Ben-Ze'ev, 1987).

When invasion occurs, the migrating cells are confronted with the extracellular matrix which is a dense latticework of collagen and elastin, embedded in a viscoelastic ground substance composed of proteoglycans and glycoproteins. It is a

supporting scaffold which isolates tissue compartments, mediates cell attachment and influences the tissue architecture (Kleinman et al, 1981; Wicha et al, 1980). The matrix acts as a selective macromolecular filter and plays a role in mitogenesis and differentiation. Altered interactions between tumour cells and the matrix may have an effect on tumour cell proliferation and invasion (Liotta et al, 1983).

In vertebrate organisms, tissue compartments are bordered by basement membrane and intersitial stroma (Hay, 1982). The basement membrane is composed of type IV collagen, which forms the structural framework, glycoproteins, such as laminin and entactin/nidogen, and heparan sulphate proteoglycans (Timpl et al, 1981; Vracko, 1974; Kefalides et al, 1979). In most tissues, the parenchymal cells secrete and assemble the basement membrane. The epithelial basement membrane undergoes widespread changes during the transition from benign tumour to invasive carcinoma (Siegal et al, 1981; Barsky et al, 1983). Benign pathological disorders with epithelial disorganisation or proliferation are usually characterised by a continuous basement membrane which separates the epithelium from the stroma. By comparison, invasive carcinomas consistently exhibit a defective basement membrane adjacent to the invading tumour cells in the stroma, and it is also defective around tumour cells in lymph nodes and metastatic deposits (Burtin et al, 1982). In some regions of well-differentiated carcinomas, the production of basement membrane by differentiated structures can be identified; however it is often abnormal in that it is either discontinuous or focally reduplicated. Electron microscopy has revealed focal defects in the continuity of the lamina densa of carcinomas in situ (Liotta, 1986). Such defects in the basement membrane may be the earliest stages of the tumour progression process, as in zones of microinvasion, the basement membrane is fragmented or absent (Burtin et al, 1982). This may be due to decreased synthesis, or to the abnormal assembly of the secreted components, or alternatively, the loss of basement membrane may be due to increased breakdown by host-derived proteases (Liotta, 1986). Normal epithelial cells are thought to require a basement membrane for anchorage and growth (Hay, 1982; Kleinman et al, 1981; Wicha et al, 1980), and it may be that invasive tumour cells lack such a requirement.

When tumour cells detach from the primary tumour they must interact with host basement membranes at many stages in the metastatic cascade. During intravasation or extravasation, the tumour cells must penetrate the subendothelial basement membrane and the ability of tumour cells to cross the vascular basement membrane is the rate-limiting step in the exit of tumour cells from the circulation to initiate metastatic colonies (Liotta et al, 1987). The majority of newly released tumour cells arrest in the first capillary bed they encounter (Liotta et al, 1979), with the mechanism of arrest due to impaction, or embolization of the tumour cell clump, or the direct adherence of the tumour cell to the endothelial surface (Fidler et al, 1978; Fidler & Hart, 1982). The presence of the arrested tumour cell causes the underlying endothelium to actively retract, exposing the subendothelial basement membrane. The kinetics of tumour cell adhesion to endothelial cell monolayers varies among tumour subpopulations, and it often correlates with the invasive abilities of the cells as observed in vivo (Kramer & Nicolson, 1981; Varani et al, 1980; Varani et al, 1985). Analysis of the adhesive characteristics of cloned cell lines derived from a rhabdomyosarcoma revealed that the majority of clones which had low lung colonizing ability showed low attachment rates to endothelial cells, while all of the highly colonizing clones had high attachment rates (Korach et al, 1986).

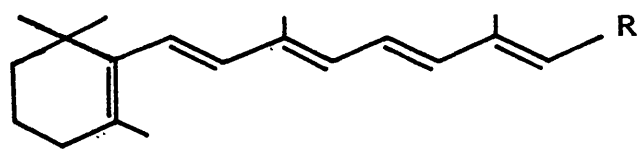
In general, the subendothelial matrix provides a better adhesive substrate for most tumour cells as compared to endothelial cell surfaces (Kramer et al, 1980). However, differences in metastatic potential are not usually reflected by different adhesion rates (Vlodavsky et al, 1983; Nicolson et al, 1981), although correlations between the metastatic potential of various tumour cells and their adhesion to particular extracellular matrix components have been reported (Nicolson, 1988; McCarthy et al, 1985). Once tumour cells attach to the basement membrane, the retracted endothelial cells extend over the tumour cell surface covering the cell. After a time, local dissolution of the basement membrane occurs immediately below the tumour cell, followed by protrusion of tumour cell pseudopodia through the area of dissolution (Wallace et al, 1978). Following this, the tumour cell fully extravasates to form a metastatic colony. At the site where the colonies are initiated,

the extravasated tumour cells must migrate through the perivascular interstitial stroma before tumour colony growth can occur in the organ parenchyma (Wallace et al, 1978).

2.The Retinoids.

Retinoids are a group of vitamin A metabolites and synthetic analogues which have been shown to suppress carcinogenesis and promote the differentiation of several types of tumour cells (Lotan, 1980; Sherman,1986; Lippman et al, 1987). Neoplastic cell types classified as epithelial, mesenchymal and cells derived from the neural ectoderm and primitive ectoderm have all been shown to respond to treatment with retinoids.

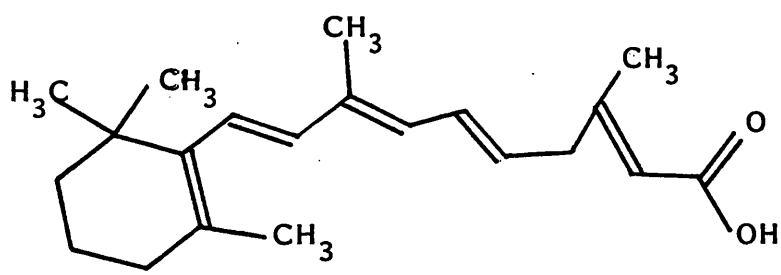
The parent substance trans - vitamin A alcohol, or trans retinol, and its naturally occurring oxidation products trans - retinal (vitamin A aldehyde) and trans - retinoic acid (vitamin A acid) have a common structure consisting of a cyclic end group, a polyene chain and a polar end group.



CYCLIC END GROUP | POLYENE CHAIN | POLAR END GROUP

from Sporn & Roberts,1984.

Each region of the retinoid molecule can be chemically modified in many ways, resulting in an almost unlimited number of compounds, eg for retinoic acid.



from Sporn & Roberts,1984.

Synthetic vitamin A analogues may share some, or all of the biological activities of the naturally occurring compounds. Some analogues are both less toxic and more potent than the naturally occurring retinoids while others are inactive, indicating that there are certain specific structural requirements for activity (Sporn et al, 1976).

Although it has been shown that retinoic acid may play a role in cellular differentiation, the possible mode of action could not be investigated until a suitable model was developed. This utilised cells in cultures derived from embryonal carcinoma cells of testicular origin (Strickland & Mahdavi, 1978). These cells, especially the F9 clonal cells, show very little spontaneous differentiation into the two extraembryonic cell types, parietal and visceral endoderm. But the addition of small amounts of retinoic acid (10^{-8}mol/L) induces their differentiation into an early embryonic cell type (Strickland & Mahdavi, 1978; Strickland et al, 1980). The synthesis of some proteins has been shown to be influenced specifically by the addition of retinoic acid to embryonal carcinoma cells or other cells in culture, eg cytoskeletal proteins and glycoproteins such as collagen (Strickland et al, 1980; Forest et al, 1982), fibronectin (Grover et al, 1982) and laminin (Strickland et al, 1980; Grover et al, 1982; Carlin et al, 1983), enzymes such as glycosyltransferase (Lotan, 1985) and plasminogen activator (Strickland et al, 1980), and glycosaminoglycans (King & Tabiowo, 1981).

Retinoic acid appears to affect gene transcription, and specific gene sequences influenced by retinoic acid have been cloned (Wang et al, 1985). The suggestion that retinoids have an effect on the formation of mRNA is supported by the fact that retinol affects the terminal differentiation of cultured keratinocytes by differential expression of specific keratin mRNAs (Fuchs & Green, 1981). Additionally, cloning of cDNA specifying retinol-responsive human keratins (Eeckert & Green, 1984), and co-ordinative control of keratin gene expression in human keratinocytes (Gilfix & Eeckert, 1985) has also been reported.

Most of the effects of retinoids on gene expression have been determined after exposing the cells to the retinoids for several hours, and as most of the gene products are cytoskeletal proteins, suggests that the appearance of these gene products represent a late event in retinoid action (Chytil, 1986). However, animal studies have shown that retinoic acid simultaneously activates, and inhibits gene expression (Omori & Chytil, 1983), and that this occurs within one hour of administration. Furthermore, chromatin structure is also influenced, and this may account for the large number of genes affected.

Many normal and malignant tissues contain both the cellular retinoic acid-binding protein (CRABP) and the cellular retinol-binding protein (CRBP) (Chytil, 1986). These proteins appear to transport their non-covalently bound ligands, either extracellularly or intracellularly, to sites of action or metabolism. Both sets of proteins are characterised by a sediment coefficient of apparent value 2S corresponding to a molecular weight of 14.6kDa, and by a high binding specificity and affinity for their respective ligands. As well as binding all-trans retinoic acid, CRABPs can also bind 13-cis-retinoic acid (Chytil & Ong, 1984), the acid form of etretinate (Chytil & Ong, 1976) and some arotinoids (Sherman et al, 1983). There has been shown to be a correlation between the ability of these proteins to bind retinoic acid derivatives and the biological activity of these derivatives (Chytil & Ong, 1976; Jetten & Jetten, 1979; Trown et al, 1980). Furthermore, the availability of the free carboxyl group at the C15 position appears to be a necessary condition for the binding to CRABP to occur (Trown et al, 1980; Sani & Hill, 1974; Lotan et al, 1980).

In humans, two CRABPs have been isolated and characterised - CRABP-I and CRABP-II (Astrom et al, 1991) - and been shown to be highly homologous to the murine, avian and rat forms. CRABP-II was demonstrated to be highly selectively and markedly induced after topical retinoid treatment to the skin, which suggests that it might function to regulate the cutaneous actions of retinoic acid (Astrom et al,1991).

Two families of nuclear retinoic acid receptors have, to date, been identified - retinoic acid receptors (RARs)(Petkovich et al, 1987; Giguere et al, 1988; Krust et al, 1989) and retinoid X receptors (RXRs)(Mangelsdorf et al, 1990). Both

consist of three receptor types - α, β, and γ - and belong to the superfamily of steroid hormone receptors. RAR is the prominent receptor mRNA expressed in both murine and human skin (Krust et al, 1989; Elder et al, 1991; Zelent et al, 1990), while RXR has been seen predominantly in the skin, liver and digestive tract epithelia of the adult mouse (Mangelsdorf et al, 1992). RARs bind with high affinity to both all-trans retinoic acid and its stereoisomer 9-cis retinoic acid, however, although RXRs bind to both compounds, binding with high affinity only occurs with 9-cis retinoic acid (Allenby et al, 1993). Both sets of receptors are ligand-inducible trans-regulators which control transcription initiated from the promotors of retinoic acid target genes by interacting with specific DNA sequences termed retinoic acid response elements (RAREs), which are generally located upstream from the sites of transcriptional initiation (Glass et al, 1991; Leid et al, 1992).

Although the mechanisms by which retinoids exert their effects are unknown, it is thought that changes in the growth potential in responsive cells most likely results from a block or retardation of the progression of the cells through the cell cycle or from enhanced differentiation or maturation of the cells (Sachs, 1978; Sachs, 1980). Other effects of retinoids which have been observed include inhibition of cell growth in monolayer cultures, inhibition of anchorage-independent growth in semi-solid medium which is generally thought to correlate with tumourigenicity (Kahn & Shin, 1979; Cifore & Fidler, 1980), and the promotion of terminal differentiation of certain fully neoplastic embryonal carcinoma and promyelocytic leukaemia cells to non-neoplastic differentiated cell types. Many of the effects of retinoids on the growth of transformed cells are reversible, and thus probably represent the suppression of certain aspects of the transformed phenotype. However, their effects on the neoplastic embryonal carcinoma and promyelocytic leukaemia cells represents a stable alteration of the transformed phenotype to a non-neoplastic phenotype (Roberts & Sporn, 1984).

The response of melanoma cells to retinoic acid has been well documented. Unlike other cell types where irreversible terminal differentiation is induced, melanoma cells treated with retinoic acid respond with a reversible

inhibition of growth (Lotan et al, 1981), which is accompanied by an increase in melanogenesis (Edward et al, 1988). When protein synthesis was investigated it was found that retinoic acid treatment of Cloudman S91 murine melanoma cells brought about only minor quantitative changes in the proteins expressed (Lotan et al, 1982). This is in marked contrast to murine embryonal carcinoma cells in which retinoic acid-induced differentiation was accompanied by qualitative changes in protein synthesis (Strickland et al, 1980).

A variety of melanoma cells, both human and murine, have been treated with retinoic acid, and in every case where growth inhibition was observed, it was dependent on the concentration of retinoic acid and independent of cell density (Lotan et al, 1978; Meyskens & Fuller, 1980; Maniglia & Sartorelli, 1981), with 48-72h treatment required before a decrease in cell growth rate could be detected. Additionally, both anchorage-dependent and anchorage-independent growth of these cells was affected by continuous exposure to retinoic acid. It may be that retinoic acid affects the cells in the G_1 phase of growth and slows their entry into S phase (Lotan et al, 1981; Lotan et al, 1982). Retinoic acid was not found to be cytotoxic to the cells as demonstrated by the restoration of the growth rate to control levels 48-72h after removal of the retinoic acid (Lotan et al, 1978).

Melanoma cell lines from various origins, and even subclones derived from the B16 murine melanoma respond in a heterogeneous manner to retinoic acid, with the murine melanoma cell line Cloudman S91 being the most responsive (Lotan et al, 1978; Lotan & Nicolson, 1979; Meyskens & Fuller, 1980; Hoal et al, 1982). However, the most variable response was found in clones of B16 melanoma where the parental clone was inhibited by 40%, whereas several of the cloned derivatives showed no inhibition at all (Lotan & Nicolson, 1979). As yet the basis for this variability is not understood.

Retinoic acid can stimulate melanogenesis as measured by tyrosinase activity and melanin production in some murine and human melanoma cell lines (Lotan & Lotan, 1980; Lotan & Lotan, 1981; Meyskens & Fuller, 1980). In direct contrast to this, melanogenesis is inhibited by exposure to retinoids in some human

melanoma cell lines (Hoal et al, 1982). The induction of melanogenesis by retinoic acid is not accompanied by an increase in cAMP levels, unlike melanogenesis induced by α-MSH (Lotan & Lotan, 1980; Lotan & Lotan, 1981). Retinoic acid inhibition of cell growth was not a result of increased melanogenesis, as inhibition of tyrosinase activity had no effect on growth inhibition (Lotan & Lotan, 1981).

Specific changes in cell surface glycoproteins can also be induced by retinoic acid (Lotan, 1980; Lotan et al, 1983; Lotan et al, 1984). It is possible that some of these alterations in cell membrane components are related to the antiproliferative action of retinoic acid, with support for this coming from the finding that retinoic acid augments sialyltransferase activity. Additionally, retinoic acid has been shown to affect the cell surface glycosaminoglycans, thus it is possible that there is a correlation between these changes, the effects retinoic acid exerts on the adhesion of tumour cells, and the inhibition of the formation of lung tumours.

The process of invasion can also be affected by retinoids. In a system utilising a reconstituted basement membrane, invasion by human melanoma cells was inhibited by retinoid treatment in a time-dependent manner (Wood et al, 1990). Cells treated with retinoic acid were also shown to secrete lower levels of collagenolytic activity and tissue-type plasminogen activator activity. B-actin levels and the expression of mRNA for various oncogenes were also decreased (Wood et al, 1990). From this it was concluded that the inhibitory effect of retinoic acid was caused by the suppression of type IV collagenase and plasminogen activator activity, which may have been triggered through a complex series of oncogene trans-acting factors which induce effects on the cytoskeleton, and ultimately have an effect on cell motility.

1:2. THE EXTRACELLULAR MATRIX.

A three-step theory of invasion has been proposed to explain the biochemical events in the metastatic process (Liotta et al, 1977). The first step is the attachment of tumour cells to the extracellular matrix, and in the case of epithelial tumours such as melanoma, the first matrix encountered is the basement membrane. The interaction of cells with macromolecular components of the extracellular matrix

plays a critical role in the regulation of cell morphology, growth and differentiation and in morphogenetic processes during development (Edelman, 1984; Yamada et al, 1985; Yamada, 1983). Alterations in these cell-matrix interactions have been implicated in the pathogenesis of cancer (Poste & Fidler, 1980; Nicolson, 1984; Liotta, 1986).

The existence of cell-matrix interactions was suspected for some time, but validation was only begun when the biochemical interactions of the process of invasion were investigated. The most significant development was the identification of glycoproteins capable of promoting cell adhesion and spreading in vitro, and which seemed to play a role in the organisation of the extracellular matrix. The first major glycoprotein identified was fibronectin (Yamada et al, 1985), followed by laminin, vitronectin and a variety of tissue-specific nectins (Enestein & Furcht, 1984; Hynes & Yamada, 1982). The adhesion-promoting glycoproteins are often found as part of the insoluble extracellular matrix material, with laminin being confined mainly to the basal lamina of the basement membrane and fibronectin and vitronectin being found in a soluble form in blood plasma and other tissue fluids (Yamada et al, 1985). These proteins are large complex molecules, each of which has a variety of functional activities, but share a common functional pattern comprising a series of proteinase-stable functional domains, displaying binding activities for other macromolecules linked by proteinase-sensitive interdomain regions (Juliano, 1987).

1.Laminin.

Laminin is a glycoprotein originally isolated from the EHS sarcoma (Timpl et al, 1979), with a molecular weight of 90kDa and an unique cross-shaped structure composed of three short arms and one long arm which was revealed by rotary shadowing (Engel et al, 1981). It is composed of three large polypeptide chains designated B_1, B_2 and A which are linked to each other by disulphide bonds (Cooper et al, 1981). The short arms of the molecule are 37nm in length and consist of two

globular domains connected by rod-like elements, while the long arm appears as a 77nm rod terminating in a complex globular domain (Engel et al, 1981), which appears to consist of three smaller globules (Paulsson et al, 1985).

Proteolytic fragmentation and immunological techniques have revealed that the three short arms of the molecule are formed from individual N-terminal segments of the B_1,B_2 and A chains respectively. The three chains are then aligned in parallel through the rod of the long arm, where the B_1 and B_2 chains terminate with an internal disulphide bridge. The globular end of the long arm is formed from the C-terminal segment of the A chain (Timpl, 1989). The globular end domains of the short arms exhibit type IV collagen binding ability (Laurie et al, 1986), as does the globular domain of the long arm (Charonis et al, 1986b). However, this ability to bind to type IV collagen has been shown to occur only when the laminin is in the form of a complex with nidogen/entactin, another basement membrane component (Aumailley et al, 1989). Heparan sulphate and heparin have also been shown to bind to laminin (Sakashita et al, 1980), with at least two different binding sites having been identified. A major binding site has been mapped at the end of the long arm, with some weaker binding activity located in the short arm structures. Cell binding activity has also been identified, with cell binding domains being located on the short arms of the molecule (Graf et al, 1987; Kleinman et al, 1989).

The cell adhesion receptor, located on the rod shaped intersection of the short arms, has been shown to play a role in metastasis (Barsky et al, 1984). Using animal models, tumour cells selected for their ability to attach via laminin will produce increased numbers of metastases after i.v. injection (Terranova et al, 1982). The intact laminin molecule attached to the tumour cell surface will stimulate hematogenous metastasis (Barsky et al, 1984; Varani et al, 1983), but cells treated with the receptor-binding fragment of laminin will be inhibited in the formation of lung colonies after i.v. injection (Barsky et al, 1984). The deduction from this is that the laminin receptor can play a role in hematogenous metastasis through at least two mechanisms. If the receptor is occupied, the cell can use the laminin as an attachment bridge through the globular end domains, however, if the receptor is

unoccupied, the cell can bind directly to the host laminin. Both mechanisms can be blocked by the fragment of laminin capable of binding to the receptor but which lacks the globular end domains (Liotta, 1986).

Retinoic acid has been shown to modulate the attachment of cells to laminin. Mouse fibroblasts treated with retinoic acid were shown to have increased attachment to laminin-coated dishes (Kato & DeLuca, 1987). In contrast to this, B16 melanoma cells treated with retinoic acid were found to exhibit decreased attachment to laminin-coated dishes (Edward et al, 1989). This highlights the diverse effects retinoic acid can have on the same process.

2.Type IV Collagen.

The highly insoluble nature of basement membranes, and the fact that they possess a stability against mechanical forces, has been correlated with the presence of large amounts of collagenous protein, which was shown to be different from the interstitial or fibre-forming collagens (Kefalides, 1973). Type IV collagen is specific for basement membranes and can account for as much as 60% of the total matrix protein (Kefalides et al, 1979; Timpl & Dziadek, 1986). It has a triple helical structure composed of two genetically distinct chains, and studies have revealed that its most likely structural composition is that of a heterotrimer $[\alpha 1(IV)_2]\,\alpha_2(IV)$ (Trueb et al, 1982; Mayne & Zettergren, 1980). The typical collagenous Gly-X-Y repeats of the triple helical region are frequently interrupted by several non-collagenous sequences (Oberbaumer et al, 1985; Pihlajaniemi et al, 1985), and it is these interruptions which give flexibility to the molecule, but also makes it more susceptible to proteolytic attack (Timpl & Dziadek, 1986). This is important in the subsequent degradation of the basement membrane by tumour cells, as the ability to produce enzymes capable of breaking down type IV collagen can be related to the breakdown of the structural support unit of the membrane, and as such, expose the other components of the membrane to proteolytic attack.

Early work revealed the shape of the type IV collagen molecule to be that of a long hockey stick (Timpl et al, 1982). Although the molecules may occur singly, more frequently they are combined by association of their C-terminal globules or less frequently four molecules can combine to form tetramers by joining at their N-teminals (Leblonde & Inoue, 1989). A third mode of association has also been observed where two or three collagen molecules join along part of their length (Yurchenco & Furthmayr, 1984), producing a lateral association and giving the molecule some rigidity. In addition to this a fourth mode has been identified in vitro in which the globular end of free monomers bind to sites at intervals of about 100nm along other collagen molecules (Tsilbary & Charonis, 1986).

Type IV collagen can interact with laminin and the other major component of the basement membrane, heparan sulphate proteoglycan. Laminin has a higher affinity for native type IV collagen than for denatured type IV collagen or other collagen types but it is a weak affinity. Pepsin digestion of the native type IV collagen, which removes the globular domain NCI and about 100nm of the helix, inhibited laminin binding, indicating that the binding site is in the NCI domain or close to it (Rao et al, 1985). Conversely, it has been reported that isolated NCI domain did not bind to laminin but pepsin treated material did (Charonis et al, 1986a).

3.Interstitial Collagens.

Interstitial collagens are fibre-forming collagens which have, in principle, the same molecular structure, the majority of which consists of a continuous triple helical domain. They can interact directly with cells via specific receptors at the cell surface (Mollenhauer & Von der Mark, 1983), or the interaction can be mediated by other extracellular matrix components, with fibronectin having a particularly high affinity for denatured collagens. The collagen binding region has been identified (Kleinman et al, 1976) as a region which overlaps with the cleavage site of vertebrate collagenase with the integrity of this collagenase-sensitive region being required for fibronectin binding (Kleinman et al, 1981).

4. Proteoglycans.

Proteoglycans are proteins which have one or more glycosaminoglycan chains covalently attached to them. They have been implicated in the control of certain biological processes such as adhesion (Rapraeger et al, 1985), migration (Klebe et al, 1984) and cell proliferation (Martin et al, 1984; Uhlrich et al, 1986). Alterations in proteoglycan metabolism in tumour cells or in the adjacent host cells may contribute to the altered adhesiveness and abnormal assembly of the extracellular matrix (Iozzo, 1988). Additionally, malignant cells can secrete factors which are able to modulate the proteoglycan metabolism of the host (Iozzo & Muller-Gauser, 1985).

Heparan sulphate proteoglycans have been isolated and characterised from several basement membranes (Hassell et al, 1980; Dziadek et al, 1985; Hassell et al, 1985) and this has, initially, provided conflicting results on their size and composition. Studies on heparan sulphate proteoglycan isolated from the EHS sarcoma have indicated the existence of at least two forms of the molecule, which differ in size and heparan sulphate content, and thus in their buoyant density (Fujiwara et al, 1984; Hassell et al, 1985; Kato et al, 1987). The low density form has been examined using biosynthetic studies, and found to have a uniform protein core precursor of about 400 kDa (Ledbetter et al, 1985) and antibodies to the protein core have shown a widespread occurrence in cultured cells and tissues, but limited to their basement membranes (Dziadek et al, 1985; Fenger et al, 1984).

The protein core probably plays a crucial role in the integration of low density heparan sulphate proteoglycan into basement membranes, as it cannot be dissociated by high salt concentrations which would interfere with ionic interactions. This strong binding may include self assembly of the molecule to oligomers (Yurchenco et al, 1987), additionally, association to dimers and to more complex stellate clusters occurs by binding between terminal domains of the core protein which are most distal to the heparan sulphate attachment region. Proteolytic removal of these domains abolishes self assembly (Timpl, 1989).

Heparan sulphate chains have been shown to bind weakly to laminin and the NCI domain of type IV collagen (Fujiwara et al, 1984), but this binding was non specific, as dextran sulphate and to some extent chondroitin sulphate could displace it from its ligands, with the binding properties varying according to the sulphate content of the glycosaminoglycan (Pejler et al, 1987). Some heparan sulphate proteoglycans may also be involved in the control of serine proteinase activity, which may be important in the maintenance and remodelling of basement membranes (Pejler et al, 1987), and also in the invasion of tumour cells through the basement membrane by proteolytic degradation.

Evidence that heparan sulphate proteoglycans promote cell adhesion when incorporated into basement membranes is not extensive. However reports have indicated that heparin, and sulphated glycosaminoglycans in general, can support the attachment and spreading of a wide variety of cell types including neural crest (Erickson & Turley, 1983) and neuronal cells (Stamatoglou & Keller, 1983) with the effect, in some cases, being caused by the sulphate group (Erickson & Turley, 1983; Turley, 1980). Also, highly metastatic cells release a heparinase, and this may possibly alter basement membrane architecture or the adhesive properties which affect cell attachment to it (Nicolson, 1982).

5.Fibronectin.

Fibronectin is a dimeric glycoprotein composed of two similar 240kDa disulphide bonded subunits and occurs in two forms - the soluble form which is found in high concentrations in plasma, other body fluids and the conditioned medium of cultured cells, and the insoluble form, in which high molecular weight disulphide-bonded multimers are organised into an extracellular fibrillar matrix (Mosher, 1980; Mosher, 1984; Hynes, 1981; Hedman & Varani 1989). The insoluble form provides a substrate for cell migration during embryogenesis and wound healing (Thiery et al, 1989), and is capable of promoting the attachment, spreading and migration of a variety of cell types including tumour cells (Ruoslahti, 1988b; Ruoslahti & Pierschbacker, 1987). The molecule contains two distinct

cell-binding domains, one located in the centre of the molecule and the other in a region which is alternatively spliced from precursor mRNA termed the type III connecting segment (IIICS) (Yamada, 1988).

1:3. CELL ADHESION.

1. The Integrins

Cell attachment to fibronectin is mediated, in part, by the interaction of the Arg-Gly-Asp-Ser (RGDS) sequence with a specific cell surface fibronectin receptor ($\alpha_5\beta_1$) which is a member of the noncovalent heterodimer integrin receptor group (Pytela et al, 1985; Hynes, 1987; Ruoslahti, 1988). The integrins form a family of related matrix receptors (Buck & Horwitz, 1987) which are membrane glycoproteins consisting of two subunits - α and β - both of which have a large extracellular domain, a transmembrane domain and a short cytoplasmic domain (Ruoslahti & Giancotti, 1989). The α and β subunits are non-covalently bound to one another and the association is promoted by divalent cations (Ginsberg et al, 1988).

The integrins are divided into at least five subfamilies, each being defined by a common β subunit (Hynes, 1987), with the specificity for ligand binding being determined by the particular combination of α and β subunits. The best characterised integrins are those belonging to the β_1 subfamily, which includes receptors for laminin, fibronectin and collagen; to the β_2 subfamily found on leukocytes which includes receptors mediating cell-cell interactions; and the β_3 subfamily, which includes the platelet glycoprotein 11b/111a complex and the vitronectin receptor which also binds fibrinogen, thrombospondin and von Willebrand's factor (Cheresh & Spiro, 1987; Lawler et al, 1988).

In vitro and in vivo comparisons of control and malignant cells have suggested that changes in integrin expression may be part of malignant transformation, but no uniform pattern of change has been demonstrated. Rodent cells transformed with Rous sarcoma virus (Plantefaber & Hynes, 1989), basal cell and squamous cell carcinomas (Peltonen et al, 1989), exhibit a reduction in the expression of integrins from the β_1 subfamily. In contrast to this, chemically

transformed, tumourigenic human osteosarcoma cells display an increase in β_1 integrins when compared to nontumourigenic osteosarcoma cells (Dedhar & Sauliner, 1990), while other transformed cells show an alteration in the distribution of integrins but no changes in their expression (Akiyama et al, 1990).

Despite the lack of evidence outlined above, functional studies suggest that integrins are important in the metastatic process. Synthetic peptides containing the RGD sequence which block the binding of many integrins to their extracellular matrix ligands (Pierschbacher & Ruoslahti, 1984; Yamada & Kennedy, 1984), inhibit the movement of human melanoma cells through an amniotic basement membrane in an experimental model of invasion (Gehlsen et al, 1988). Such peptides also reduced the number of metastatic deposits found in the lungs of mice following i.v. injection of B16F10 metastatic melanoma cells (Humphries et al, 1988; Saiki et al, 1988). Consistent with these findings is the observation that antibodies against the β_3 subfamily prevent the establishment of tumours when human melanoma cells are implanted into nude mice (Boukerche et al, 1989). Studies have shown that melanoma cells, both in tissues and in culture, express a wide variety of integrins in a relatively heterogeneous pattern, but in tissue sections the expression of the β_3 subfamily was restricted to only the tumorigenic cells (Albelda et al, 1990).

However, perhaps the most consistent adhesion marker for metastatic potential is the expression of the $\alpha_v \beta_3$ integrin which recognizes several ligands including vitronectin, fibrinogen and thrombospondin. Melanoma cells that do not express this integrin have been demonstrated to be less invasive, in vivo, than those cells which do express $\alpha_v \beta_3$ (Felding-Habermann et al, 1992). The expression of $\alpha_v \beta_3$ has also been shown to affect the expression and secretion of type IV collagenase (Seftor et al, 1992). These findings would suggest that the integrins expressed by melanoma cells are relevant to their invasive phenotype.

2.Cell-Cell Adhesion.

One of the earliest and most important, adhesive events in invasion is the adhesion of tumour cells to the vascular endothelium. Although many tumour cells become trapped in the first vascular bed they encounter, passive cell trapping is not sufficient to promote tumour metastasis (Hart & Fidler, 1980; Hart, 1982; Tarin, 1985; Nicolson, 1988). In many cases tumour cells will lodge in the first organ encountered, but metastatic colonies will only be found in organs downstream of the site of first arrest. From this it has been speculated that a specific cellular adhesive interaction may initiate the formation of metastatic deposits at a particular secondary site (Green & Harvey, 1964; Zetler, 1990). In vitro work has revealed that, in many cases, metastatic tumour cells adhere selectively to capillary endothelial cells derived from their preferred secondary site (Alby & Auerbach, 1986; Auerbach et al, 1987). This has been confirmed by using melanoma cells which preferentially metastasise to the lung, and lymphoma cells which preferentially metastasise to the liver (Roos et al, 1984; Nicolson, 1988).

Most of the information on adhesion of tumour cells to endothelial cells has come from studies on lymphocytes which home to specific endothelial cells in lymph nodes or Peyers patches (Stoolman, 1989), and a set of cell adhesion molecules on endothelial cells that interact with a set of adhesive receptors on the lymphocyte cell surface have been identified (Yednock & Rosen, 1989), and named vascular addressins. Antibodies to these receptors can inhibit experimental metastasis of certain lymphoid tumours (Sher et al, 1988), which implies that they may be important in the metastatic cascade.

A cell membrane proteoglycan, CD44, has also been identified and implicated in various cell processes. One of its major functions would appear to be in the regulation of lymphocyte adhesion to cells from endothelial venules during lymphocyte migration (Gallatin et al, 1987; Jalkanen et al, 1986). This process has many similarities to the metastatic spread of solid tumours (Sher et al, 1988). CD44 has also been shown to be the principal cell surface receptor for hyaluronate (Aruffo

et al, 1990; Miyake et al, 1990), a glycosaminoglycan that plays a major role in diverse cellular processes which include cell-cell adhesion, embryonic development, cell migration and angiogenesis (Toole, 1981; West et al, 1985).

3. Retinoids and Cell Adhesion.

The effect of retinoids on the ability of cells to attach varies depending on the type of cell under investigation. They can enhance the attachment of 3T3 cells to both laminin and type IV collagen, but if this cell line is virally transformed, attachment is unaffected (Kato & De Luca, 1987). 3T3 cells have been shown to adhere poorly to the two substrates, but the transformed cells adhere readily. This is supported by the finding that laminin receptor expression is increased in highly metastatic cells (Liotta et al, 1986b).

Retinoic acid has also been shown to have an effect on cell-cell adhesion. The adhesion of a human epithelial cell line to plastic is reduced by treatment with retinoic acid, as is the ability of these cells to form clusters (Shapiro & Poon, 1979). BHK21/C13 cell-cell adhesion has also been shown to be reduced by retinoids, but cell-substrate adhesion is increased (Kamei, 1983). This illustrates the diverse effects retinoids can have on the same process.

The synthesis of extracellular matrix molecules which may be involved in cell adhesion can also be affected by retinoids. Glycosaminoglycan synthesis can be increased in some cell types while being decreased in others, suggesting that the action of these compounds may be cell specific. Certain tumour cell lines have been shown to express heparan sulphates with a reduced degree of sulphation following treatment with retinoids (Robinson et al,1984). This would suggest that one possible function of retinoids is to alter the surface molecules involved in cell adhesion.

1:3. DEGRADATION OF THE EXTRACELLULAR MATRIX.

When invading tumour cells attach themselves to the basement membrane, they must degrade it before further dissemination can occur. The presence of proteolytic enzymes in the process of matrix degradation associated with

tumour invasion has been well documented (Nicolson, 1982; Liotta et al, 1983; Saksela, 1985), and reports indicate an increase in the proteinase activity in malignant tumour tissue as compared to that in normal tissues. Indeed, a direct correlation between metastatic potential and the production of certain proteinases has been established for some tumour cell lines - sublines of the B16 murine melanoma have been shown to have an increased production of type IV collagenase (Liotta et al, 1980; Liotta et al, 1979), and an endoglycosidase capable of degrading heparan sulphate (Nakajima et al, 1983), which correlate with increased metastatic potential. The origin of the proteinases has been traced to both the tumour cells and the host cells (Bauer et al, 1979), thus it would appear that the degradation of the extracellular matrix in tumour invasion is a highly complicated process, involving the abnormal expression of proteinase genes in cells of the tumour and the host (Tryggvason et al, 1987).

Proteinases are enzymes which are capable of hydrolysing peptide bonds as either exopeptidases or endopeptidases. They can be divided into four main classes depending on criteria such as their catalytic site, optimum pH, cation requirements and susceptibility to inhibitors. The proteinases most often associated with the malignant phenotype are the serine proteinases such as plasminogen activator, the cysteine or thiol proteinases which include the cathepsins, and the metalloproteinases.

1.Plasminogen Activators.

These are a group of serine proteolytic enzymes which specifically convert plasminogen to the active enzyme plasmin (Saksela, 1985; Dano et al, 1985). The inactive proenzyme plasminogen is abundant in the vasculature, in most body fluids and in the basal layers of the epidermis (Isseroff & Rifkin, 1983), while the active enzyme, plasmin, is the primary effector enzyme in thrombolysis as it effectively cleaves fibrin. Plasmin, however, can also participate in the extravascular

breakdown of extracellular matrix and basement membrane glycoproteins, and it may also be involved in the activation of procollagenase to collagenase, which is a requirement for collagen degradation in the matrix (Salo et al, 1982).

Two different types of plasminogen activator are produced - tissue type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (Nielsen et al, 1983; Golds et al, 1983). They differ from each other with respect to tissue distribution, and in catalytic, molecular and immunological properties. Additionally, it has been established that they are independent gene products (Rajput et al, 1985). Both types are secreted as single chain polypeptides (Saksela, 1985; Dano et al, 1985), but in this form u-PA has little or no enzyme activity (Eaton et al, 1984; Kasai et al, 1985). Activation occurs by the cleavage of peptide bonds which results in the formation of a two chain structure, and this process can be mediated in the extracellular space by plasmin (Dano et al, 1985). This may be an important regulatory step in the process, however, it is not known if specific enzymes for the initial proenzyme activation exist on the cell surface. Initial activation of plasminogen may occur through the interaction of single chain u-PA and plasminogen when the macromolecules are present on the cell surface or in the matrix (Miles & Plow, 1985; Miles and Plow 1987; Hajjar et al, 1986). These interactions may lead to alterations in plasminogen configuration, which favours the formation of a complex with the single chain form, and eventually to the generation of catalytic amounts of plasmin capable of converting the single chain u-PA to the active two chain form. Unlike u-PA, single chain t-PA is active and readily activates plasminogen in the presence of fibrin.

Using immunological methods, u-PA has been identified in fibroblast-like cells in vivo (Skriver et al, 1984; Kristensen et al, 1985), whereas t-PA has been localised to the vascular endothelium of many tissues (Kristensen et al, 1985). Cultured cells produce mainly u-PA (Saksela, 1985; Dano et al, 1980; Vetterlein et al, 1979), but there are examples of cell lines which produce only t-PA (Strassburger et al, 1983; Paranjpe et al, 1980) or both u-PA and t-PA (Levin & Loskutoff, 1982; Vetterlein et al, 1980). Plasminogen activators have also been found in cells of

neoplastic origin, with u-PA being found in the conditioned media of cells derived from endometrial carcinomas (Swanberg & Astedtl, 1979), and a number of cell lines of neoplastic origin have been reported to produce u-PA (Strickland et al, 1983; Azzarone et al, 1983; Dano et al, 1985). t-PA is also produced by a number of neoplastic cells, the most notable being a number of melanoma cell lines (Rifkin et al, 1974; Orenstein et al, 1983; Strickland et al, 1983).

2.Plasminogen Activator Inhibitors.

Secreted plasminogen activator activity is effectively controlled by specific inhibitors produced by a number of cell types and, indeed, two proteinase inhibitors specific for plasminogen activators have been identified and purified. Plasminogen activator inhibitor type-1 (PAI-1) has been shown to be released by endothelial cells, some neoplastic cell lines including those derived from melanoma (Andreasen et al, 1986; Emeis et al, 1983; Levin, 1983; Loskutoff et al, 1983), and it is also present in thrombocytes and blood plasma (Erickson et al, 1985; Wiman et al, 1984). Plasminogen activator inhibitor type-2 (PAI-2) was initially purified from placental extracts, but has been found to be released by cultured monocytes-macrophages (Astedt et al, 1985; Chapman & Stone, 1985; Saksela et al, 1985). Although both inhibitors belong to the serine proteinase inhibitor family - the serpins - they differ in immunological reactivity and in some physiological characteristics (Hekman & Luskutoff, 1985; Ny et al, 1986; Pannekoek et al, 1986). A third plasminogen activator inhibitor has been identified as the proteinase nexin 1 (PN1) which has been isolated from fibroblasts, but is also produced by other cultured cell types (Baker et al, 1980). However, unlike PAI-1 and PAI-2, PN1 also inhibits plasmin, thrombin and other trypsin-like serine proteinases (Scott et al, 1985).

3.Metalloproteinases.

Metalloproteinases are a family of enzymes which usually act at a neutral pH, have tightly bound Zn^{2+}, require Ca^{2+} for activity and cleave internal as opposed to terminal peptide bonds (Khokha & Denhardt, 1989). They include interstitial

collagenases, type IV collagenase (gelatinase) and a stromelysin (proteoglycanase, collagenase-activating proteinase, transin), and are typically secreted as proenzymes which require activation. Both intracellular and membrane-bound metalloproteinases have been described (Bond & Beynon, 1985; Chen & Chen, 1987; Harris et al, 1984).

a/ Interstitial Collagenases.

The interstitial collagens are highly resistant to attack by proteinases, but they can be degraded by highly specific metalloproteinases which have been isolated from a variety of cells and tissues. Interstitial collagenases have been purified from a number of sources including rabbit synovial fibroblasts (Werb & Reynolds, 1975), rabbit V2-carcinoma (Woolley & Grafton, 1980) and human skin (Stricklin et al, 1978). They require Ca^{2+} and Zn^{2+} as cofactors for maximum activity, and are secreted as proenzymes which must be activated. Activation can be achieved by a variety of proteinases such as trypsin, chymotrypsin, plasmin, cathepsin B, kallikrein and mast cell proteinases (Paranjpe et al, 1980; O'Grady et al, 1981; Werb et al, 1977; Woolley, 1984; Nagase et al, 1982; Eckhout & Vaes, 1977). Additionally, some endogenous neutral proteinases (Horwitz et al, 1976) and serine proteinases (Woessner, 1977) may be able to activate latent collagenases, either directly or after they themselves have been activated (Eckhout & Vaes, 1977). Procollagenase secreted by human skin fibroblasts can also be autoactivated without any detectable change in molecular weight (Stricklin et al, 1977)- the endogenous activator isolated from the medium of cultured human skin and rat uterus activates collagenase without any measurable change in the molecular weight of the zymogen (Tyree et al, 1981). Activation can also be initiated chemically, especially by organic mercurials and chaotropic agents, again without any apparent change in molecular weight, and this may occur by the formation of alternative disulphide bridges in the molecule (Goldberg et al, 1986).

The activated interstitial collagenases cleave the native triple-helical molecules at a single site, with both soluble and fibrillar collagen molecules being cleaved. Types I, II and III collagen are degraded in a similar fashion, although the cleavage of type II collagen is slower than that of other types (Woolley & Grafton,

1980; Cawston et al, 1981). The enzymes cut the polypeptide chains at a Gly-Leu or Gly-Ile sequence depending on the type of chain (Woolley, 1984; Woolley et al, 1978). The triple helix has also been reported to be unstable at this sequence and as such it makes the molecules, particularly type III collagen, susceptible to the action of trypsin and thermolysin (Gross et al, 1980). When the molecule has been cleaved, the degradation fragments, which are thermally labile, are denatured and can be further degraded by collagenase itself or by other proteinases (Woolley, 1984).

In 1965, ultrastructural evidence was provided for the breakdown of collagen fibres by tumour cells (Birbeck & Wheatley, 1965), and this was supported by reports of increased collagenolytic activity in breast carcinoma cells (Keiditsch & Strauch, 1976), as well as in a number of tumour tissues, and in cultured tumour cells (Woolley & Grafton, 1980; Hashimoto et al, 1972; Bauer et al, 1979; Biswas, 1982). However, a direct correlation between the secretion of interstitial collagenases and the metastatic potential of tumour cells has not been established. The collagenases may often be secreted by host tissue cells (Biswas & Dayer, 1979), and it is likely that the specific activities of the different tumour collagenases, as well as their diffusibility within tissues can vary greatly and can, in turn, affect the invasive potentials of different tumours.

b/ Type IV Collagenase.

Type IV collagen is specifically degraded by a metalloproteinase-type collagenase (Liotta et al, 1979; Salo et al, 1982), and is resistant to attack by interstitial collagenases (Babel & Glanville, 1984). Type IV collagenase has been found in malignant tumour tissue (Liotta et al, 1979; Salo et al, 1982), cultured tumour cells (Salo et al, 1985) and also in proliferating human fibroblasts (Salo et al, 1985). The enzyme has been purified and identified as a glycoprotein with a molecular weight of 60-70kDa (Salo et al, 1982; Salo et al, 1985; Garbisa et al, 1986). Serine and sulphydryl proteinase inhibitors do not inhibit type IV collagenase whereas metal chelators do, which confirms that the enzyme is a metalloproteinase. However, it can be activated, in vitro, by pretreatment with plasmin, trypsin or organomercurials and in this respect it resembles the interstitial collagenases. It

cleaves the native type IV collagen molecule at a single site in the helical domain (Fessler et al, 1984), and in this way the enzyme may be able to decompose the collagen network of basement membranes and as such solubilize the structure.

The association of type IV collagenase and neoplasia has been confirmed by the demonstration that type IV collagenolytic activity is secreted by tumour tissue and numerous tumour cells in culture (Salo et al, 1982; Eisenbach et al, 1985; Starkey et al, 1984). Enzyme activity detected in the culture media of variants of the B16 murine melanoma has been shown to correlate with the metastatic potential of the cells (Poste and Fidler, 1980). In contrast, type IV collagenase activity has been detected in several other tumour cell lines, but no correlation determined between enzymatic activity and metastatic potential (Starkey et al, 1984). The reason for the discrepancy in the observations of enzyme activity are not clear, but changes in the cellular phenotypic properties may be responsible.

Work with melanoma cells has demonstrated that the secretion of type IV collagenase was enhanced by laminin but was unaffected by fibronectin (Turpeenniemi-Hujanen et al, 1986), and this agreed with in vivo work which demonstrated that laminin can potentiate the metastatic capacity of tumour cells (Terranova et al, 1982; Barsky et al, 1984). Laminin binds to a specific cell surface protein which does not bind fibronectin or any of the collagens, and it was found that fragments of laminin which contain the receptor-binding domain, but none of the other matrix attachment domains, had a much greater effect on type IV collagenase production than the whole molecule (Turpeenniemi-Hujanen et al, 1986). Monoclonal antibodies to the laminin receptor completely blocked the effects of laminin on type IV collagenase, and although the possibility that laminin may affect type IV collagenase production through channels other than the laminin receptor cannot be excluded, the evidence would suggest this to be the case. This would link the adherence of a tumour cell to laminin in the basement membrane with the production of a degradative enzyme in the process of invasion.

c/ Stromelysin.

Unlike the collagenases, stromelysin is not specific for any one component of the extracellular matrix. It can degrade a broad spectrum of substrates including proteoglycans, nonhelical regions of collagen, elastin, fibronectin and laminin (Chin et al, 1985; Khokha & Denhardt, 1989). It is secreted as a proenzyme which can be activated by trypsin or mercurials (Chin et al, 1985), and the molecule itself has been shown to resemble the interstitial collagenases in that there is a significant homology in the amino acid sequences of both molecules (Tryggvason et al, 1987). Production of stromelysin can be induced in fibroblasts by the same agents capable of inducing collagenase production, which suggests that secretion of stromelysin may be associated with malignant transformation (Chin et al, 1985).

4.Metalloproteinase Inhibition.

Metalloproteinases must be tightly regulated at the enzyme level because of the potential to degrade collagens at a neutral pH. As stated, these enzymes are secreted in latent forms which can be activated either by factors inducing conformational changes, by limited proteolysis, or by a catalytic process involving another metalloproteinase (Harris et al, 1984; Murphy & Reynolds, 1985; Peppin & Weiss, 1986; Okada et al, 1988). An important control is exerted on the metalloproteinases by inhibitors of enzyme activity (Tryggvason et al, 1987; Cawston, 1986), which have been identified in serum and several body tissues including skin, bone, tendon and uterus from various animal species. α_2-Macroglobulin accounts for much of the anti-metalloproteinase activity in serum where it mainly acts against proteinases that enter the blood stream. Smaller cationic proteins are another class of tissue inhibitors which prevent events such as tumour-induced vascular proliferation, bone resorption and new vessel growth into cartilage (Harris et al, 1984; Cawston, 1986). However, perhaps the most important inhibitors are the highly effective tissue inhibitors of metalloproteinases (TIMPs) found in many body fluids.

TIMP, also called collagenase inhibitor, is not active against the serine, cysteine or aspartic proteinases (Murphy & Reynolds, 1985; Cawston, 1986; Welgus et al, 1985), but it can inhibit the matrix metalloproteinases. It regulates the breakdown of collagen and basement membrane components in a number of situations (Jeffrey, 1986) by forming a 1:1 complex with the target metalloproteinase which is effectively irreversible, with small changes in the ratio having a dramatic effect on enzyme activity (Welgus et al, 1985; Harris et al, 1984).

There is strong correlative, as well as direct, evidence implicating TIMP in controlling the invasive potential and metastatic proficiency of cells, with an inverse correlation between the levels of TIMP production of various intracranial tumours (Halaka et al, 1983), and a series of metastatic variants derived from NIH7T1/2 cells (Hicks et al, 1984). In contrast to this, human cirrhotic livers possess high levels of metalloproteinase inhibitors and are able to resist invasion by metastasizing cancers (Barsky & Gopalakrishna, 1988). Purified TIMP was able to partially inhibit the invasion of human amnion by M5076 cells (Thorgeirsson et al, 1982), and it was shown that recombinant human TIMP blocked invasion of the amnion at a step after adhesion. Frequent intraperitoneal infusions of TIMP produced a significant inhibition of colonization of the mouse lung by subcutaneously injected B16F10 melanoma cells. This effect was specific for invasion since TIMP treatment did not alter the growth rate of the primary tumour, or the size of the metastatic nodules that formed in the lung.

5.Endoglycosidases.

The proteoglycan heparan sulphate is a major constituent of basement membranes and, as such, its degradation is important in the process of metastatic invasion. Heparan sulphate-degrading activity has been identified in metastatic melanoma sublines and, indeed, this activity correlates with their lung colonizing potentials (Nakajima et al, 1983). The melanoma heparan sulphate degrading enzyme is an endo-β-D-glucuronidase (heparanase) that cleaves heparan sulphate at specific intrachain sites (Nakajima et al, 1984). If melanoma cells are treated with

heparanase inhibitors, the number of experimental lung metastases produced is decreased, which indicates that heparanase plays an important role in melanoma metastasis (Irimura et al, 1986).

Endoglycosidase activity is not unique to metastatic tumour cells, and it has been found in a variety of normal tissues and cells including liver (Hook et al, 1984), skin fibroblasts (Klein et al, 1976) and inflammatory macrophages (Naparstek et al, 1984; Savion et al, 1984). In some cases these normal cell enzymes have been shown to be different from tumour enzymes - a platelet derived endo-β -D-glucuronidase which is different from melanoma heparanase in that the normal cell enzyme is capable of degrading both heparan sulphate and heparin, whereas heparin is a potent inhibitor of melanoma heparanase (Oosta et al, 1982; Nakajima et al, 1984).

There have been several reports indicating that plasminogen activator can have an effect on the action of heparanase (Bar-Ner et al, 1986). Using several normal and malignant cell types, it has been shown that degradation of heparan sulphate proteoglycans in the subendothelial extracellular matrix is accelerated by a proteolytic activity which is thought to make the heparan sulphate side chains more accessible to the action of heparanase (Bar-Ner et al, 1985). Plasmin has been shown to digest various extracellular matrix components such as fibronectin, laminin and proteoglycans (Liotta et al, 1981; Jones & De Clerck, 1980). Thus it is possible that the effects observed are due to plasmin-mediated degradation of various matrix constituents, not necessarily heparan sulphate proteoglycan, and this would allow a better interaction between the heparanase and the extracellular matrix heparan sulphate. Another possibility is that a limited degradation of the heparan sulphate proteoglycan core protein could occur which would lead to the release of soluble high molecular weight fragments with heparan sulphate side chains which would be more readily cleaved by the heparanase than those still bound to the extracellular matrix.

6. Effect of Retinoids on Matrix Degradation.

Retinoids have been shown to have an inhibitory effect on tumour cell invasion. Indeed, retinoic acid has been demonstrated to inhibit invasion by human melanoma cells and rat mammary adenocarcinoma cells through a basement membrane in an in vitro assay system, with the inhibition of invasion being correlated to a decrease in type IV collagenolytic activity (Hendrix et al, 1990; Nakajima et al, 1989). Retinoic acid inhibition of invasion was found to be dose and time dependent, but the inhibitory effect was found to be reversible on removal of the retinoid from the assay system (Hendrix et al, 1990). In this system, the presence of the retinoic acid in the basement membrane model had no effect on invasion, but pretreatment of cells suppressed invasion which would suggest that an effect of retinoic acid on the cells, rather than on the matrix, is responsible for alterations in cell invasion (Hendrix et al, 1990). This is in contrast to a study examining the effect of retinoic acid on the invasion of virally transformed rat cells through a chick chorioallantoic membrane (Fazely et al, 1985). Although invasion was inhibited, it was dependent upon pretreatment of the chorioallantoic membrane with retinoid and on the continuous presence of retinoid during the assay. Thus, the inhibitory effect was attributed to the ability of retinoic acid to enhance the differentiation of the chorioallantois rather than the tumour cells (Fazely et al, 1985).

The ability of retinoids to inhibit invasion of melanoma cells and rat mammary adenocarcinoma cells through a basement membrane system has been correlated to a decrease in type IV collagenolytic activity (Hendrix et al, 1990; Nakajima et al, 1989). In contrast to this, reports have also indicated that retinoids have no effect on type IV collagenase activity (Oikarinen & Salo, 1986). This illustrates, again, the diverse effects retinoids can have on the same processes in different cells.

Retinoic acid has also been shown to have an effect on t-PA production by human melanoma cells (Hendrix et al, 1990). A decrease in t-PA was observed when human melanoma cells were exposed to retinoic acid. Various reports have shown that tumour cell invasion through a basement membrane matrix is dependent upon a proteolytic cascade in which plasminogen activators have important roles, one

of which being the activation of procollagenase type IV (Tryggvason et al, 1987; Saksela & Rifkin, 1988). Thus, inhibition of invasion by retinoids may be due to reduced levels of plasminogen activators rather than directly on type IV collagenolytic activity.

In human skin fibroblasts, retinoids have been shown to have an inhibitory effect on type I collagenase activity (Bauer et al, 1983). However, there is little evidence in the literature to suggest that retinoids have any effect on other degradative enzymes such as the heparanases.

1:5. PROTEOGLYCANS.

Proteoglycans are complex macromolecules which contain a protein core to which one or more glycosaminoglycan (GAG) chains are covalently bound. They are present in all mammalian tissue, are most prominent in connective tissues, and occupy highly strategic positions such as on the cell surface, the basal lamina and the extracellular matrix where most of the cell-cell interaction events take place. They are dynamic macromolecules which can influence fundamental biological processes including cell proliferation, cell recognition and cytodifferentiation, with most of these effects being due to the polyanionic nature of proteoglycans, their expanded configuration in tissues and their ability to interact with a variety of extracellular matrix components. Experimental evidence suggests that in pathological processes in which tissue remodelling occurs such as atherosclerosis, inflammation and tumour invasion, there are concurrent alterations in proteoglycans.

Glycosaminoglycans are unbranched chains with characteristic repeating disaccharide units which contain hexosamine, either D-glucosamine or D-galactosamine, and uronic acid - either D-glucuronic acid or L-iduronic acid - with the exception of keratan sulphate, in which a galactose residue replaces uronic acid. There are four main types of glycosaminoglycan - heparin/heparan sulphate, chondroitin sulphate/dermatan sulphate, keratan sulphate and hyaluronic acid. With the exception of hyaluronic acid, all glycosaminoglycans are O-sulphated to varying degrees, with heparin and heparan sulphate also having N-sulphated groups. Although

other macromolecules such as some basement membrane glycoproteins contain sulphate, the density of the sulphate groups in these compounds is generally much less than that of the glycosaminoglycans, in which the maximum is from three to four sulphate groups per disaccharide as found in some of the repeating units of heparin. Because of their sulphate and carboxyl groups, the glycosaminoglycans have an unusually high charge density, and this contributes significantly to their biological properties and governs some of their interactions with other molecules (Hook et al, 1984; Lindahl & Hook, 1978).

The glycosaminoglycan portions of chondroitin sulphate, dermatan sulphate, heparan sulphate and heparin proteoglycans contain additional sugars at their reducing end which include one xylose and two galactose molecules covalently linked to the hydroxyl group of serine on the protein core (Lindahl & Roden, 1966; Lindahl & Roden, 1972).

1.The Glycosaminoglycans.

a/ Hyaluronic Acid.

This is the largest glycosaminoglycan with molecular weights ranging from 100kDa to 1000kDa. It is made up of repeating disaccharide units of N-acetylglucosamine and D-glucuronic acid but, unlike the other glycosaminoglycans it is not covalently linked to a protein core (Mason et al, 1982) and is nonsulphated. It is found in nearly all connective tissues and in some instances, such as umbilical cord, synovial fluid and vitreous humour, it is the principal glycosaminoglycan. Although it is abundant in embryonic tissue, it is later replaced by other glycosaminoglycans; thus it may represent the earliest evolutionary form of these macromolecules (Toole, 1973;Toole, 1981). A primary role of hyaluronic acid is to retain water, and it is an effective lubricant for the synovial and mesothelial surfaces as well as being a resilient buffer against mechanical stress.

During wound repair, and in normal development, hyaluronic acid is produced in large quantities. In both of these processes a major reassembly of cellular and extracellular elements occurs, and it has been shown that the enzymatic removal

of hyaluronic acid coincides with the cessation of cell migration (Toole, 1981). However, in cartilage of various kinds, hyaluronic acid represents only a minor proportion of the total uronic acid present (Muir, 1980), but it plays an essential role in the aggregation of proteoglycans and is mostly present as a component of proteoglycan aggregates rather than in the free state.

Hyaluronic acid is also the primary constituent of the pericellular coat surrounding a variety of cells (Goldberg & Toole, 1984; McBride & Bard, 1979; Underhill & Toole, 1982), and its interaction with the cell surface can influence proteoglycan synthesis and secretion (Handley & Lowther, 1976; Solursh et al, 1980; Wiebkin & Muir, 1973). Even at relatively low concentrations, hyaluronic acid can have a profound influence on its environment by, for example, affecting the movement of macromolecules across tissues (Ogston & Preston, 1966). In physiological solution, hyaluronic acid is polyanionic and assumes a stiff, randomly kinked coil structure which occupies large solution domains. Individual molecules can entangle through specific associative interactions to form networks which can, perhaps, contribute to the properties of the matrix (Wight et al, 1991).

b/ Chondroitin Sulphate.

Chondroitin sulphate consists of repeating units of D-glucuronic acid and N-acetylgalactosamine. Two common isomers can be distinguished containing a sulphate group either on the fourth or sixth carbon of the galactosamine residue - chondroitin-4-sulphate and chondroitin-6-sulphate. The size of the chondroitin sulphate chain varies greatly, with an average of about 40 repeating disaccharide units for the cartilage proteoglycan, which corresponds to a molecular weight of about 20 kDa, also the number of sulphate ester groups varies with an average of 0.8 per disaccharide. The chondroitin sulphates are found in a variety of tissues, but most often in cartilage and intervertebral discs where it can reach a concentration of up to 10% of the total wet weight.

c/ Dermatan Sulphate.

Dermatan sulphate may be considered as an isomer of chondroitin sulphate in which a variable proportion of D-glucuronic acid has been reconverted to L-iduronic acid. However, formation of L-iduronic acid is generally incomplete, therefore dermatan sulphate chains are hybrid molecules, with several alternating sequences, each containing one to several disaccharide units of either type (Fransson & Havsmark, 1970; Fransson & Malmstrom, 1971). The formation of L-iduronic acid occurs by C5 epimerization of the D-glucuronic acid already incorporated into the growing polymer (Malmstrom et al, 1975), and the proportion of disaccharide units containing L-iduronic acid varies widely from a few percent to over 90%. The average amount of sulphate per disaccharide is usually higher than for chondroitin sulphate, mainly because of an additional ester sulphate group at the two position of the L-iduronic acid. Dermatan sulphate is widely distributed among tissues and occurs in relatively high concentrations in fibrous connective tissues such as skin, tendon and blood vessels.

d/ Heparin and Heparan Sulphate.

These are structurally related glycosaminoglycans with repeating disaccharide units of D-glucosamine and either L-iduronic acid or D-glucuronic acid, with a unique feature of both polysaccharides being the presence of N-sulphated groups on the glucosamine residues. However, there are significant structural and functional differences between the two. In heparan sulphate, D-glucuronic acid is the predominant uronic acid and about half of the glucosamine residues are N-sulphated with an average of one or less sulphate groups per disaccharide. It also contains block structures of N-acetylated or N-sulphated disaccharides which results in the production of fragments of various sizes after nitrous acid treatment - nitrous acid removes the N-substitution and cleaves the exposed bond thus producing fragments. In contrast, heparin contains a relatively high proportion of L-iduronic and N-sulphated glucosamine residues, thus nitrous acid generates small fragments which are predominantly disaccharides. Additionally, most iduronic acid and glucosamine

residues are sulphated at the C2 and C6 positions respectively, thus heparin is the most charged glycosaminoglycan with as many as three or four sulphate and one carboxyl group per disaccharide.

Heparan sulphate is produced by a variety of cells, and is generally present in the form of a high molecular weight proteoglycan on cell surfaces, basement membranes and/or in the extracellular matrix (Rapraeger & Bernfield, 1983). The intracellular forms of heparan sulphate are generally small chains which are either free or linked to short peptides, and they represent the degradation products of a larger precursor proteoglycan (Iozzo, 1984). In contrast, heparin is limited mainly to mast cells and, intracellularly, it tends to occur as a high molecular weight proteoglycan (Robinson et al, 1978), which is stored in mast cell granules from which it is released in response to specific signals.

e/.Keratan Sulphate.

This is the only glycosaminoglycan that contains a hexose (D-galactose) instead of uronic acid in the repeating disaccharide unit. It usually has a relatively low molecular weight with the sulphate groups on the C6 positions of the D-galactose and D-glucosamine residues being present in roughly equal amounts (Bhavanandan & Meyer, 1968). It has limited distribution among tissues, but is present in cornea and tissues of skeletal origin including cartilage and intervertebral discs. However, it differs from other glycosaminoglycans in the linkage region to the protein core which lacks the xylose-serine linkage. Corneal keratan sulphate is bound to the protein core via a N-glycosidic linkage between N-acetylglucosamine and asparagine (Baker et al, 1975; Nilsson et al, 1983). Skeletal keratan sulphate is linked to the core protein via an O-glycosidic linkage between N-acetylgalactosamine and the hydroxyl groups of serine or threonine residues (Bray et al, 1967; Seno & Toda, 1970).

2.Cell-Associated Proteoglycans.

Proteoglycans associated with the cell surface may be integral or peripheral plasma membrane components and, in both cases, the glycosaminoglycan chains can be deeply embedded in the extracellular matrix or free to interact with

proteins in the extracellular fluid. The core protein has a hydrophobic segment which is intercalated into the lipid bilayer of the membrane, while the extracellular protein core and attached GAG chains can interact with other molecules in the matrix.

Many heparan sulphate proteoglycans serve as multivalent matrix receptors (Hook et al, 1984; Rapraeger et al, 1986). Syndecan, a cell surface proteoglycan, contains both heparan sulphate and chondroitin sulphate GAG chains (Rapraeger et al, 1985), and it has been found to bind to several extracellular matrix components via its heparan sulphate chains - it binds, with high affinity, to interstitial collagens (Koda et al, 1985), fibronectin (Saunders & Bernfield, 1988), thrombospondin (Sun et al, 1989) and basic fibroblast growth factor (Bernfield & Saunders, 1990). Other heparan sulphate-containing proteoglycans such as fibroglycan and glypican are also capable of binding matrix macromolecules. Additionally, proteoglycans can also align with actin bundles, with the suggestion that the cytoplasmic portion interacts with the cytoskeleton (Fransson, 1987). Thus, the proteoglycan could link the extracellular matrix to the cytoskeleton and both stabilise cell morphology and organise the matrix. An alternative peripheral association could be via a membrane receptor (Hook et al, 1984). Both of these receptors could participate in the endocytosis of extracellular proteoglycans. At the surface of chondrocytes another form of peripheral association has been described - the large cartilage proteoglycan binds via the N-terminal globular domain of the core protein to chains of hyaluronate, which are actively synthesised on the intracellular side of the plasma membrane (Sommarin & Heinegard, 1986).

Much evidence points towards a role for heparan sulphate proteoglycans in the control of cell growth. Many growth factors have a strong affinity for heparin-like molecules (Thomas & Gimenez-Gallego, 1986), also cell surface heparan sulphate proteoglycans may sequester growth factors and thereby regulate their effect. Heparin-like chains have direct antiproliferative effects and can also interfere with nuclear activities (Hook et al, 1984; Gallagher et al, 1986). It is possible that both growth factors and heparan sulphate fragments have nuclear sites of action (Ishihara et al, 1987) - in a hepatocyte cell line which exhibits contact inhibition in

confluent monolayers, changes in the amount and structure of the nuclear pool of heparan sulphate are observed when growing cells reach confluence. If entry of heparan sulphate into the nucleus of these cells is prevented, then contact inhibition is lost (Ishihara et al, 1987).

3.Extracellular Matrix Proteoglycans.

Heparan sulphate and chondroitin sulphate proteoglycans are integral components of the extracellular matrix of basement membranes and indeed all basement membranes contain heparan sulphate proteoglycan (Dziadek et al, 1985; Hassell et al, 1985; Paulsson, 1987; Paulsson et al, 1987). Basement membrane heparan sulphate proteoglycan can be subdivided into three groups which differ from each other in respect of their density, size of their heparan sulphate side chains and size of the protein core, although some of them do share immunological properties. The smallest high-density, low-molecular weight form has four heparan sulphate side chains, which each have a molecular weight of about 3kDa, and a very small protein core (Paulsson et al, 1987).

The large, low-buoyant density form has a large core protein which, by rotary shadowing, has been shown to be made up of six globular domains (Paulsson, 1987; Paulsson et al, 1987), with the three heparan sulphate side chains asymmetrically located at one end of the protein core (Ledbetter et al, 1987; Paulsson et al, 1987).

A third population of basement membrane-derived heparan sulphate proteoglycan has a relatively higher buoyant density and is of intermediate size. They have a shorter protein core than the low-buoyant density, high-molecular weight proteoglycans, but have identical glycosaminoglycan side chains, and are derived from the high-molecular weight, low-buoyant density heparan sulphate proteoglycan (Ledbetter et al, 1985).

The most widely studied basement membrane proteoglycan is the large heparan sulphate proteoglycan isolated from the Engelbreth-Holm-Swarm (EHS) tumour. Structural information about this macromolecule has come from the

sequencing of cDNA from two large nonoverlapping cDNA clones (Noonan et al, 1988). One of the clones contains two cysteine-rich domains and two domains which lack cysteine. The latter are homologous to each other and may represent two globular domains of the molecule. The cysteine-rich domains display homology to the cysteine-rich domains of the B1 and B2 chains of laminin (Sasaki et al, 1987;Sasaki & Yamada, 1987). The second cDNA clone consists of eight internally homologous repeats, each of which have two cysteines capable of forming a disulphide bond. These repeats show homology with neural-cell adhesion molecule (N-CAM), a member of the immunoglobulin gene superfamily. This heparan sulphate proteoglycan can interact with itself and with other basement membrane macromolecules, including type IV collagen and laminin. Laminin also binds type IV collagen, and this shared property with this heparan sulphate proteoglycan may be a functional reflection of the structural homologies (Laurie et al, 1986). Antibody studies suggest that the core proteins of these proteoglycans are anchored in or at the surface of the lamina densa via specific interactions that may contribute to the architecture of the basement membrane (Schittny et al, 1988).

Some of the potential functions of heparan sulphate proteoglycans have been examined by studying their GAG chains. Heparan sulphate isolated from several different basement membranes has been shown to have more extensive O-sulphation than N-sulphation (Pejler et al, 1987; Gallagher & Lyon, 1989).

Basic fibroblast growth factor (bFGF) binds to heparan sulphate glycosaminoglycan chains of basement membrane heparan sulphate proteoglycan, and is released by heparinase, an enzyme which degrades heparan sulphate (Folkman et al, 1988), additionally proteinases such as plasmin, can release bFGF still complexed with the heparan sulphate chains. In this form, bFGF retains its biological activities, but is protected from inactivation by other proteinases (Rifkin & Moscatelli, 1989; Klagsbrun, 1990). Thus heparan sulphate proteoglycans can sequester and/or concentrate particular growth factors important in the regulation of a variety of cellular processes (Roberts et al, 1988).

Heparan sulphate chains can also act directly with certain cell types, influencing their growth properties. Heparan sulphate from the proteoglycan synthesised by vascular endothelial cells can inhibit vascular smooth muscle cell proliferation (Castellot et al, 1981; Marcum et al, 1987). Heparan sulphate proteoglycans synthesised by pulmonary endothelial cells also exhibit growth inhibitory effects on smooth muscle cells (Benitz et al, 1990). Thus, heparan sulphate proteoglycans in endothelial basement membranes may regulate smooth muscle cell growth during development and vascular remodelling and/or cell proliferation, cell migration and matrix production in response to vascular damage (Wight, 1989).

Many basement membranes also contain chondroitin sulphate proteoglycans. A chondroitin sulphate proteoglycan with a protein core of about 150kDa and approximately 20 chondroitin sulphate chains has been isolated from Reichert's membrane (McCarthy et al, 1989). Immunolocalization of this proteoglycan with specific antibodies has stained basement membranes of nerve fibres, blood vessels, dermal-epidermal junctions, striated muscles, kidney tubules and glomerular mesangium, but not glomerular basement membrane (McCarthy et al, 1989).

4.Proteoglycans and Cell Adhesion.

Proteoglycans have also been shown to play a role in cell adhesion. Extracellular matrices and most of their individual components, when insolubilized on a surface, enhance cell attachment. Many of their cell attachment-promoting activities are related to the tripeptide recognition sequence RGD which is the cell recognition site of a variety of extracellular matrix components. These RGD-containing proteins and their cell surface receptors (integrins) constitute the primary mechanism whereby many cells attach to extracellular matrices (Ruoslahti & Pierschbacher, 1987). However, in general, proteoglycans do not promote cell attachment in the same way as the RGD containing proteins. The proteoglycans at the cell surface which reinforce cell attachment are probably of the heparan sulphate variety (Lark & Culp, 1984; Cole et al, 1985; Izzard et al, 1986; Wightman et al,

1986; Woods et al, 1986; Farach et al, 1987; Saunders & Bernfield, 1988), because the attachment of cells to various glycosaminoglycan-binding substances can be prevented by treating the cells with an enzyme which specifically degrades heparan sulphate (Ruoslahti, 1988a). Chondroitin sulphate/dermatan sulphate proteoglycans existing in solution or bound to extracellular matrix have the opposite effect on cell adhesion.

Chondroitin sulphate proteoglycans such as the large cartilage proteoglycan, serglycin, and the decorin-related small dermatan sulphate proteoglycans are able to inhibit attachment of cells to insoluble fibronectin or type I collagen (Knox & Wells, 1979; Ts'ao & Eisenstein, 1981; Brennan et al, 1983; Schmidt et al, 1987). Certain malignant cells express increased amounts of chondroitin sulphate proteoglycans (Iozzo, 1985b), which suggests that the decreased adhesiveness of such cells could be, at least, partly due to the inhibitory effect of these proteoglycans. The cell attachment-inhibiting effect may also underlie the ability of certain proteoglycans to inhibit cell migration (Funderburg & Markwald, 1986; Perris & Johansson, 1987), which requires cell attachment (Ruoslahti & Pierschbacher, 1987), and is accompanied by changes in proteoglycan synthesis (Kinsella & Wight, 1986). The interaction of cell surface proteoglycans with immobilised extracellular matrix molecules facilitates the organisation of actin in stress fibres and the formation of adhesion plaques in the attaching cells (Izzard et al, 1986; Woods et al, 1986), all of which have profound effects on the cells.

5.Proteoglycans and Neoplasia.

Several studies have reported changes in the amounts and types of glycosaminoglycans and proteoglycans associated with neoplasms. High levels of hyaluronic acid have been found in the blood and urine of patients with nephroblastoma, and tumour tissue or cells derived from such tumours synthesise high levels of this glycosaminoglycan (Morse & Nussbaum, 1967; Hopwood & Dorfman, 1987). Surgical removal of the affected kidney results in the blood levels of hyaluronic acid returning to normal levels (Powars et al, 1972). This work would

suggest that Wilm's tumours synthesise and release large amounts of hyaluronic acid in vivo. Support for such transformation-related changes comes from the findings that cells infected with oncogenic viruses (Hopwood & Dorfman, 1977), or cells isolated from breast carcinoma (Angello et al, 1982a), hyperplastic mammary nodules (Angello et al, 1982b) and human gliomas (Glimelius et al, 1978) also show a marked increase in the synthesis of hyaluronic acid.

A variety of epithelial and mesenchymal neoplasms express abnormal levels of chondroitin sulphate, and attempts have been made to establish a relationship between this glycosaminoglycan and tumour cell properties (Iozzo, 1984; Iozzo, 1985). Chondroitin sulphate is generally found in only trace amounts in most adult and normal non-cartilaginous tissue (Iozzo, 1985), but is present in elevated concentrations in tumours of the breast (Takeuchi et al, 1982), lung (Iozzo et al, 1981), liver (Kojima et al, 1975), colon (Iozzo et al, 1982; Iozzo & Wight, 1982) and prostate (De Klerk et al, 1984). The elevated amounts of both sulphated and unsulphated glycosaminoglycans may contribute either directly or indirectly to the growth of the cancer cells. The findings suggest that the loss of control over cell proliferation, at least in some transformed cells, may be associated with changes in hyaluronic acid and chondroitin sulphate.

Much evidence links alterations in heparan sulphate proteoglycan to the neoplastic phenotype. One of the most consistent qualitative abnormalities in heparan sulphate structure that has been proposed to be directly related to the transformed phenotype is a reduced degree of sulphation in the heparan sulphate chains (Underhill & Keller, 1975). Over production of heparan sulphate can also be linked with neoplasia as seen in patients with multiple myeloma (Khoory et al, 1980; Palmer et al, 1984).

6.Retinoids and Proteoglycans.

The literature suggests that the effects of retinoids on glycosaminoglycan synthesis are inconsistent, however several general points can be made. Retinoids have been reported to decrease glycosaminoglycan synthesis in corneal fibroblasts

(Dahl & Axelsson, 1980), primitive mesenchyme (Pennypacker et al, 1978) and chondrocytes (Shapiro & Poon, 1976), whereas an increase in synthesis was observed in cells derived from the dermis and the epidermis (Shapiro & Poon, 1976; King & Tabiowo, 1981) and in mouse fibroblasts (Jetten et al, 1979), suggesting that the effect on glycosaminoglycan production is cell type specific. If the production of these macromolecules is measured in the culture medium, the cell matrix and inside the cell, a differential effect may be observed. It has been shown that retinoid treatment of cultured dermal cells results in an increase in glycosaminoglycan levels in the cellular and matrix components, however a reduction was observed in the medium fraction. In contrast, the glycosaminoglycan levels were increased in all three fractions in epidermal cells (Shapiro & Poon, 1976; Shapiro & Poon, 1979). The different glycosaminoglycan types can also be independently affected by retinoid treatment. For example, if rat chondrocytes are treated with retinoic acid, there is a dose dependent increase in heparan sulphate levels, a decrease in chondroitin 4-sulphate, but the amount of chondroitin 6-sulphate remains the same (Shapiro & Poon, 1976).

The sulphation of the molecules may also be affected by retinoids. This is the case in intestinal cells in which retinoic acid treatment changes the ratio of N-sulphate to O-sulphate in heparan sulphate without a significant change in the total sulphate incorporation. Changes in the levels of glycosaminoglycans can be detected within 4-8h of treatment and the direction of the changes in the glycosaminoglycan synthesis may vary with the time of treatment (Maniglia & Sartorelli, 1981). These changes occur before any changes in cell growth and adhesion are seen (King & Tabiowo, 1981).

1:6 Experimental Plan.

The reported ability of retinoids to inhibit the growth of some tumour cells would suggest that they may be useful agents in the prevention of metastatic spread. By using retinoic acid within the murine melanoma cell system B16F1M2 it is possible that an insight into the mode of action of the retinoid and the mechanisms of metastatic spread may be achieved.

One of the first steps in the metastatic cascade is the adhesion of the neoplastic cells to the extracellular matrix which, in the case of melanoma cells, is the basement membrane. Thus initial experiments were designed to investigate B16F1M2 cell adhesion after pretreatment with retinoic acid to various extracellular matrix components and eventually to an intact subendothelial extracellular matrix. Time and dose dependency were investigated as was the effect of cell density. Alterations in cell morphology both on the different substrates and on plastic after retinoic acid-pretreatment were also considered.

In vivo, the melanoma cells do not exist in isolation, thus cell-cell interactions of both a homotypic and heterotypic nature were examined. The effects of retinoic acid on these processes would perhaps give an indication of the strength of the interactions as well as adhesive events leading to degradation of any underlying extracellular matrix.

Degradation of the basement membrane must occur to allow the cells access to the circulation. Thus the production of degradative enzymes by the melanoma cells or the host cells will be of importance in the metastatic cascade. The degradation of intact subendothelial extracellular matrix was examined by monitoring the release of radio-labelled fragments from the matrix. Also, the ability of B16F1M2 cells to produce specifically collagenases, endoglycosidases and plasminogen activators was investigated and any effects retinoic acid may have on this production determined.

The bulk of the project was centred around the adhesion of B16F1M2 cells to extracellular matrix or other cells, thus the molecules by which such adhesion occurs are also of importance. To this end the production of cell surface

glycosaminoglycans by the melanoma cells was determined both in the presence and absence of retinoic acid. It is possible that any changes detected in the synthesis of cell surface glycosaminoglycans may be related to alterations demonstrated in the adhesion patterns of the cells. Both a qualitative and a quantative analysis of glycosaminoglycans was performed.

As stated above, tumour cells in the primary tumour do not exist in isolation within the host. It is probable that they produce a factor or factors which can influence the surrounding host cell population. To this end the effect of B16F1M2-conditioned medium (both with and without retinoic acid) on fibroblasts suspended within collagen gels was examined. The factors produced by the tumour cells are thought to stimulate host fibroblasts to modify the extracellular matrix such that cellular migration is aided. Stimulation of the host fibroblasts to increase the amount of hyaluronic acid present in the tumour environment is of importance in the movement of cells during tumour cell invasion.

MATERIALS AND METHODS.

2:1. Cell Lines.

B16F1M2 murine melanoma cells were derived from the poorly metastatic B16F1 cell line by injecting 5 x 10^4 cells in 0.2ml of 0.15M NaCl into the tail vein of syngeneic C57BL mice and isolating the lung colonies three weeks later. Cells from the lung colonies were cultured and passaged through the lungs once more to yield a highly metastatic cell line. The cells were checked for metastatic capacity by re-injecting into mice and comparing the number of lung colonies produced with those observed with control cells (B16F1). These were the only melanoma cells used in the project and as such the findings related only to this particular derivative of the parent cell line.

Cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum, 2mM L-glutamine, 100 units/ml penicillin and 100µg/ml streptomycin (growth medium) at 37°C in a humidified atmosphere of 5% $C0_2$ in air. Cells were seeded at a density of 1.4 x 10^5 cells per 75cm^2 flask and cultured for 4 days with a medium change on day 3.

Calf pulmonary arterial endothelial cells (CPAE) were maintained in Dulbecco's minimal essential medium with 1000mg/L glucose (DMEM) supplemented with 10% foetal calf serum, 2mM L-glutamine, 100 units/ml penicillin and 100µg/ml streptomycin at 37°C in a humidified atmosphere of 5% $C0_2$ in air. The cells were seeded at a density of 3.2 x 10^5 cells per 75cm^2 flask, and cultured until confluent (10-14 days) with a medium change every 3 days.

All tissue culture media, sera, supplements and plastics were obtained from Life Technologies, Paisley, Scotland.

All cell lines used in this project were tested for the presence of mycoplasma using the method of Chen (Chen, 1977). The method is as follows:-

Cells were seeded at the usual seeding densities on cover slips and incubated until 20% - 50% confluent, at which time the medium was then removed and the monolayer rinsed with BSS. A 1:1 solution of BSS:acetic methanol was added and the rinse discarded, followed by pure acetic methanol which was again discarded.

Fresh acetic methanol was added and the cultures allowed to stand for 10 min after which the acetic methanol was removed and discarded. A stock solution of Hoechst 33258 stain (Sigma) (1µl in 20ml BSS) was added, and the cells allowed to stand at room temperature for 10 min. The stain was then removed and the monolayer rinsed with distilled water. The cover slip was mounted and examined by epifluorescence.

2:2. Retinoic Acid.

All-trans retinoic acid (retinoic acid) (Sigma) was dissolved in ethyl alcohol under subdued light to give a stock solution of 10^{-2}M. Serial dilutions were made from this to give a concentration range of 10^{-2}M - 10^{-7}M. These stock solutions were then diluted 1:1000 into culture medium, while control cultures received ethyl alcohol to a final concentration of 0.1%. Stock solutions were stored in the dark at -20°C and fresh solutions prepared every three months.

2:3. Determination of Relative Melanin Content (Lotan & Lotan, 1980)

B16F1M2 cells were grown in the presence and absence of retinoic acid. Once confluent, the monolayers were removed by scraping and counted. The cell pellet containing 5 x 10^6 cells was placed in triplicate tubes then resuspended and lysed in 0.5ml distilled water and subjected to 2 cycles of freezing and thawing.

Perchloric acid (BDH) was added to a final concentration of 0.5M and the suspension kept on ice for 10 min, then centrifuged at 5000 x g for 5 min. The pellet was then extracted 2x with 0.5M perchloric acid followed by 2x with a cold mixture of ethyl alcohol/ether (both BDH) 3:1 v/v, and then a final extraction with ether. The pellets were air dried and 1ml of 0.85M KOH (BDH) added and the pellets dissolved by heating to 100°C for 10 min. The insoluble material was pelleted, the supernatant allowed to cool, and the absorbance measured at 400nm.

2:4. Adhesion Assays.

1. Preparation of Substrates.

(a) Fibronectin (Miekka et al, 1982).

Citrated human blood was centrifuged, the plasma collected and 0.78g/L benzadinium chloride (BDH) added to the plasma which was then centrifuged at 10,000 x g for 30min. 100ml of the supernatant was applied to a Sepharose-4B (Pharmacia) column (2.6 x 6cm) equilibrated with 0.05M Tris (Boehringer Mannheim)/HC1 pH 7.4 containing 0.005M benzadinium chloride and 0.02% $^{w}/_{v}$ sodium azide (both BDH). The protein was eluted with equilibration buffer and the unbound proteins applied to a gelatin-Sepharose (Pharmacia) column (2.6 x 7cm) equilibrated in the above buffer. The column was washed with 0.1M NaC1, 0.05M 6-aminohexanoic acid, 0.003M sodium azide (All BDH), 0.05M Tris/HC1 pH 7.4 and unspecifically bound proteins were eluted with 1M NaC1, 0.005M benzadinium chloride in equilibration buffer, followed by 50ml of 0.05M Tris/HC1 pH7.4 containing 0.001M EDTA. The fibronectin was eluted with 3M Urea (BDH), 0.001M EDTA, 0.05M Tris/HC1 pH 7.4 and dialysed extensively against 0.15M NaC1, 0.05M Tris/HC1 pH 7.4.

(b) Type I Collagen (Schor, 1980).

Rat tail tendon (1g) was dissolved in 0.5M acetic acid (250ml) (BDH) by stirring for 48h at 4^{o}C, followed by centrifugation at 10,000 x g for 30 min. The pellet was discarded and an equal volume of 10% $^{w}/_{v}$ NaC1 was added to the supernatant. The collagen-rich salt-insoluble material was recovered by centrifugation (10,000 x g, 30 min) and resuspended in 0.25M acetic acid, then dialysed against several changes of 1:1000 acetic acid before centrifugation (20,000 x g, 2h) to sterilise the solution. The collagen concentration was determined by freeze drying a set volume of the solution, then weighing it.

(c) Type IV Collagen (Kleinman et al, 1986).

EHS sarcoma tissue was removed from lathyritic mice and washed in 3.4M NaC1, 0.05M Tris/HC1 pH 7.4 containing 0.002M N-ethylmaleimide (Sigma) and 0.008M EDTA (protease inhibitors). The insoluble residue was homogenised in

0.5M NaCl, 0.05M Tris/HCl, pH 7.4 containing protease inhibitors and then extracted twice overnight at 4°C. The residue was then extracted with 2M guanidine (Sigma) in 0.05M Tris/HCl, pH 7.4 containing protease inhibitors, followed by a further extraction with the same buffer containing 0.005M dithiothreitol (DTT) (Sigma) to solubilize the type IV collagen.

The extract was dialysed against 4M urea, 0.05M Tris/HCl, pH 7.4 containing 0.25M NaCl and 0.005M DTT, then chromatographed on a DEAE-cellulose DE52 (Whatman) column (2.5 x 25cm) equilibrated in the same buffer. The unbound material was dialysed against 4M urea, 0.05M Tris/HCl pH 7.4 containing 0.005M DTT and applied to a second DEAE-cellulose column equilibrated in the same buffer. The unbound material was retained and dialysed into 0.5M acetic acid prior to use.

(d) Laminin/Nidogen (Paulsson et al, 1987).

EHS tumour tissue was excised from mice and homogenised in 0.15M NaCl, 0.05M Tris/HCl pH 7.4 containing 0.0005M phenylmethylsulfonyl fluoride (PMSF) (Sigma) and 0.0005M N-ethylmaleimide as protease inhibitors. The homogenate was centrifuged (8000 x g, 20min) and the supernatant discarded while the pellet was re-homogenized in the same buffer with the addition of 0.01M EDTA. After stirring for 1h at 4°C, the extract was centrifuged (8000 x g, 20 min) and the supernatant stored at -20°C. Aliquots of the Tris/NaCl/EDTA extract were chromatographed on a Sepharose CL-6B (Pharmacia) column (1 x 120cm) equilibrated in 0.15M NaCl, 0.05M Tris/HCl pH 7.4 containing 0.002M EDTA and protease inhibitors. The laminin/nidogen complex was collected in the void volume fraction and concentrated by filtration through an Amicon PM10 filter unit (Amicon) to about 1mg/ml, and stored at -20°C.

2. Protein Estimation (Bradford, 1976).

With the exception of type I collagen, the concentration of the substrates was determined as follows. A range of volumes of the protein of unknown concentration were adjusted to a volume of 0.1ml with the appropriate buffer and to

this, 5ml of protein reagent was added. The tubes were mixed and the absorbance at 595nm measured. The protein concentration was determined from a standard curve generated from set concentrations of bovine serum albumin (Sigma).

3. Preparation of Protein Solutions.

Protein solutions were diluted to the required concentrations with phosphate buffered saline (PBS) and 1.5ml aliquots used to coat 35mm tissue culture plastic petri dishes. The dishes were incubated at 37°C for 1h, then washed twice with 1.5ml PBS. Any remaining protein absorption sites were blocked by a further incubation of 2h at room temperature with bovine serum albumin (BSA) (10mg/ml).

4. Preparation of Cell Monolayers.

B16F1M2 or CPAE cells were seeded at 5×10^4 and 8×10^4 cells/35mm petri dish respectively, with care being taken to ensure that the cells were evenly distributed over the surface of the dish. Medium was changed every 3 days and the cells maintained until fully confluent (4 and 8 days respectively).

5. Preparation of Subendothelial Extracellular Matrix (Vlodavsky et al, 1983).

CPAE cells were seeded in 35mm petri dishes at a density of 4×10^4 cells/dish in CPAE growth medium supplemented with 5% dextran (Sigma) and cultured until confluent (10-14 days), with a medium change every 3 days. Confluent cultures were washed once with PBS, then exposed to 0.5% Triton X-100 (Sigma) in PBS(v/v) and agitated gently. The cell layer was dissolved, and remaining nuclei and cytoskeletons removed by a brief exposure (2 min) to 0.025M NH_4OH (BDH) followed by four washes with PBS.

6. Adhesion Assays.

(a) Cell-Substrate Adhesion.

B16F1M2 melanoma cells grown in the presence or absence of retinoic acid for 4 days, or 2 days for low density control cultures, were washed twice with PBS without Ca^{2+} and Mg^{2+}, and detached by treatment with 0.05% trypsin in PBS/EDTA (0.02% EDTA in PBS without Ca^{2+} Mg^{2+}). The cells were harvested in complete growth medium, centrifuged (400 x g, 5min), washed three times in serum-free medium and then resuspended in the same serum-free medium. Cells were added to the prepared 35mm petri dishes in 1.5ml of serum-free medium at a density of 2 x 10^5 cells/dish, and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for various times. Unattached cells were removed, and the dishes washed twice with 1.5ml PBS. The number of attached cells were determined using a Coulter Counter (Model Dn with coincidence correction) following detachment with trypsin/EDTA.

(b) Cell-Cell and Cell-Extracellular Matrix Adhesion.

B16F1M2 cells were grown in 75cm^2 flasks in the presence or absence of retinoic acid (10^{-6}M) for 4 days, or 2 days for low density controls. On day 3, or day 1 for low density controls, the medium was removed and replaced with growth medium containing ^{51}Cr (403mCi/mg Cr) (ICN Flow) (100μCi/flask). Following a 24h incubation in the presence of ^{51}Cr, the cells were processed as previously outlined. Adhesion was terminated by the removal of the medium containing unattached cells, followed by careful washing of the monolayer three times with PBS and finally the addition of 1.5ml, 0.1M NaOH (BDH) to solubilise the cells. 2 x 0.5ml samples were taken from each dish for counting using a gamma counter.

2:4. Glycosaminoglycan Analysis.

1. Qualitative Analysis.

B16F1M2 cells were seeded in 75cm^2 flasks as follows:-

Control flasks at 1.9 x 10^5 cells, retinoic acid treated cells at 2.7 x 10^5 cells and low density controls at 1.9 x 10^5 cells. 48h after initiation of the cultures, or at initiation in the case of the low density controls, retinoic acid-treated cells and low density controls were incubated with [^{3}H] glucosamine (5μCi/ml) (32 Ci/mmol) and subconfluent control cultures with [^{14}C] glucosamine (2μCi/ml) (58.7mCi/mmol) (both Amersham). The medium was then removed and the cell layer washed twice with 10ml PBS, without Ca^{2+} & Mg^{2+}, then detached with trypsin/EDTA. Cells were harvested in MEM supplemented with 10% foetal calf serum, pelleted and washed twice with PBS, yielding three fractions - medium, trypsin releasable and cell-associated. All fractions were boiled for 10min, then the medium fraction from the retinoic acid-treated cultures was mixed with the medium from the subconfluent control cultures, and the medium fraction from the low density control mixed with another subconfluent control medium fraction. The appropriate trypsin-releasable and cell-associated fractions were similarly combined. The fractions were concentrated by rotary evaporation and then dialysed against 0.05M Tris/HCl pH 7.6 prior to incubation with Pronase (Sigma) (0.5mg/ml) for 24h at 50°C with a second addition of Pronase after 6h incubation. The samples were boiled for 10min to heat-inactivate the Pronase and then dialysed extensively against 0.05M NaCl, 0.01M Tris/HCl pH 7.6.

The glycosaminoglycans were separated using ion-exchange chromatography on DEAE cellulose columns (1 x 8cm) equilibrated with 0.05M NaCl, 0.01M Tris/HCl pH 7.6 and eluted at room temperature with a linear gradient of NaCl (0.05M - 0.65M) in a total volume of 250ml and at a flow rate of 20ml/h. 2.5ml fractions were collected and 0.3ml samples taken for scintillation counting. The peaks corresponding to the sulphated glycosaminoglycans were pooled, dialysed against distilled water, split into two equal portions and freeze dried. One half was treated with chondroitinase ABC (Sigma) (0.25units in 0.05M Tris/acetate buffer, pH 8.0 containing 0.15M NaCl and 0.1% (w/v) bovine serum albumin), while the other half was treated with nitrous acid for 90min at room temperature by the addition of 1.8M acetic acid containing 0.25M sodium nitrite (BDH) followed by neutralisation

with sodium carbonate (BDH) (Lindahl et al, 1973). All samples were then dialysed against 0.05M NaCl, 0.01M Tris/HCl pH 7.6 and rechromatographed on ion-exchange columns as described above.

2. Quantitative Analysis

B16F1M2 cells were seeded and incubated as outlined in section 4-1 above, but all cultures were radiolabelled with [^{3}H] glucosamine (32Ci/mmol) (5μCi/ml) and $^{35}SO_4$ (1154Ci/mmol) (20μCi/ml) (both Amersham). Similarly, the cells were harvested, digested with Pronase and the sulphated glycosaminoglycans isolated by DEAE ion-exchange chromatography as previously outlined. The pooled glycosaminoglycan fractions were concentrated by rotary evaporation, freeze dried, then dissolved in 0.25M NaCl. The samples were chromatographed on a Sephadex G-50 (Pharmacia) column (0.5 x 60cm), and the void volume peak pooled, dialysed, concentrated then digested with chondroitinase ABC as previously described. The digest was re-applied to the Sephadex G-50 column, with the chondroitinase ABC susceptible material being determined from the included peak. The void volume peak was further treated with nitrous acid and rechromatographed.

2:5. Degradative Assays.

1. Extracellular Matrix Degradation.

(a) Preparation of Radiolabelled Extracellular Matrix.

CPAE cells were seeded at a density of 4 x 10^4 cells/35mm petri dish and maintained in CPAE growth medium containing 5% dextran. On day 3, $^{35}SO_4$ in sulphate-depleted growth medium or [^{3}H]-proline (ICN flow) in proline-deficient medium (30μCi/ml) was added to the cultures with a second addition of radiolabel on day 7 without changing the medium. 5-7 days after reaching confluence (10-12 days after seeding), the matrix was prepared as previously described.

(b) Degradation Assay.

B16F1M2 cells previously treated with retinoic acid were suspended in serum-containing medium with the appropriate concentration of retinoic acid (10^6 cells/35mm petri dish) and seeded onto the radiolabelled extracellular matrix. After 2h the cells had firmly attached to the matrix and unattached or floating cells were removed along with the medium. The cell monlayers were washed twice with serum-free medium and 1ml of serum-free medium containing the appropriate concentration of retinoic acid added to the dishes. The dishes were further incubated for 48h, after which the medium was collected and centrifuged at 12,000 x g for 5min. 100ul samples of the supernatants were taken for total radioactivity measurements.

2.Glycosidase Assay (Niedbala et al, 1987).

Intracellular enzyme activity was determined by removing cells from the culture dishes by trypsinization, washing in growth medium containing 20% ($^v/_v$) heat inactivated foetal calf serum, centrifuged at 500 x g for 10 min at 20°C and subsequently washed three times with cold PBS. The cell number was determined and the cell pellet homogenised in four volumes of 0.1% Triton X-100 with 30 strokes in a Dounce homogeniser at 4°C. The amount of glycosidase activity present in the cellular homogenates was determined at pH 4.3 using the commercially available p-nitrophenyl sugar derivative p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma). The enzyme assay was carried out in the presence of 0.5ml citrate buffer (0.2M, pH 4.3), 0.5ml 0.006M p-nitrophenyl-N-acetyl-β- D-glucosaminide and 0.02ml of the cellular homogenate. The reaction mixture was incubated for 1h at 37°C and the reaction terminated by the addition of 2ml 0.4M glycine/NaOH buffer pH 10.5 (BDH). The absorbance of the hydrolyzed p-nitrophenyl was measured at 420nm.

3.Type I Collagenase Assay.

(a)Acetylation of Collagen (Gisslow & McBride, 1975).

2mg/ml type I collagen (125ml) was brought to pH 8.9 by the addition of 1M K_2HPO_4. The acetylating agent [1-^{14}C] acetic anhydride (10.2mg, 100μCi/mg) (Amersham) in 1.5ml benzene (BDH) was added dropwise over a period of 2 hours. The temperature of the reaction mixture was kept at 10°C and the pH maintained at 8.0 by the addition of 1M NaOH. The pH of the mixture was then adjusted to 4.0 with glacial acetic acid and the benzene removed. The acetylated collagen was dialyzed against distilled water at 4°C to remove [^{14}C] acetic acid, a by-product of the reaction. Dialysis was continued until no further ^{14}C was detected in the dialysate. The collagen was then freeze dried and stored at -20°C.

(b)Collagenase Assay (Bailly et al, 1990).

^{14}C-labelled type I collagen was solubilized in 0.01% acetic acid by stirring overnight at 4°C, and was used as a substrate at a concentration of 140μg/ml. Collagenase activity was measured in the culture medium which was collected at each medium change and stored at -20°C until assayed. Medium containing serum was dialyzed against 3M KSCN (Sigma) to dissociate complexes between the enzyme and serum inhibitors, and then against 0.05M Tris, 0.15M NaCl, 0.002M $CaCl_2$ pH 7.4 (test buffer) before activation. Activation of latent collagenase was performed by incubating 100μl medium with 10μg trypsin (Sigma) for 10 min at 37°C followed by the addition of a five fold excess of soybean trypsin inhibitor (Sigma). Activated medium was incubated for 16h at 25°C with 50μl of [^{14}C]-collagen. The enzymatic reaction was stopped by adding 0.002M EDTA and ethanol up to a final concentration of 18%(v/v). After 6h at room temperature, the collagen precipitate was collected by centrifugation and the radioactivity of the supernatant determined.

4.Type IV Collagenase Assay (Eisenbach et al, 1985).

(a) Preparation of [^{3}H]Proline-Labelled Type IV Collagen.

Solid EHS tumour was harvested after 3 weeks of growth, and the tissue minced, washed in PBS and incubated in 1g/ml proline-free DMEM containing 20% dialyzed foetal calf serum, 75μg ascorbate (Sigma)/ml and 50μg 3-aminopropionitrile (Sigma)/ml. After 30 min [^{3}H]proline was added to a final concentration of 5μCi/ml, and the organ culture incubated for 4-5h at 37°C. The tissue was washed, then homogenised at 4°C in ice-cold 0.5M acetic acid containing 0.02M EDTA and 0.008M N-ethylmaleimide (1g/9ml) and extracted overnight at 4°C. After centrifugation at 12,000 x g for 30 min, the supernatant was dialyzed against 0.05M acetic acid containing 10% NaCl, 0.02M EDTA and 0.008M N-ethylmaleimide. The precipitate was collected by centrifugation (12,000 x g, 30 min), and re-dissolved in 0.5M acetic acid. Impurities were removed by a further centrifugation (15,000 x g, 30 min), and re-precipitated in 10% NaCl, 0.05M Tris/HCl, pH 7.4. The precipitate was dissolved in 0.5M acetic acid and freeze dried. Prior to use, the labelled collagen was dissolved in 0.5M acetic acid and dialyzed against 0.05M Tris/HCl, pH 7.6 containing 0.2M NaCl and 0.005M $CaCl_2$ (reaction buffer).

(b)Preparation of Culture Medium for Testing.

Cell cultures in late log phase were rinsed with PBS and serum-free MEM containing L-glutamine and antibiotics added for the final 48-72h of the incubation. The medium was collected and the collagenase degradation activity enriched by precipitation in 25-50% ammonium sulphate (Sigma) - media proteins were directly precipitated by slowly adding ammonium sulphate to saturation at 25% at 4°C. The precipitate was collected by centrifugation (27,000 x g, 60 min), and solid ammonium sulphate was similarly added to the supernatant to achieve a final saturation of 50%. The 25%-50% ammonium sulphate precipitate was collected by centrifugation (27,000 x g, 60 min) dissolved in and dialyzed against reaction buffer.

(c)Preparation of Cell Extracts.

Cells in late log phase were collected in 0.1% PBS/EDTA, washed, counted and lysed in 0.5% Triton X-100 in water (2 x 10^6 cells/ml) at 0^oC. The lysates were centrifuged (5000 x g, 20 min) before being used.

3. Plasminogen Activator Assay.

(a) Preparation of cells.

B16F1M2 cells were seeded in a 24 well plate at a density of 10^5 cells/well in complete growth medium containing the appropriate concentration of retinoic acid, and incubated at 37^0C for 24h. The medium was then removed and replaced with serum-free medium containing 200µg/ml BSA and retinoic acid at the required concentration. The dishes were further incubated for 48h, after which the medium was removed and clarified by low speed centrifugation (400 x g). The cell monolayers were removed by scraping, concentrated, washed, counted and lysed in 0.5% Triton X-100 in water.

(b) Preparation of Fibrin Plates (Eisenbach et al, 1985).

A fibrinogen solution (250µl) containing 27µg non-radioactive fibrinogen (Sigma) and 0.1µCi [^{125}I] fibrinogen (ICN Flow) was added to each well of a 24 well plate and the plates air dried. Prior to the assay, conversion of fibrinogen to fibrin was achieved by incubating the plate at 37^oC for 2h with 0.5ml MEM containing 0.5% foetal calf serum. Each well was then washed twice with 0.1M Tris/HCl pH 8.1.

(c) Fibrin Plate Assay (Eisenbach et al, 1985).

Each well contained 300µl of reaction mixture consisting of 250µl Tris/HCl pH 8.1, 75µg BSA, 6µg human plasminogen (Sigma) and 50µl of the test sample. Trypsin was included as a control for the maximum releasable counts. The reaction was initiated by incubating the plate at 37^oC for the required time. 50µl aliquots were removed from each well and counted for soluble [^{125}I]-labelled fibrin degradation products.

2:6 Effect of Retinoic Acid on Fibroblasts Within a Collagen Lattice.

1. Preparation of Collagen Gels.

Adult forearm fibroblasts were grown to confluence, trypsinized and counted. Aliquots containing 7.5 x 10^5 cells were pelleted and resuspended in 1ml of serum (either foetal calf or newborn calf). 7ml of 3mg collagen/ml 1/1000 acetic acid was placed in an universal. The collagen solution was combined with 2ml 10 x MEM containing 1ml 0.34M NaOH, the pH adjusted to pH 7.2, and the serum/cell mixture added (all solutions were maintained at 4^oC). The preparation was well mixed to ensure that the cells were evenly distributed and 3ml aliquots dispensed into 35mm petri dishes which were then incubated at 37^oC. After the gels had solidified, 1.5ml of medium was added to each gel, followed by gentle detachment of the gels from the dishes. Contraction of the gel was measured on each subsequent day.

2. Preparation and Collection of Tumour-Conditioned Medium.

B16F1M2 cells were seeded at the usual density and incubated until almost confluent. The medium was then removed, the monolayers washed twice with serum-free MEM to remove any traces of serum and a set volume of serum-free MEM with or without 10^{-6}M retinoic acid added to the flask. The cells were incubated for another two days after which the medium was removed, gently centrifuged (400 x g, 5 min). The media were then placed in petri dishes on ice and irradiated for 2 hours with a U.V. source to eradicate any residual retinoic acid. Conditioned media were stored at -70^oC until required.

2:7 Fibroblast Glycosaminoglycan Analysis.

Adult forearm fibroblasts were seeded in 25cm^2 flasks and grown to near confluence (6 days). B16F1M2 murine melanoma-conditioned medium was prepared containing 10% foetal calf serum and with or without 10^{-6}M retinoic acid and this was added to the near confluent fibroblast cultures along with [^{3}H] glucosamine (5μCi/ml) and the cultures incubated for 48h. Controls of 10% FCS containing medium +/- 10^{-6}M retinoic acid, incubated for 2 days, irradiated then added to the

fibroblasts were also included. After the incubation, the medium was collected and the monolayers washed with PBS. 2ml of trypsin/EDTA was added to each flask and the cells allowed to detach, then 5ml of complete medium was added and 2 x 0.5ml samples taken for cell counting. The cell suspension was added to the medium/wash and the flasks washed with 2 x 5ml PBS. Each sample was boiled, dialysed against distilled water followed by 0.05M Tris/HCl pH 7.6 then treated with Pronase. The fractions were boiled to inactivate the Pronase, dialysed against distilled water then 0.05 M NaCl, 0.01M Tris/HCl pH 7.6 before being subjected to ion-exchange chromatography as previously described. Samples were taken for counting and the peaks pooled, rotary evaporated, dialysed against distilled water and the volume measured. 3 x 0.3ml samples were taken to determine the total amount of radioactivity in each peak.

The peaks corresponding to hyaluronic acid were treated with Streptomyces hyaluronidase (Sigma) and then rechromatographed on a gel filtration column as previously outlined. The sulphated GAG peaks were treated with chondroitinase ABC and similarly chromatographed. Each peak was again pooled, dialysed against distilled water and samples taken from each known volume to determine the total radioactivity in each peak.

The figures presented in the results section represent the average of triplicate samples taken from three separate cultures +/- S.D.. Standard deviations were calculated using the triplicate values obtained from the three separated experiments i.e. n = 9.

RESULTS.

3:1. EFFECT OF RETINOIC ACID ON MELANOMA CELLS.

1.Dose Response of B16F1M2 Melanoma Cells to Retinoic Acid.

B16F1M2 cells were seeded onto 35mm petri dishes, exposed to a concentration range of retinoic acid (10^{-10}M - 10^{-5}M) and incubated for 4 days. The results are shown in Figure 1. Retinoic acid inhibited cell growth at all tested concentrations. A significant reduction in cell numbers was demonstrated at concentrations of 10^{-8}M and greater ($p<0.01$). At a concentration of 10^{-8}M, the cell numbers were reduced to 71% of controls and 66% of controls on exposure to 10^{-6}M retinoic acid. At a retinoic acid concentration of 10^{-5}M, the cell numbers were reduced to 31% of the controls, however this was probably due, in part, to the cytotoxic effects of the retinoid at this concentration - the cells looked vacuolated and enlarged - and as such 10^{-6}M was selected as the test concentration for all subsequent work.

2.B16F1M2 Growth Curves.

Figure 2 shows the growth profile of B16F1M2 cells in both the presence and absence of 10^{-6}M retinoic acid. On day 1 there was no difference in the cell numbers, however by day 2 the number of cells present in the cultures treated with retinoic acid had been reduced by 16% when compared to control cultures. By the final day (day 4) the number of cells in the retinoic acid-treated dishes had been reduced by 36% as compared to control cells. This difference in cell numbers was found to be significant ($p<0.01$).

The dishes were seeded at a density of 2.3×10^4 cells/dish. The cell doubling time for the control cultures was approximately 33.25h, however when the cells were exposed to 10^{-6}M retinoic acid, the cell doubling time was increased to approximately 42.75h.

Figure 1. Response of B16F1M2 Cells to Retinoic Acid.

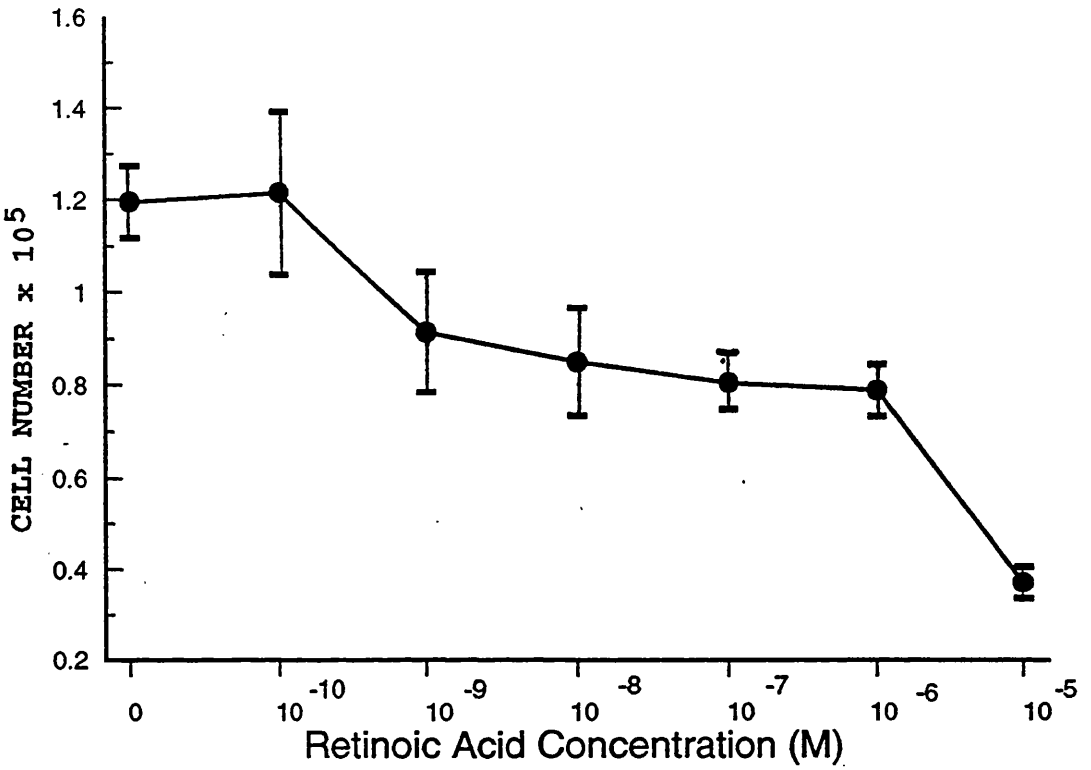


Figure 2. B16F1M2 Growth Curves +/- 10-6M Retinoic Acid.

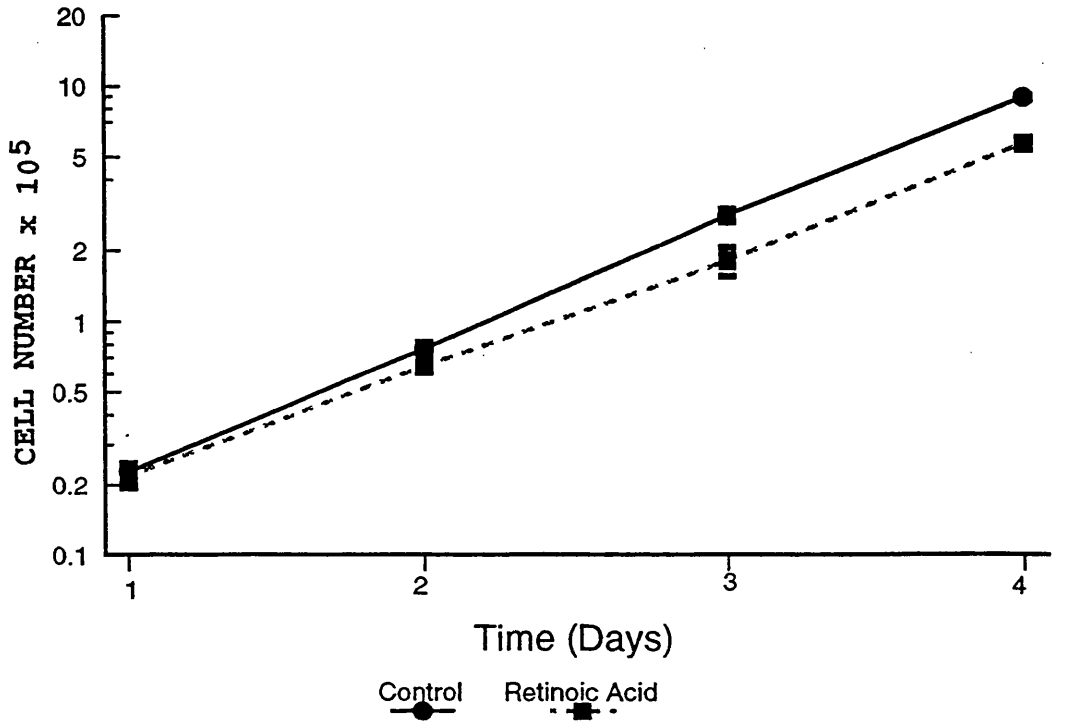
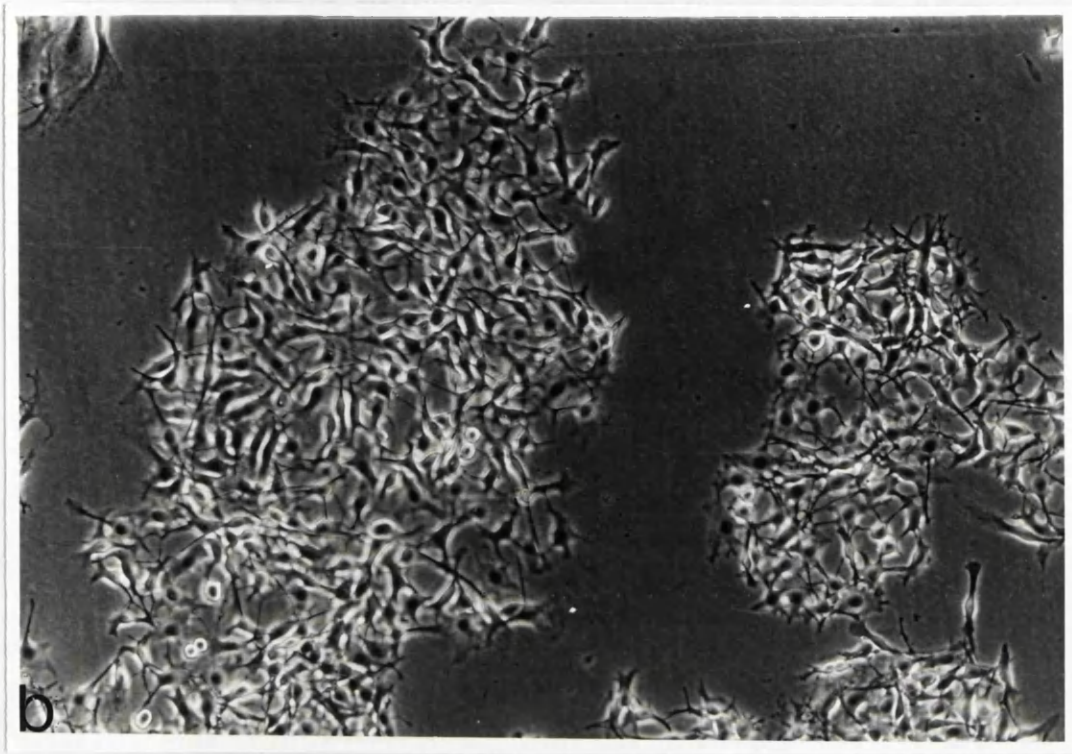


Figure 3. Effect of Retinoic Acid on B16F1M2 Cell Morphology.

a/ B16F1M2 Cells Grown in The Presence of 10^{-6}M Retinoic Acid.

b/ B16F1M2 Cells Grown in The Absence of Retinoic Acid.


b


a

3.Effect of Retinoic Acid on B16F1M2 Cell Morphology.

B16F1M2 cells were incubated in the presence and absence of 10^{-6}M retinoic acid. Cells treated with retinoic acid (fig. 3a) appeared to have a reduced tendency to form compact colonies. This supports the suggestion that retinoic acid treatment reduces the maximum cell density. Retinoic acid-treated cells also appeared less flattened and slightly more elongated than control cells (fig. 3b). In addition, exposure to retinoid caused the cells to exhibit more complex and extensive dendritic processes than those observed in control cells. When the cells were treated with trypsin for detachment, the retinoic acid-treated cells detached more readily than control cells which would suggest that retinoic acid has an effect on the adhesion processes of the cell.

4.Effect of Retinoic Acid on B16F1M2 Lung Colonization.

The lung colonizing ability of control and retinoic acid-pretreated cells is shown in Table 1. Subconfluent control cells formed many more pulmonary nodules than did the cells treated with 10^{-6}M retinoic acid. The difference in the number of lung colonies formed was found to be significant ($p<0.01$). The ability of low density control cells to form lung nodules was also investigated in an effort to eliminate any density-dependent affect being confused with the true effects of the retinoid treatment. The low density control cells formed fewer lung colonies than the subconfluent control cells but more than the retinoic acid-treated cells. Again the reduction in the number of lung colonies as compared to the subconfluent control cells was found to be significant ($p<0.01$). In subsequent experiments, control and retinoic acid treated cells were seeded such that, when harvested, the cell numbers were similar, thus eliminating any density effect.

5.Effect of Retinoic Acid on B16F1M2 Melanogenesis.

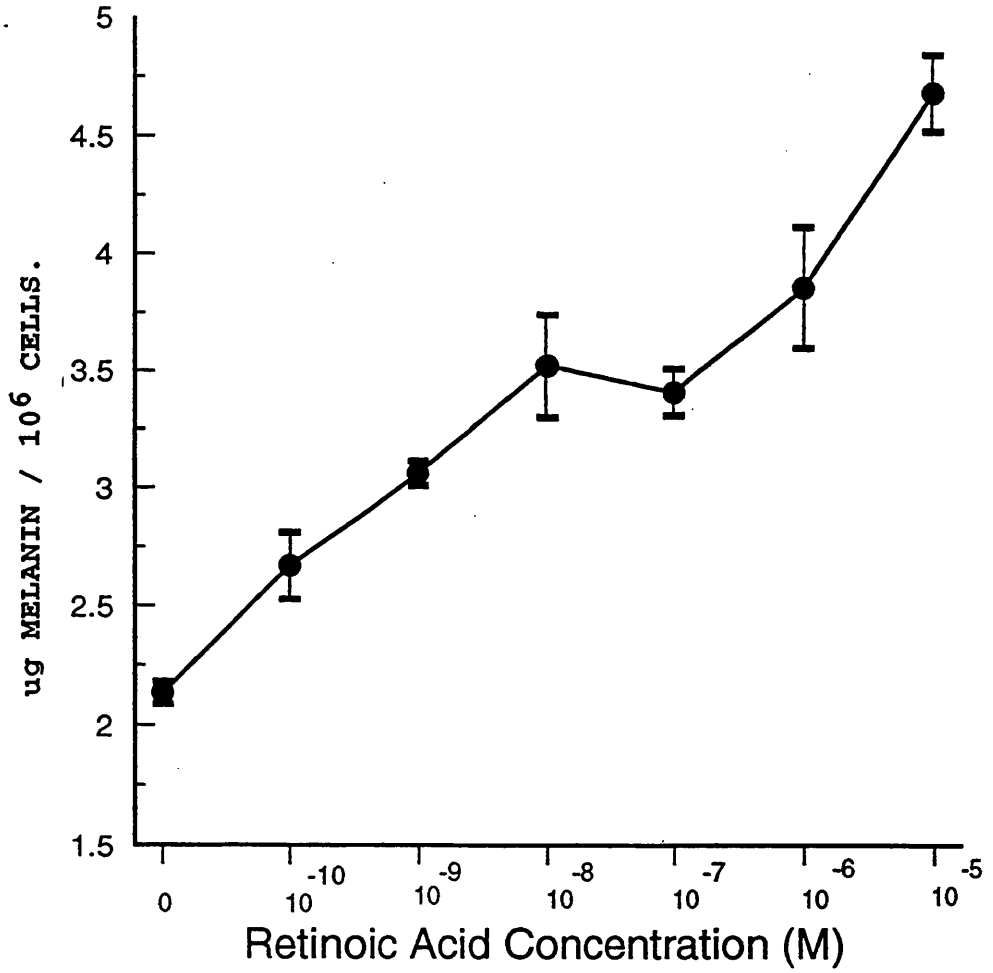
B16F1M2 cells were grown for 4 days in the presence of a range of retinoic acid concentrations (10^{-10}-10^{-5}M) and the total melanin content of the cells determined (fig.4). As can be seen, there was an increase in melanin content at all

Table 1.Effect of Retinoic Acid and Cell Density on Lung-Colonizing Ability of B16F1M2 Melanoma Cells.

	No.Lung Nodules.	Range (n=5).
Subconfluent Control	14.8 +/- 7.73*	7-24
R.A. Treated	0.4 +/- 0.89#	0-2
Low-Density	2.4 +/- 2.3#	0-6

* Mean +/- S.D.
#Significantly different from control, at $p<0.01$

Figure 4. Effect of Retinoic Acid on Melanogenesis.



tested retinoic acid concentrations - as retinoic acid concentration increased, melanin content increased. The half maximal effect was achieved at a retinoic acid concentration of 10^{-7}M. On examination of the cultures, the increased melanin content could be observed - the cells appeared darker, with the cell pellets being brown\black as compared to the white of control cells, and the culture medium was also brown (results not shown). Previous studies have included tyrosinase activity in the measurement of melanogenesis, but in this instance no measure of this enzyme activity was made.

3:2.ADHESION TO BASEMENT MEMBRANE COMPONENTS.

1.Dose Dependency of Retinoic Acid on B16F1M2 Cell Attachment.

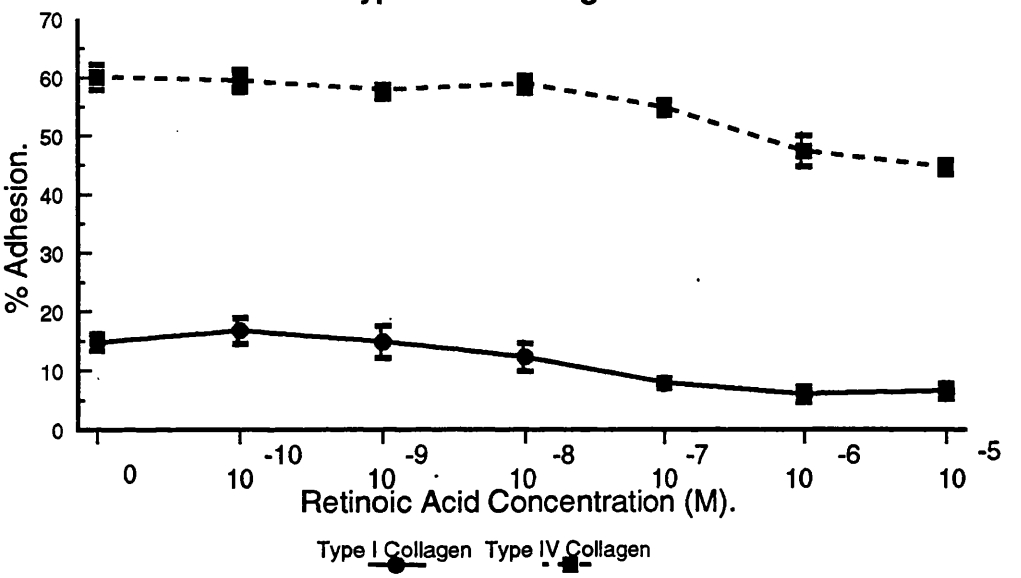
B16F1M2 cells were grown in the presence of various concentrations of retinoic acid (10^{-10}-10^{-5}M) for 4 days and their adhesion to 35mm petri dishes treated with 40µg in 1.5ml PBS or acetic acid of either fibronectin, laminin/nidogen, type I collagen or type IV collagen, investigated after 60 min incubation. The results are shown in figure 5. When the adhesion of cells to type I collagen was examined it was found that attachment to this substrate decreased as retinoic acid concentration increased. The half maximal effect was seen at a concentration of 10^{-7}M retinoic acid. Indeed, the cells, even in the absence of retinoic acid did not readily adhere to type I collagen and were easily detached from this substrate.

The cells adhered much better to type IV collagen although, as with type I collagen, there was a decrease in attachment as the retinoic acid concentration increased.

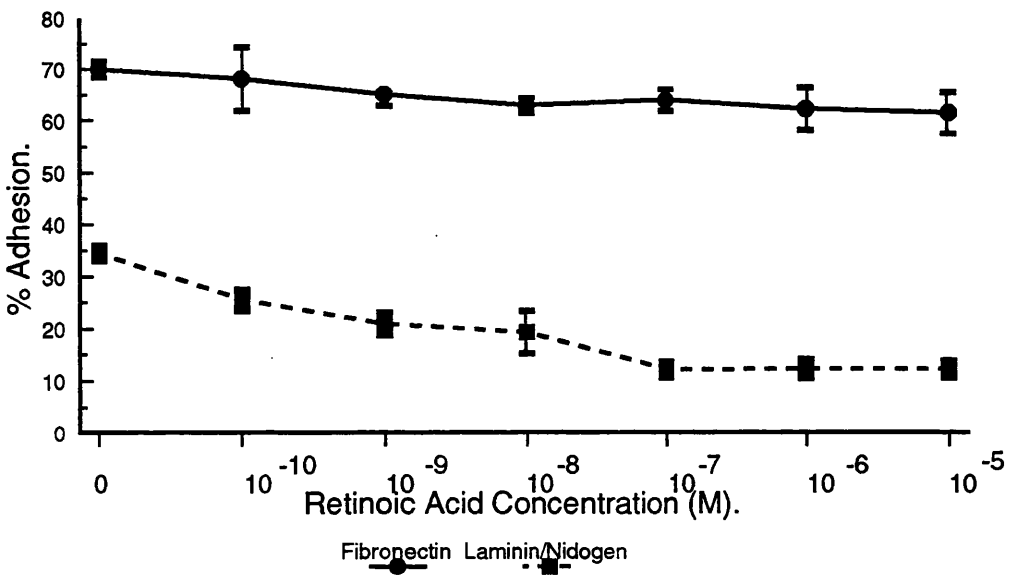
When the effect of retinoic acid treatment on adhesion to fibronectin was examined, it was found that the cells adhered readily to this substrate at all test concentrations (fig.5b). There was no difference in the percentage of adherent control cells when compared to those cells pretreated with even the highest test concentration of retinoic acid (10^{-5}M). However, adhesion to laminin/nidogen was concentration dependent - the number of adherent cells decreased as retinoic acid concentration increased (fig.5b). Half maximal adhesion occurred at a concentration of between

Figure 5. Dose Dependent Adhesion of B16F1M2 to Substrates.

a/ Type I & IV Collagens.



b/ Fibronectin and Laminin/Nidogen.



10^{-7}M and 10^{-8}M. Unlike the pattern of adhesion observed with the collagens, a reduction in adhesion to laminin/nidogen was observed at even the lowest retinoic acid concentration tested (10^{-10}M). Indeed, at concentrations above 10^{-7}M, adhesion to this substrate was reduced by approximately 60%.

2.Effect of Substrate Concentration on B16F1M2 Cell Adhesion.

(a) Fibronectin.

Melanoma cell line B16F1M2 readily attached to petri dishes coated with very low concentrations of fibronectin. Indeed, half maximum attachment was obtained following treatment of the dishes with 1.3μg protein in 1.5ml PBS (fig.6). Retinoic acid pretreatment did not increase this value and there was no significant increase in attachment at concentrations above 10μg protein in 1.5ml PBS (fig.6).

(b)Laminin/Nidogen.

B16F1M2 cells adhered to laminin/nidogen but not as readily as to fibronectin. Control cells adhered to this substrate in a concentration-dependent manner (fig.7). Very few cells attached to the lower substrate concentrations (<40μg protein in 1.5ml PBS). When the cells were pretreated with retinoic acid, there was an overall decrease in adhesion to this substrate. However, at the lower test concentrations (up to 5μg protein in 1.5ml PBS), it would appear that retinoic acid stimulates adhesion of melanoma cells to this substrate. At a concentration of 5μg protein in 1.5ml PBS approximately 24% of retinoic acid-pretreated cells adhered as compared to 9% of control cells. This was repeated three times and a similar result obtained each time. Although there was an overall decrease in adhesion to laminin/nidogen when the cells were pretreated with retinoic acid, this initial stimulation at low substrate concentrations is, perhaps, the more significant finding.

(c)Type IV Collagen.

B16F1M2 cells readily adhered to petri dishes coated with type IV collagen. After an incubation period of 60 min, half maximum adhesion was observed at a concentration of 1.8μg protein in 1.5ml PBS (fig.8). Once a concentration of 40μg type IV collagen in 1.5ml PBS had been reached, over 60% of control cells had

Figure 6. B16F1M2 Adhesion to Fibronectin.

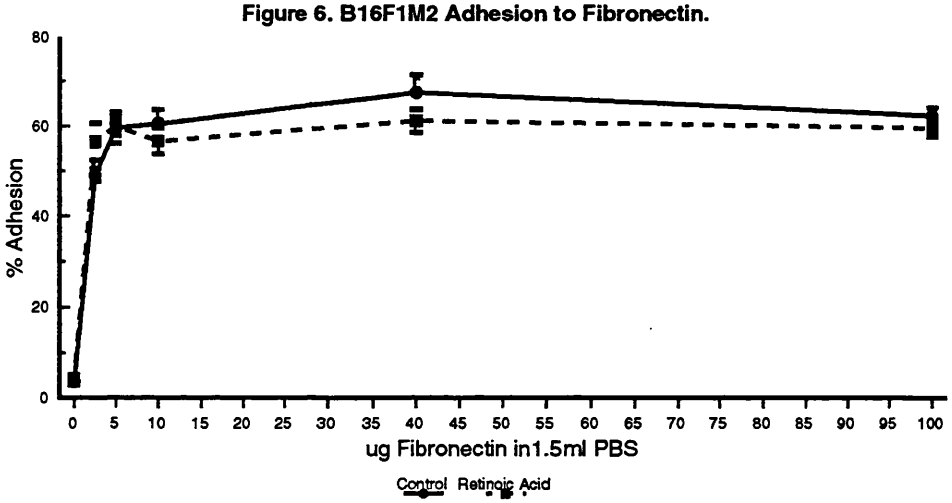


Figure 7. B16F1M2 Adhesion to Laminin/Nidogen.

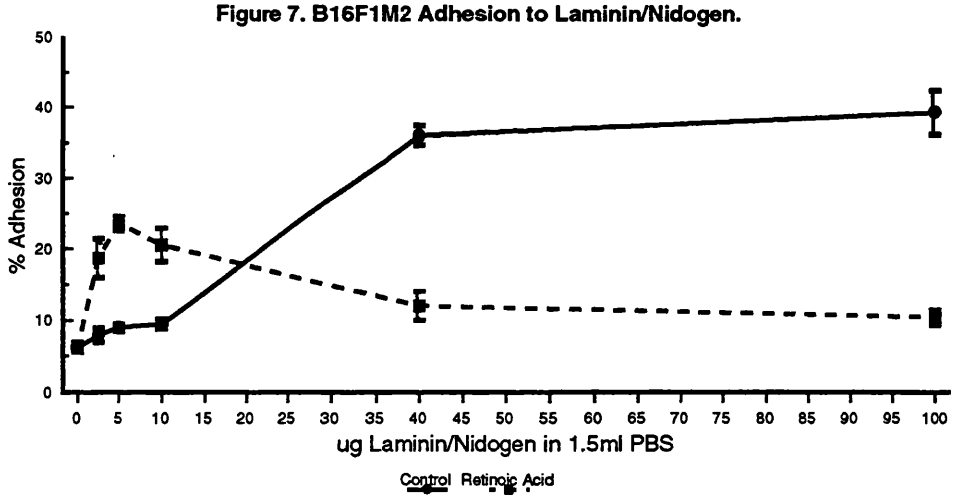
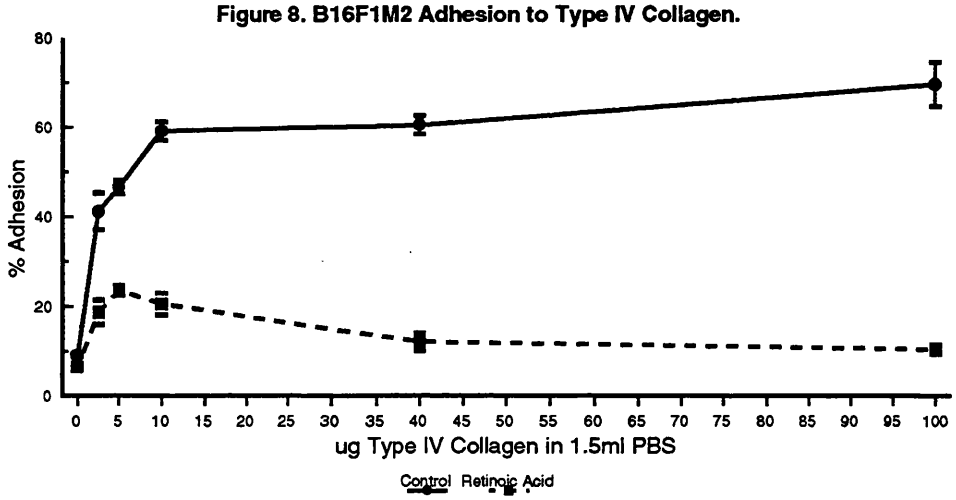


Figure 8. B16F1M2 Adhesion to Type IV Collagen.



adhered. Pretreatment of the cells with 10^{-6}M retinoic acid resulted in a significant reduction in the number of adherent cells ($p<0.01$). Although at the lower substrate concentrations the number of adherent cells appeared to be slowly increasing, at substrate concentrations above 5μg protein in 1.5ml PBS the number of cells attaching steadily decreased.

3.Time Dependence of B16F1M2 Cell Adhesion to Basement Membrane Components.

(a)Fibronectin.

B16F1M2 cells adhered very rapidly to dishes coated with 40μg fibronectin in 1.5ml PBS, with half maximum adhesion being reached by about 9 min in control cultures (fig.9). Pretreatment of the cells with 10^{-6}M retinoic acid appeared to cause only a slight reduction in the rate of adhesion.

(b)Laminin/Nidogen.

B16F1M2 cells adhered at a much slower rate to laminin/nidogen than to type IV collagen or fibronectin (fig.10). In control cultures, half maximum adhesion to this substrate was obtained after an incubation period of 30 min at which point approximately 18% of cells had adhered as opposed to 72% to fibronectin after the same incubation period. The adhesion to laminin/nidogen continued to increase until 60 min incubation after which time it would appear that a plateau was reached. When the cells were exposed to 10^{-6}M retinoic acid, there was a steady increase in the percentage of adherent cells with increasing incubation time and it is possible that, if the period of incubation had been extended, the number of adherent cells would continue to increase and a plateau achieved as for control cultures. However, it is unlikely that the number of adherent retinoic acid-treated cells would reach values similar to those obtained for the control cultures as initial experiments suggested that the number of cells which attach after a 90 min incubation are minimal.

Figure 9. Time Dependent Adhesion to Fibronectin.

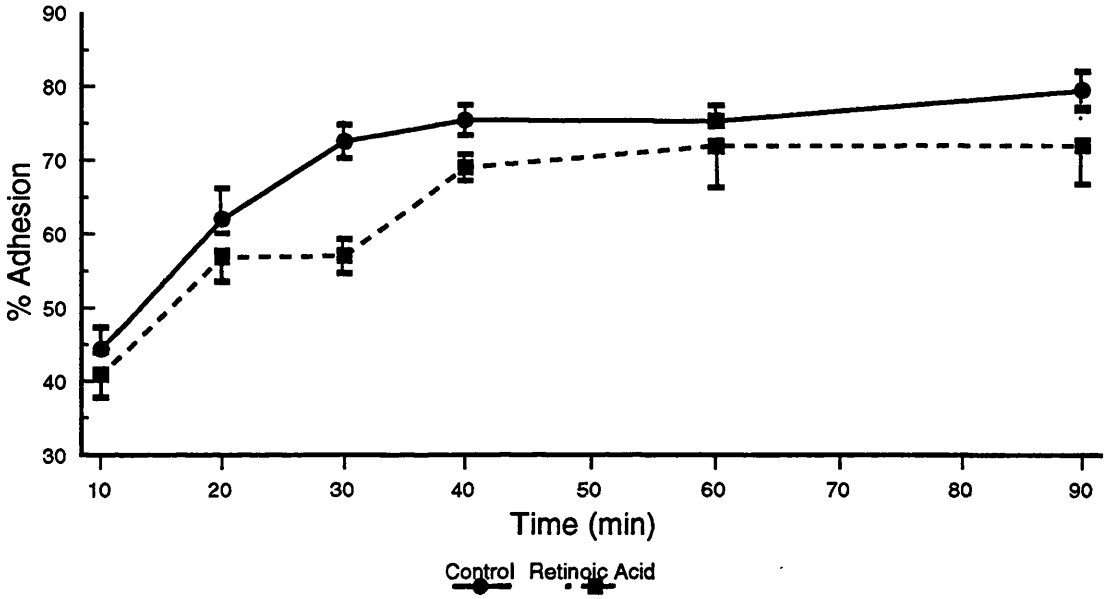
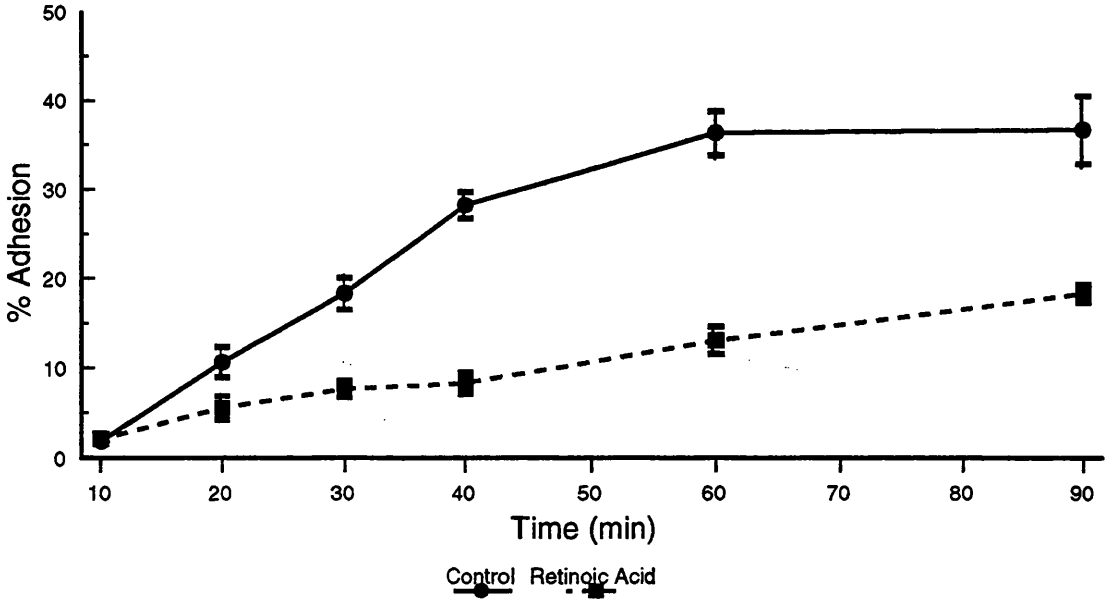


Figure 10. Time Dependent Adhesion of B16F1M2 to Laminin/ Nidogen.



(c)Type IV Collagen.

Half maximum adhesion by B16F1M2 control cells to dishes coated with type IV collagen occurred after an incubation period of 16 min (fig.11). The control cells adhered readily to this substrate, with a plateau being reached after 60 min incubation after which the increase in the number of cells attaching was minimal. Pretreatment of these cells with 10^{-6}M retinoic acid had an effect on the rate of adhesion to this substrate. The incubation period required for half maximum attachment to occur increased to 26 min. As with control cultures, cells pretreated with retinoic acid showed a steady increase in adhesion over a 60 min period, after which time the increase in the number of adherent cells was minimal. Unlike control cells, the rate of initial attachment of retinoic acid-treated cells was much slower with very few cells attached at the first time point (10 min).

(d)Type I Collagen.

B16F1M2 cells adhered very slowly to type I collagen (fig.12). Unlike type IV collagen, no plateau was reached at any point in the incubation profile, with the number of cells adhering still increasing at the final 90 min incubation time. Retinoic acid treatment again reduced the number of cells adhering to this substrate. As in the control cultures, the number of adhering cells appeared to be still increasing at the final incubation time point.

4. Time Dependence of B16F1M2 Cell Attachment to Dishes Coated With Laminin/Nidogen and Type IV Collagen.

Laminin/nidogen may mediate the adhesion of certain cell types to type IV collagen, thus dishes were treated with 40µg type IV collagen in 1.5ml PBS, unoccupied sites blocked with BSA, and the dishes then treated with 40µg laminin/nidogen in 1.5ml PBS. The adhesion profile of control and retinoic acid - treated cells is depicted in Figure 13. The control cells adhered very rapidly with 80% of the cells having attached after 60 min. After this time there was a slight decrease in the number of cells which adhered. When the cells were pretreated with 10^{-6}M retinoic acid there was a reduction in the number of cells which adhered to the

Figure 11. Time Dependent Adhesion of B16F1M2 to Type IV Collagen.

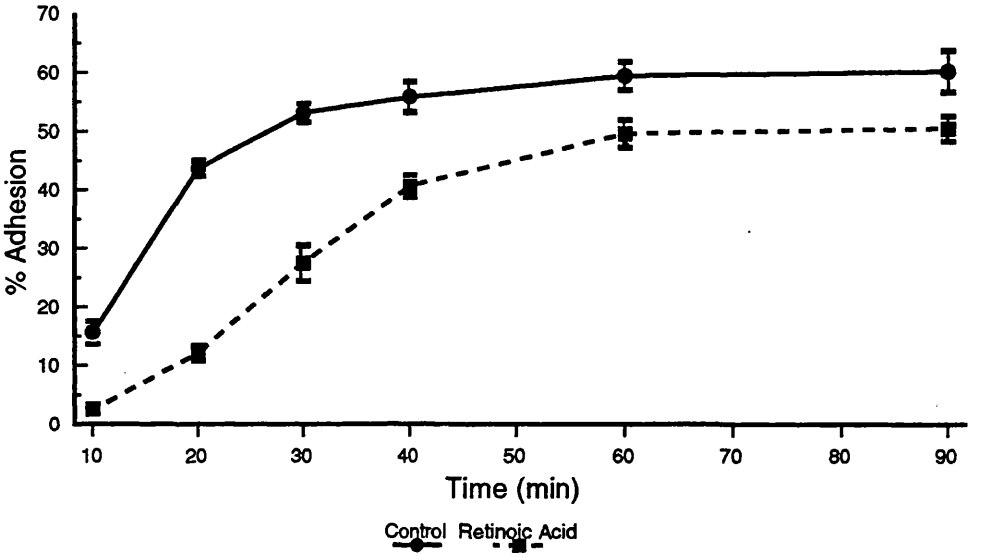
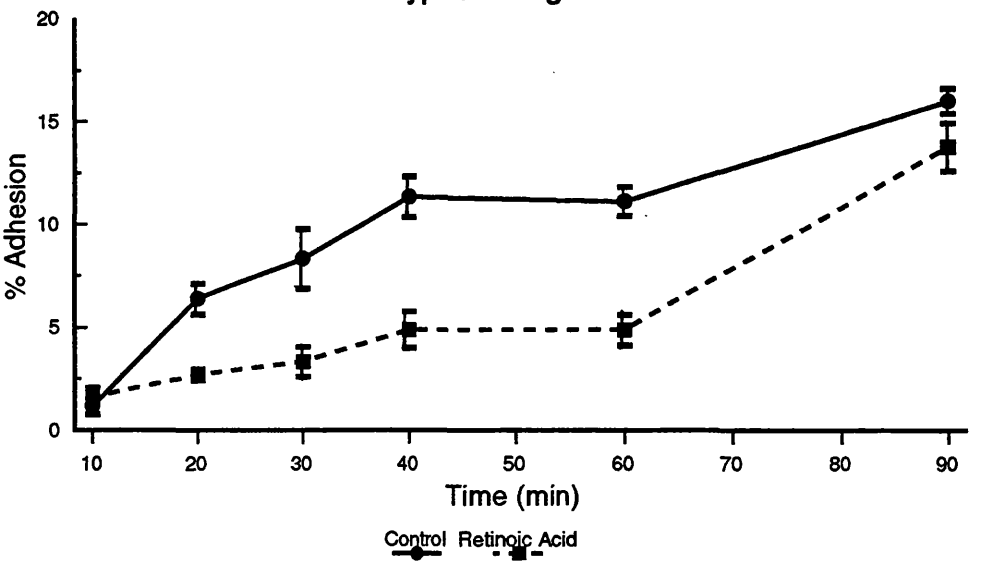


Figure 12. Time Dependent Adhesion of B16F1M2 to Type I Collagen.



substrate - after 60 min only 47% of retinoic acid-treated cells had adhered as compared to 80% of control cells. The initial rate of adhesion of the retinoic acid - treated cells was also much slower with a plateau reached after 40 min incubation.

When the rate of cell adhesion to the double substrate was compared to that of the individual substrates at the 60 min time point, the combined adhesion rate of the cells to laminin/nidogen and type IV collagen was higher than adhesion to the plates coated with both substrates. However, the number of cells which adhered to the dishes with both substrates was greater than to either of the individual substrates. Retinoic acid pretreatment of the cells had a similar effect, with the adhesion to dishes coated with both substrates being greater than adhesion to either of the individual substrates. Retinoic acid pretreatment of the cells had a similar effect with the adhesion to dishes coated with both substrates greater than the adhesion to either of the individual substrates.

5. Adhesion of B16F1M2 Cells to Bovine Serum Albumin.

As BSA was used to block any exposed adhesion sites on the petri dishes which were left untreated by the substrate under investigation, the adhesion of melanoma cells to dishes coated only with BSA was examined (fig.14). Although the cells adhered very slowly, the number of cells attaching was still increasing at 90 min incubation. Unlike the other substrates, retinoic acid pretreatment of the cells slightly increased the rate of adhesion although, as in the control cells, adhesion was very slow and still increasing at the final time point. There was no difference in the initial rate of attachment of either control or retinoic acid-treated cells.

6. Effect of Cell Density on Adhesion.

In all of the experiments the cells were seeded such that control and retinoic acid-treated cultures were of a similar density when harvested. To examine the possible effect of cell density on cell attachment, the adhesion of both low-density and subconfluent control cultures to fibronectin, laminin/nidogen and type IV collagen was investigated.

Figure 13. Time Dependent Adhesion of B16F1M2 to Type IV Collagen-Laminin/Nidogen.

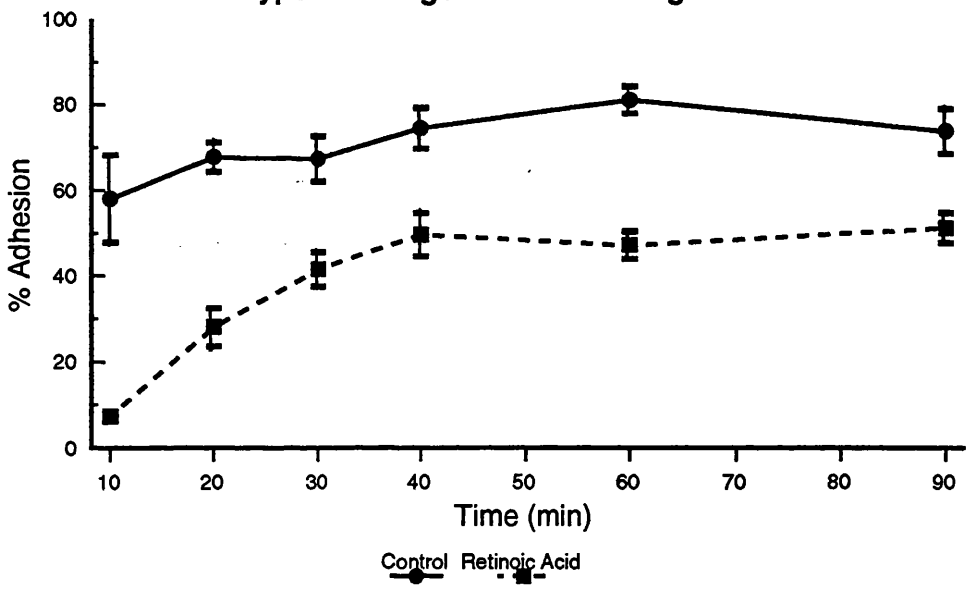
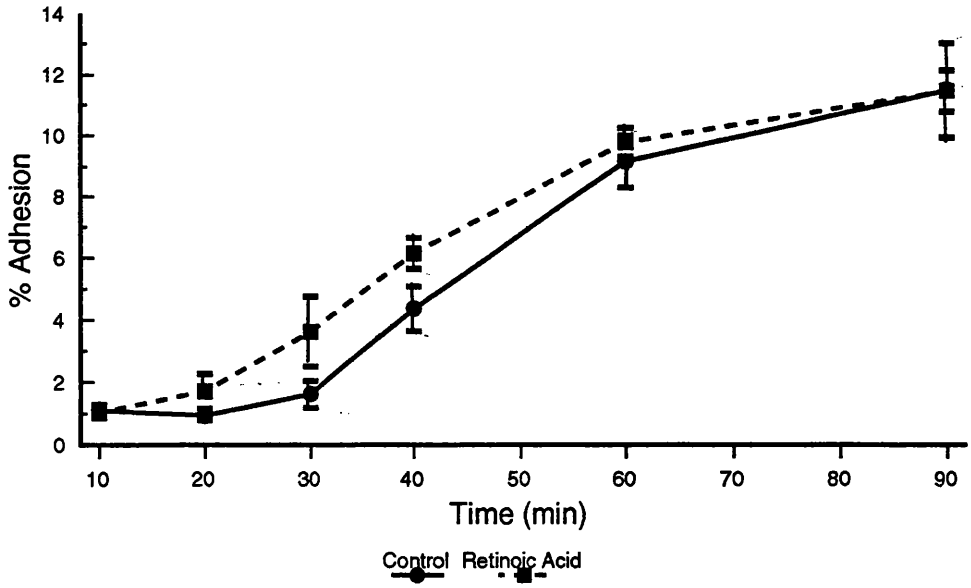


Figure 14. Time Dependent Adhesion of B16F1M2 to BSA.



(a) Fibronectin.

Low-density control cells adhered much more slowly to fibronectin than did subconfluent control cells (fig.15a). This was in contrast to retinoic acid pretreatment, which had no significant effect on the rate of adhesion to fibronectin. In this set of experiments the adhesion of the subconfluent control cells to fibronectin decreased at incubation times greater than 40 min. In previous studies, a plateau was achieved after 60 min with no significant change in the number of adherent cells. This may be due to slight differences in the prepared batches of fibronectin or, possibly, slight alterations in the adhesive properties in the cells themselves. It may also be due, in part, to partial degradation of the fibronectin which may have occurred while the substrate was stored prior to use.

b)Type IV Collagen.

The low-density cells exhibited a reduced adhesion to type IV collagen as compared to subconfluent cells, although by the 90 min time point, the number of cells attached were almost at the same levels as those of the subconfluent control cells (fig.15b). The initial adhesion of the low-density cells to type IV collagen was much slower after 10 min than that of the subconfluent control cells.

(c)Laminin/Nidogen.

Again the low-density cells had a reduced adhesion to laminin/nidogen (fig.15c). The profile of adhesion follows a very similar pattern to subconfluent control cells, but at a much reduced rate. In both cases a plateau is reached after 60 min with no significant increase in adhesion after this time.

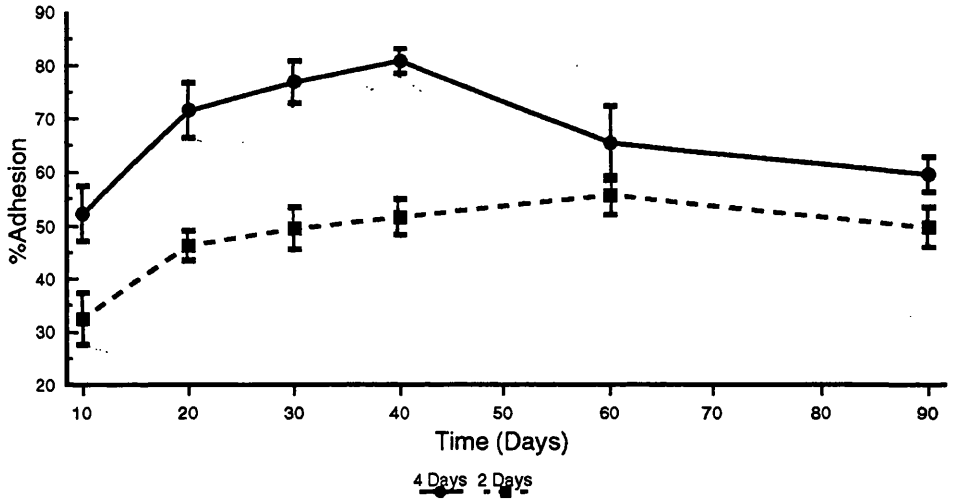
Cells seeded very thinly and incubated for 4 days were also examined for their adhesive properties to basement membrane components (VLD).

(d)Fibronectin.

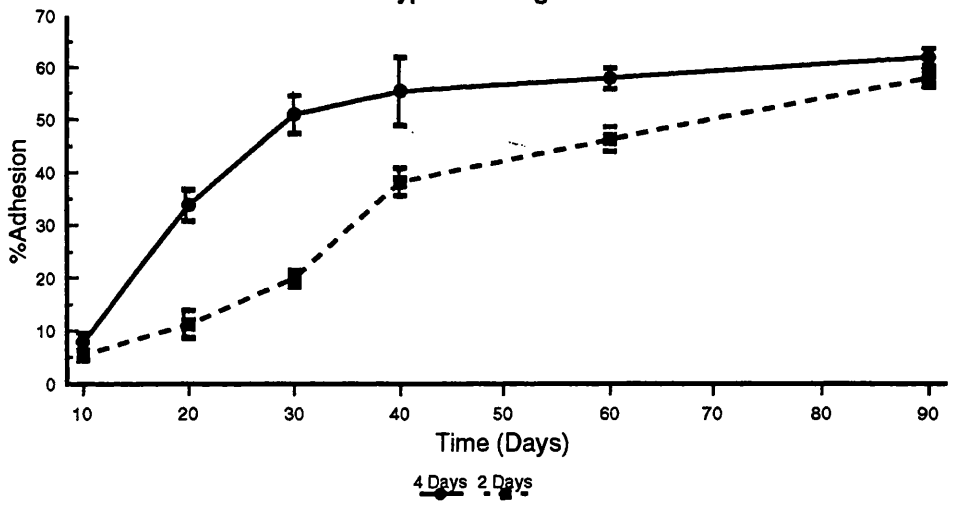
Cells seeded at a very low-density (VLD cells) also exhibited a reduction in adhesion to fibronectin but this was not as pronounced as for the cells seeded at normal seeding density but incubated for 2 days (LD cells) (fig.16a). Half maximum adhesion of VLD cells was observed at approximately 10 min. This was only slightly longer than the time required for control cells to reach this value.

Figure 15. Effect of Cell Density on Adhesion.

a/Fibronectin.



b/Type IV Collagen.



c/Laminin/Nidogen.

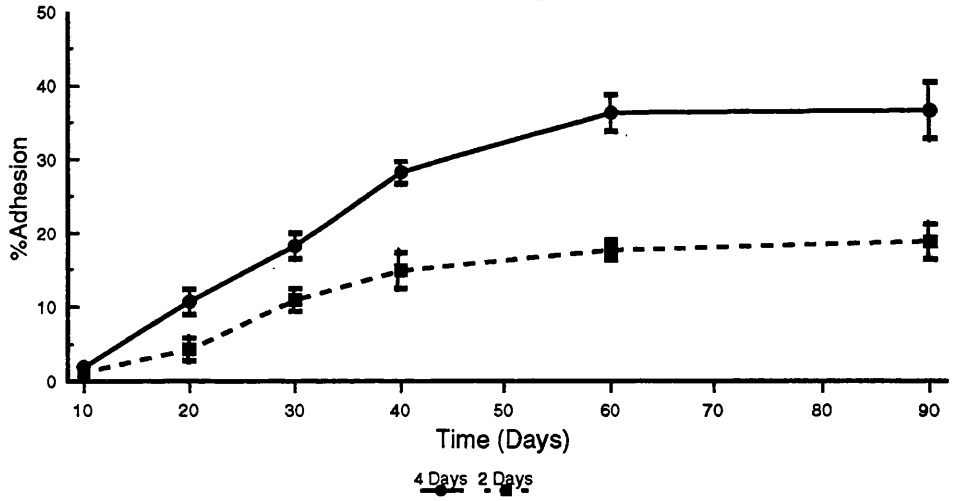
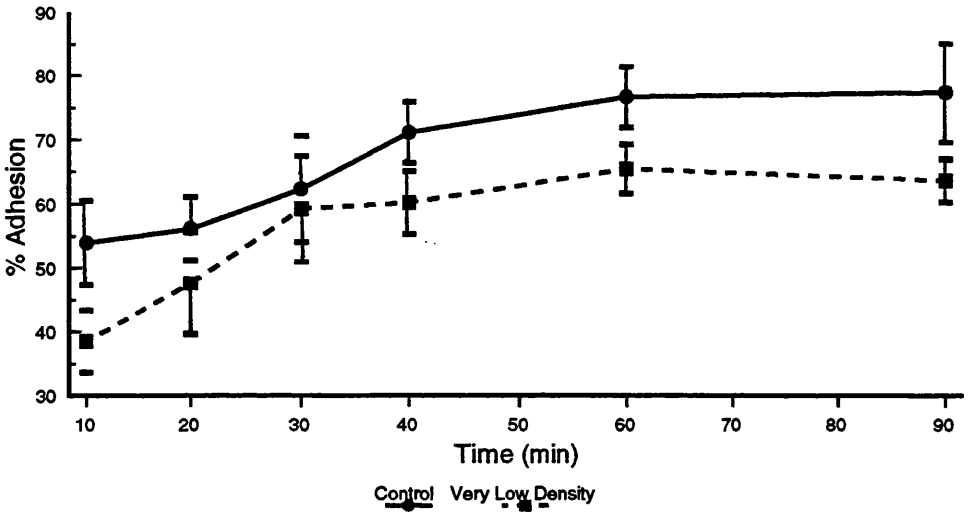
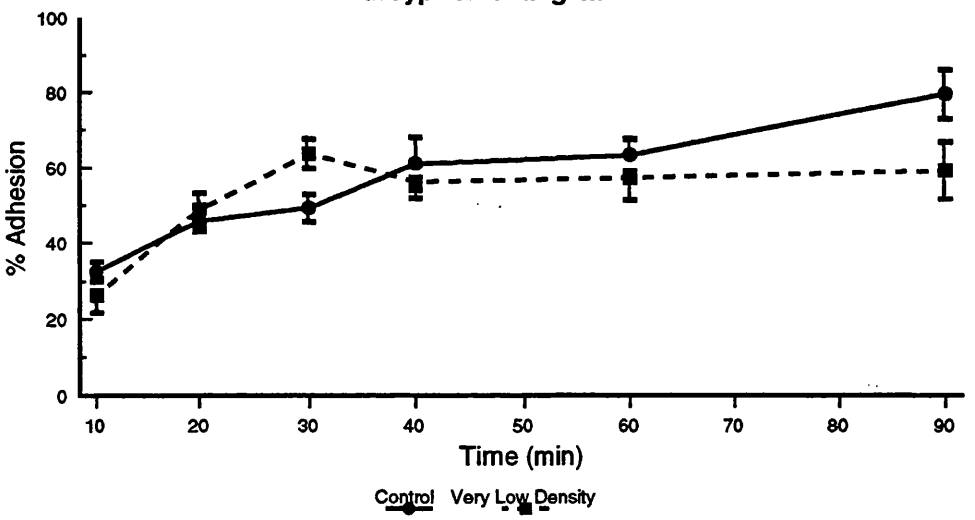


Figure 16. Adhesion of Very Low Density Cells.

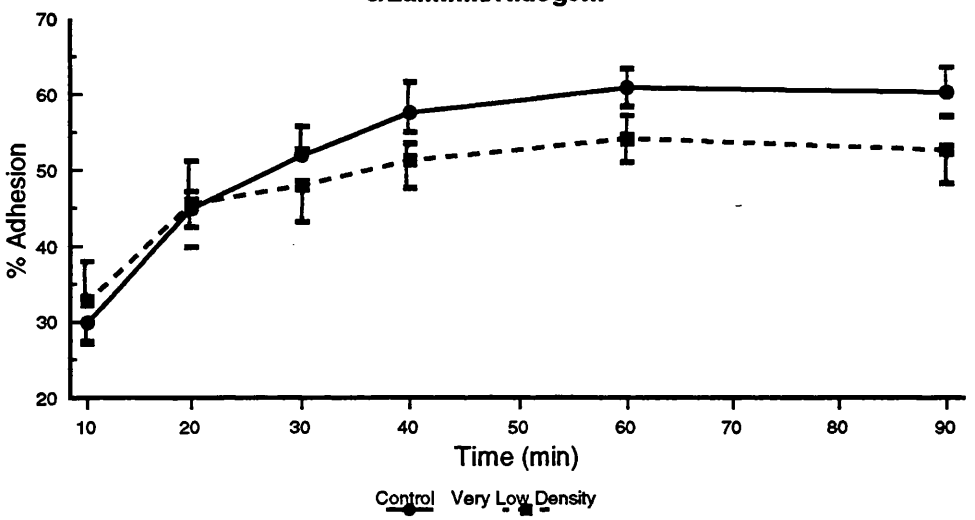
a/Fibronectin.



b/Type IV Collagen.



c/Laminin/Nidogen.



(e)Type IV Collagen.

After an initial increase in adhesion over the first 30 min, VLD cells were slightly less adhesive to type IV collagen than subconfluent control cells (fig.16b). After 40 min there was little or no increase in the number of VLD cells which adhered and unlike the LD cells, the number of VLD cells did not attain the same value as adherent subconfluent control cells. Half maximum adhesion of VLD cells occurred at about 14 min as compared to approximately 16 min for subconfluent control cells.

(f)Laminin/Nidogen.

There was very little difference in the adhesion profiles of VLD cells and subconfluent control cells to this substrate (fig.16c). Over the first 20 min, approximately the same number of cells adhered in both sets of dishes. The half maximum adhesion of both occurred by about 9 min. This is a much reduced value for the control cells as in previous experiments, half maximum adhesion did not occur until 30 min. Also, in this experiment there was an increase in the percentage of cells which attached to laminin/nidogen. This may have been due, in part, to differences in the prepared batches of laminin/nidogen.

7. Reversibility of Retinoic Acid Treatment.

The reversibility of retinoic acid-induced inhibition of B16F1M2 adhesion to laminin/nidogen and type IV collagen was examined over a period of 4 days.

(a)Laminin/Nidogen.

Over the initial 2 days of the experiment, there was a marked reduction in the number of retinoic acid-exposed cells which adhered as compared to the attachment of control cells (fig.17). There appeared to be no increase in retinoic acid-treated cells over this period. Once the retinoic acid was removed, there was an increase in adhesion from day 2 to day 3, however the increase from day 3 to day 4 was more apparent. When retinoic acid was added to control cultures on day 2, there was a marked reduction in the number of adherent cells between day 2 and day 3 which levelled out between day 3 and day 4.

Figure 17. Reversibility of RA Treatment on B16F1M2 Adhesion to Laminin/Nidogen.

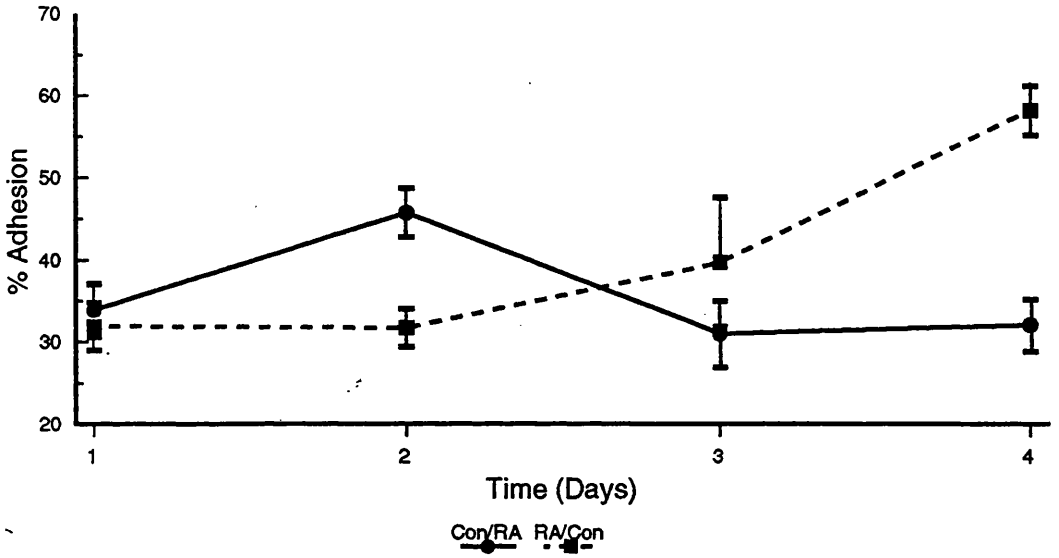
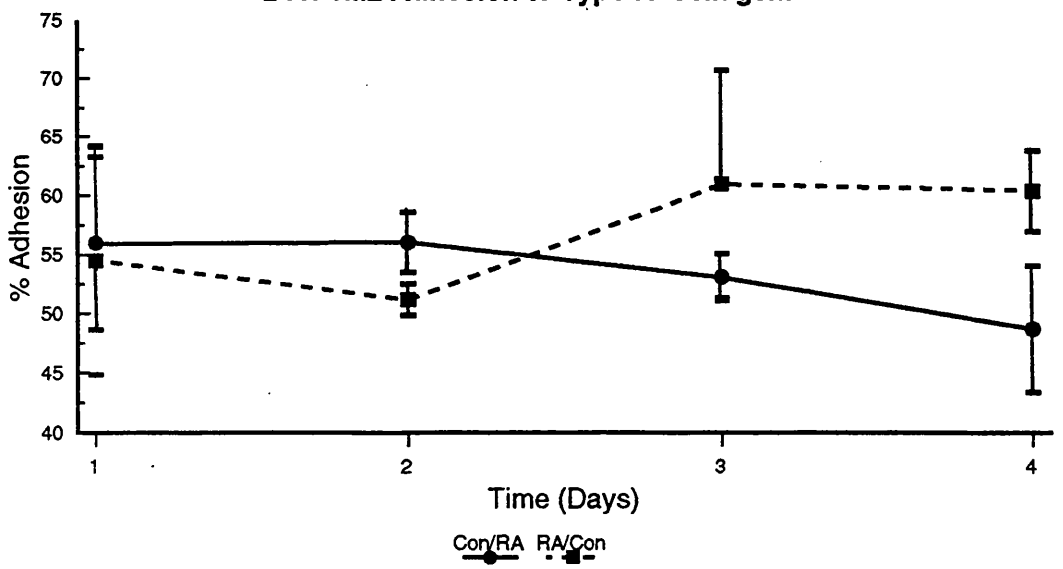


Figure 18. Reversibility of RA Treatment on B16F1M2 Adhesion to Type IV Collagen.



(b)Type IV Collagen.

During the initial 2 day control period, the number of cells which attached to type IV collagen-coated dishes remained constant. When retinoic acid was introduced into the system there was a steady decline in the number of attached cells (fig.18). In the cultures seeded in the presence of retinoic acid, the number of attached cells decreased over the initial 2 day period. Once the retinoid was removed, there was a sharp increase in cell attachment on day 2 to day 3 and this increase levelled out between day 3 and day 4 (fig.18).

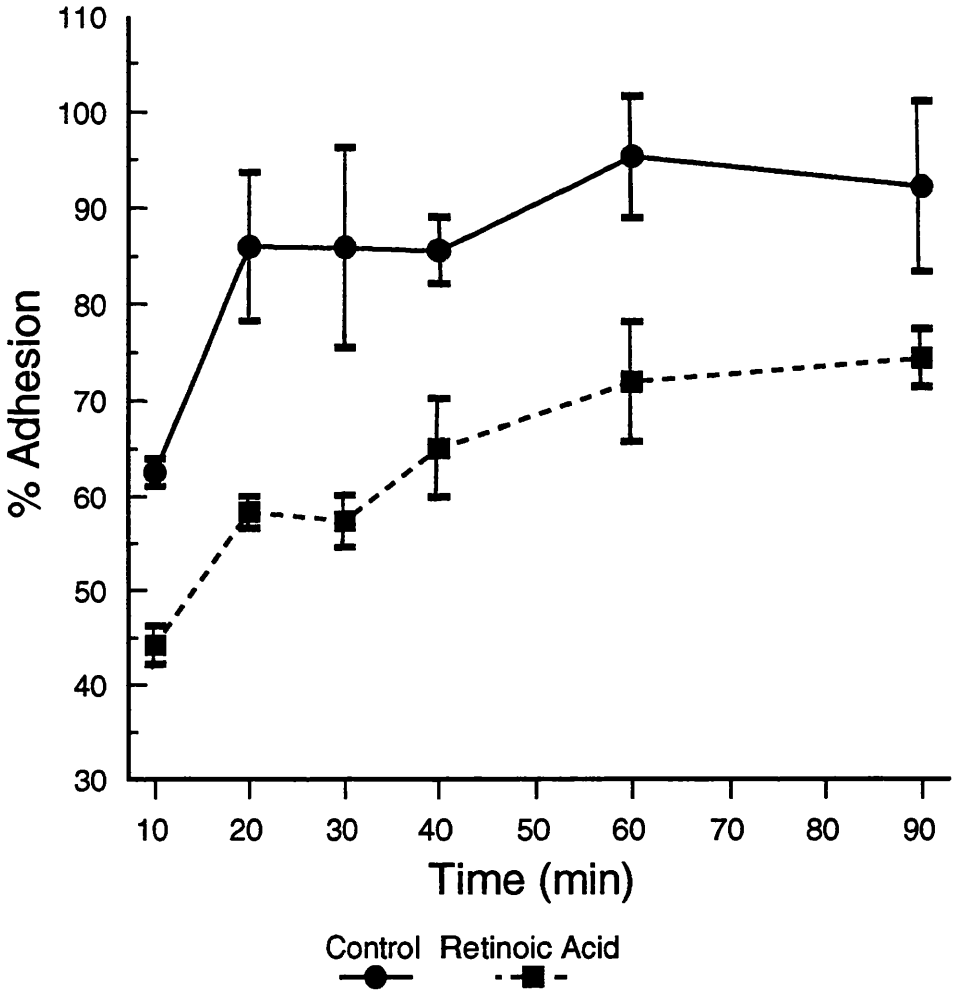
8. Adhesion to Subendothelial Extracellular Matrix.

The effect of retinoic acid pretreatment on the adhesion of B16F1M2 cells to intact subendothelial extracellular matrix (ECM) was examined (fig.19). Control cells readily adhered to the ECM with half maximum adhesion occurring after approximately 6 min. This time was almost doubled for retinoic acid-treated cells. Between 20 and 40 min, the number of adherent control cells remained relatively constant. After this period, there was an increase in adhesion before a steady level was again achieved (fig.19). With the retinoic acid-treated cells, adhesion appeared to be relatively constant between 20 and 30 min. After this period there was a slight, but steady, increase in the number of adherent cells but there was no real plateau attained following the increase.

In control cultures, the maximum number of adherent cells to ECM was greater than the observed maximum adhesion to any of the single substrates. Also, the initial rate of cell attachment to intact ECM was higher than for any of the single substrates - 62% of control cells had adhered to intact ECM after 10 min as compared to 44% to fibronectin, 2% to laminin/nidogen and 16% to type IV collagen. However, this initial rate of attachment was comparable to that observed with dishes coated with type IV collagen and laminin/nidogen (58% of cells had adhered after 10 min).

The effect of retinoic acid on B16F1M2 cell adhesion to intact ECM was to decrease it. Initial adhesion was reduced by about 18% when compared to control cultures (62% for control cells, 44% for retinoic acid-treated cells). As with control

Figure 19. B16F1M2 Adhesion to Intact Extracellular Matrix.



cultures, the maximum number of retinoic acid-treated cells which adhered to intact ECM was greater than for any of the single substrates with the exception of fibronectin to which the percentage of retinoic acid - treated cells which adhered was approaching the value obtained with ECM (72% for fibronectin and 74% for intact ECM). Again the initial rate of attachment by B16F1M2 cells pretreated with 10^{-6}M retinoic acid to ECM was much higher than for any single substrate, with the exception of fibronectin, or indeed for dishes coated with type IV collagen and laminin/nidogen - 44% to ECM, 41% to fibronectin, 2% to laminin/nidogen, 2.5% to type IV collagen and 7% to type IV collagen and laminin/nidogen.

9. Effect of Retinoic Acid on Cell Spreading.

Retinoic acid pretreatment of B16F1M2 cells would appear to inhibit their adhesion to laminin/nidogen and type IV collagen to varying degrees. It also has an effect on cell spreading. Both control and retinoic acid-treated cells spread well on fibronectin (fig.20 a & b), however spreading on laminin/nidogen and type IV collagen was inhibited by retinoic acid pretreatment (fig.20 c & d, e & f), with the majority of adherent cells remaining rounded after an incubation period of 40 min. Cells which adhered to laminin/nidogen had more dendritic processes than did cells which adhered to the other substrates.

3:3. CELL-CELL ADHESION.

1. Homotypic Adhesion.

B16F1M2 cells adhered to attached monolayers of B16F1M2 cells with a half maximum adhesion time of approximately 18 min, and by about 30 min almost 70% of the cells had attached (fig.21). Pretreatment of the cells with retinoic acid reduced the total number of cells which adhered, and the time taken for half maximum adhesion to occur increased to 28 min (fig.21). Initial attachment of retinoic acid-treated cells to the monolayer was slower than for control cells, but after 60 min incubation the percentage of adherent cells was similar in both instances.

Figure 20. Effect of Retinoic Acid on B16F1M2 Cell Spreading on Various Substrates.

B16F1M2 Control Cells (a) and Grown in The Presence of 10^{-6}M Retinoic Acid (b) on Fibronectin.

B16F1M2 Control Cells (c) and Grown in The Presence of 10^{-6}M Retinoic Acid (d) on Laminin/Nidogen.

B16F1M2 Control Cells (e) and Grown in The Presence of 10^{-6}M Retinoic Acid (f) on Type IV Collagen.

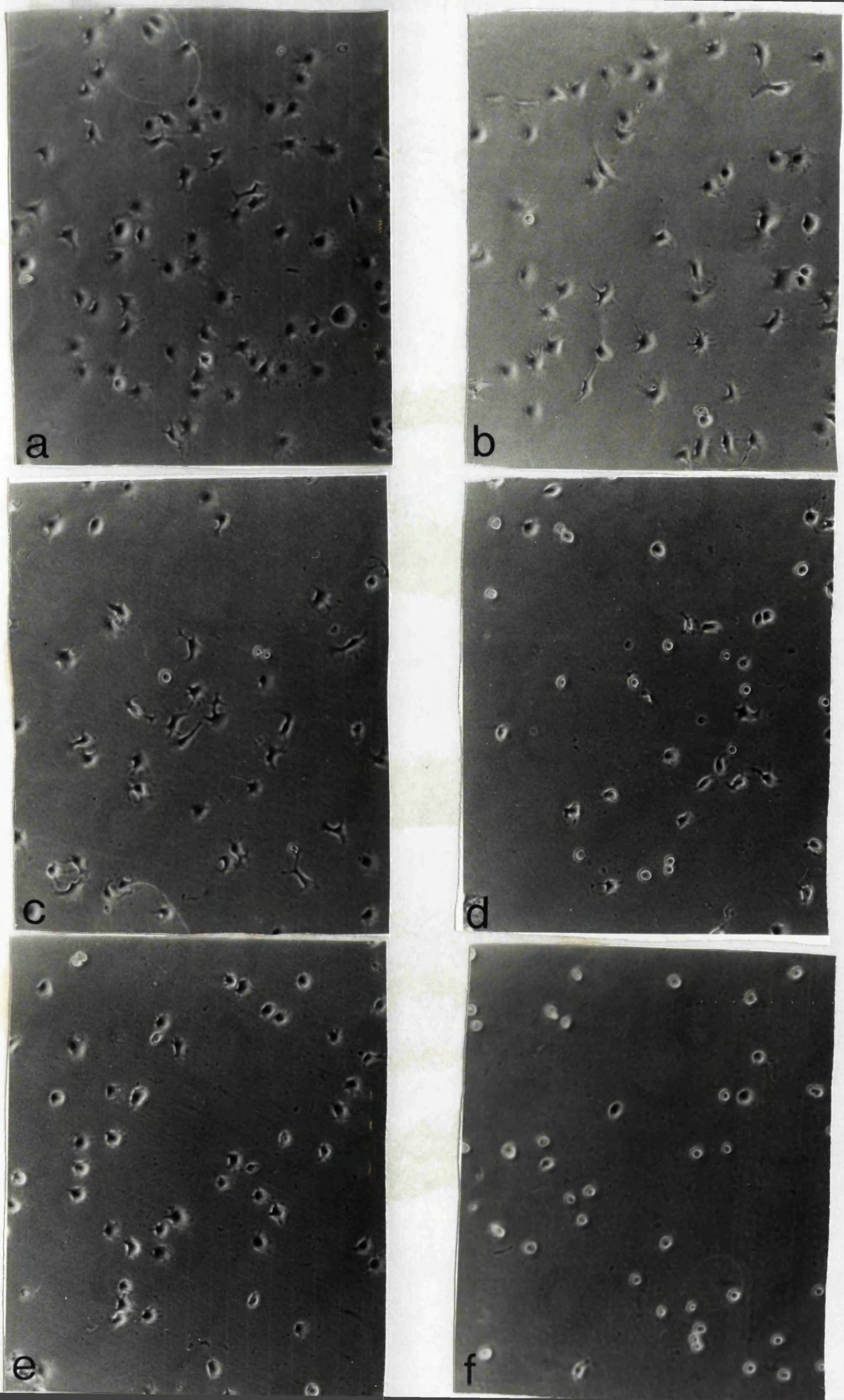

a
b
c
d
e
f

Figure 21. Homotypic Cell-Cell Adhesion.

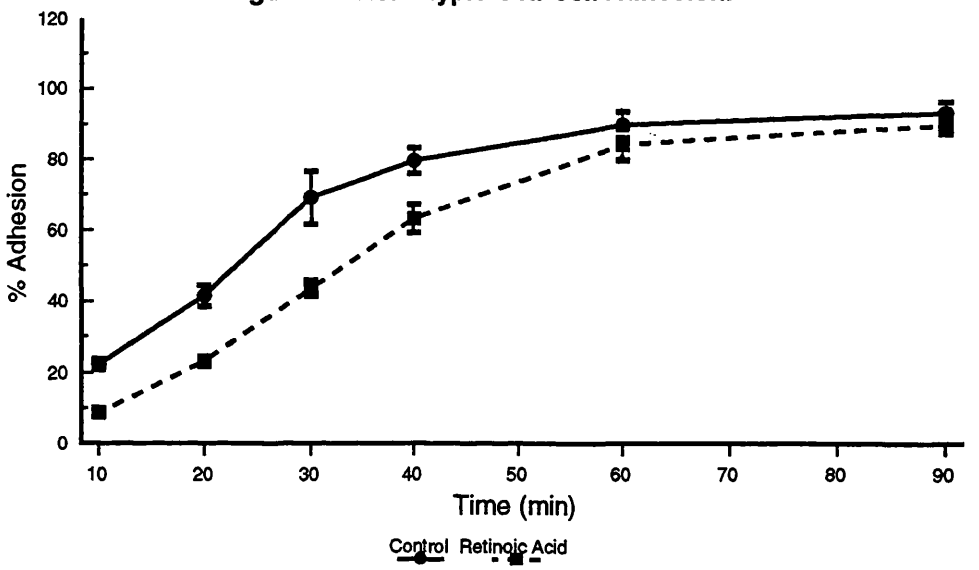
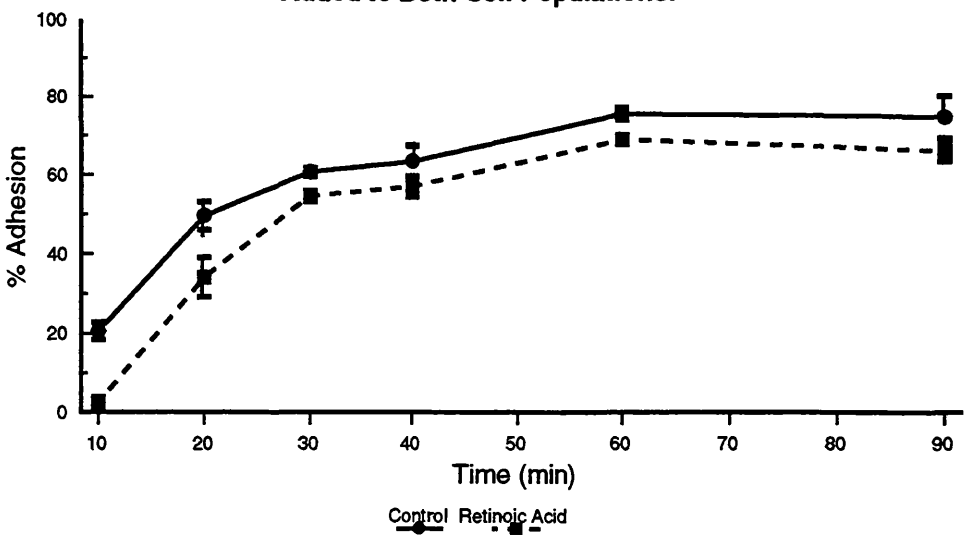


Figure 22. Homotypic Cell-Cell Adhesion - RA Added to Both Cell Populations.



The adhesion of B16F1M2 cells to monolayers of B16F1M2 cells also treated with 10^{-6}M retinoic acid was examined (fig.22). Half maximum adhesion of control cells occurred after approximately 15 min, but it took until 60 min for 70% of cells to adhere. Although the half maximum adhesion rate was slightly quicker than for untreated monolayers, fewer control cells adhered. Half maximum adhesion for the retinoic acid-treated cells occurred after 21 min. This is increased as compared to the control cell adhesion, but it is less than the value obtained for retinoic acid-treated cells adhering to untreated monolayers. However, fewer retinoic acid-treated cells adhered to treated monolayers than to untreated monolayers.

2. Heterotypic Adhesion.

CPAE cells were grown to confluence in 35mm petri dishes and the adhesion rate of B16F1M2 melanoma cells to the monolayer examined. Control cells adhered rapidly to the monolayer with over 70% of the cells attached following a 20 min incubation, with half maximum adhesion achieved by about 16 min (fig.23). Retinoic acid-pretreated cells adhered more slowly with half maximum attachment occurring by about 21 min. It required an incubation time of 40 min for 70% of retinoid-treated cells to adhere to the monolayer.

Maximum adhesion to CPAE cells by control cells occurred after 90 min incubation, by which time 95% of cells had attached. This is a similar value to that obtained for homotypic adhesion to an untreated monolayer (93%) and to the intact ECM (92%). It is much higher than the maximum adhesion values obtained for type IV collagen (60%) or laminin/nidogen (36%), and although the value obtained for maximum control cell adhesion to fibronectin was higher than for the other two, it was also lower (80%). When the cells were treated with retinoic acid, maximum adhesion after 90 min incubation was 84%. This is slightly lower than for homotypic adhesion to untreated monolayers (90%) but is slightly higher than to intact ECM (74%). However, it is much higher than the values obtained for maximum adhesion to single substrates - type IV collagen 51%, laminin/nidogen 18% and fibronectin 72%.

Figure 23. Heterotypic Cell-Cell Adhesion.

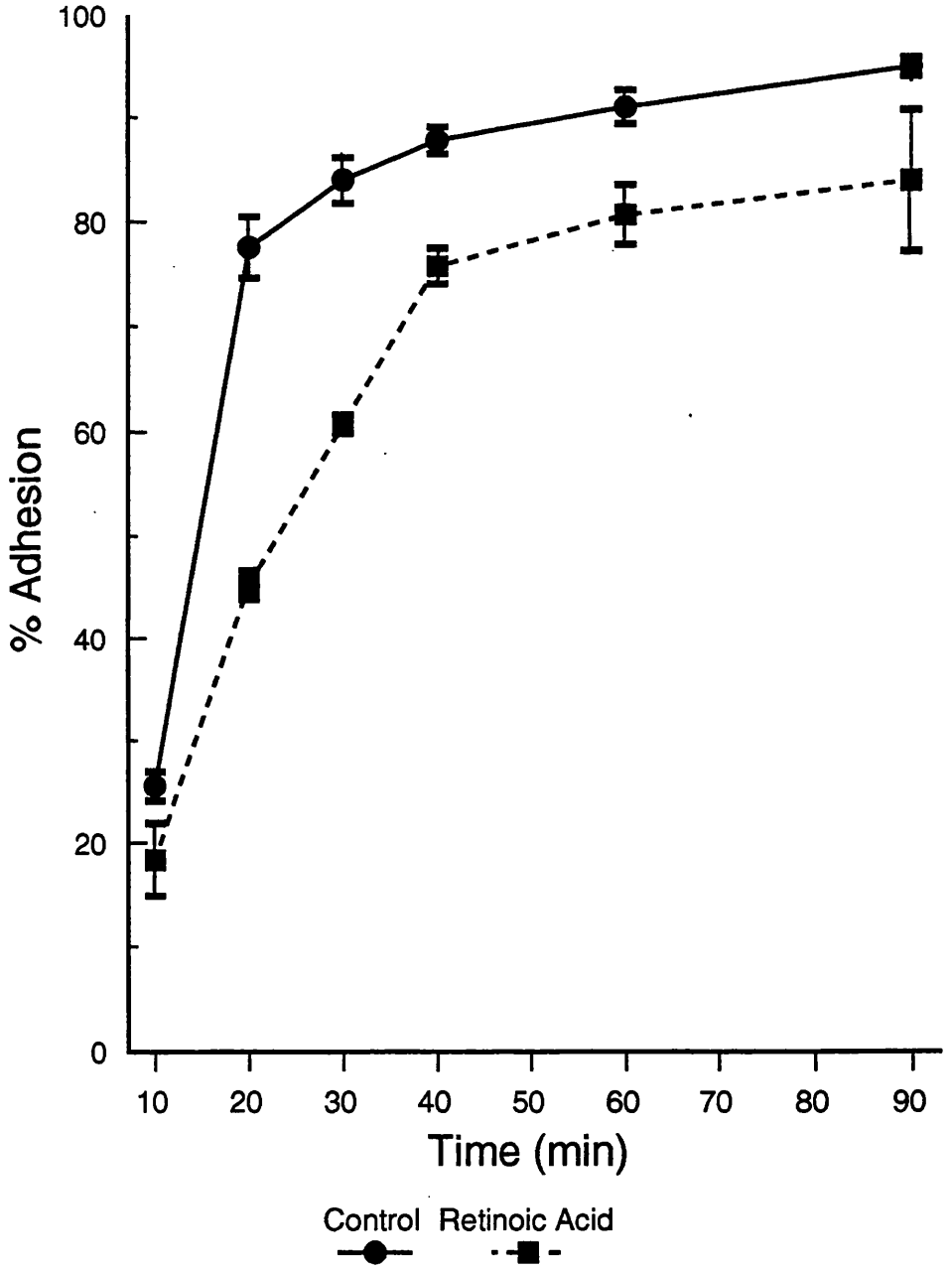
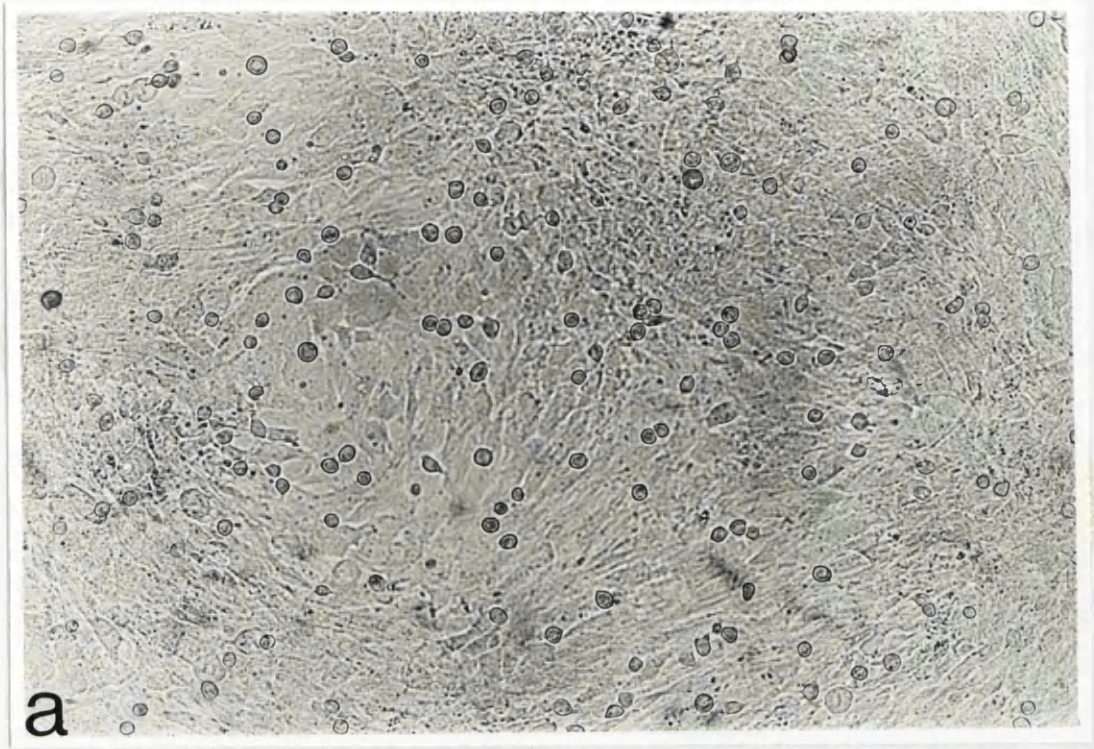
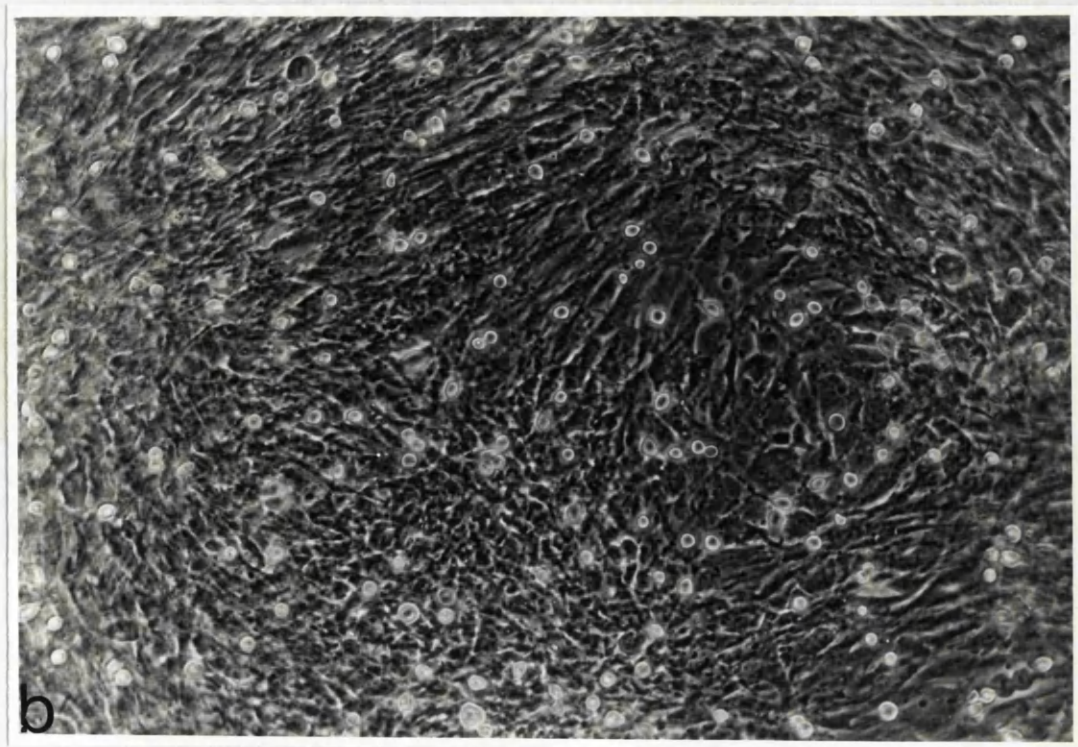


Figure 24. Heterotypic Cell-Cell Adhesion.

B16F1M2 Control Cells (a) and those Grown in The Presence of 10^{-6}M Retinoic Acid (b).


a


b

The morphology of the B16F1M2 cells on the endothelial cell monolayer was also affected by treatment with retinoic acid (fig.24 a & b). Control cells appeared more flattened and spread out than those subjected to retinoic acid pretreatment which remained rounded even after 60 min incubation.

3. Cell-Cell Aggregation.

The ability of B16F1M2 cells to form cell-cell aggregates was investigated by seeding the cells onto an agar substrate. B16F1M2 cells do not adhere to agar, thus by seeding the cells onto this substrate the formation of cell-cell aggregates can be examined. After 4 days in culture, control cells formed compact cell-cell aggregates (fig.25). Pretreatment of cells with 10^{-6}M retinoic acid resulted in the formation of smaller cell-cell aggregates. The cells also appeared to exhibit reduced cell-cell adhesion as fragmentation of the aggregates was achieved more easily than with control cell aggregates.

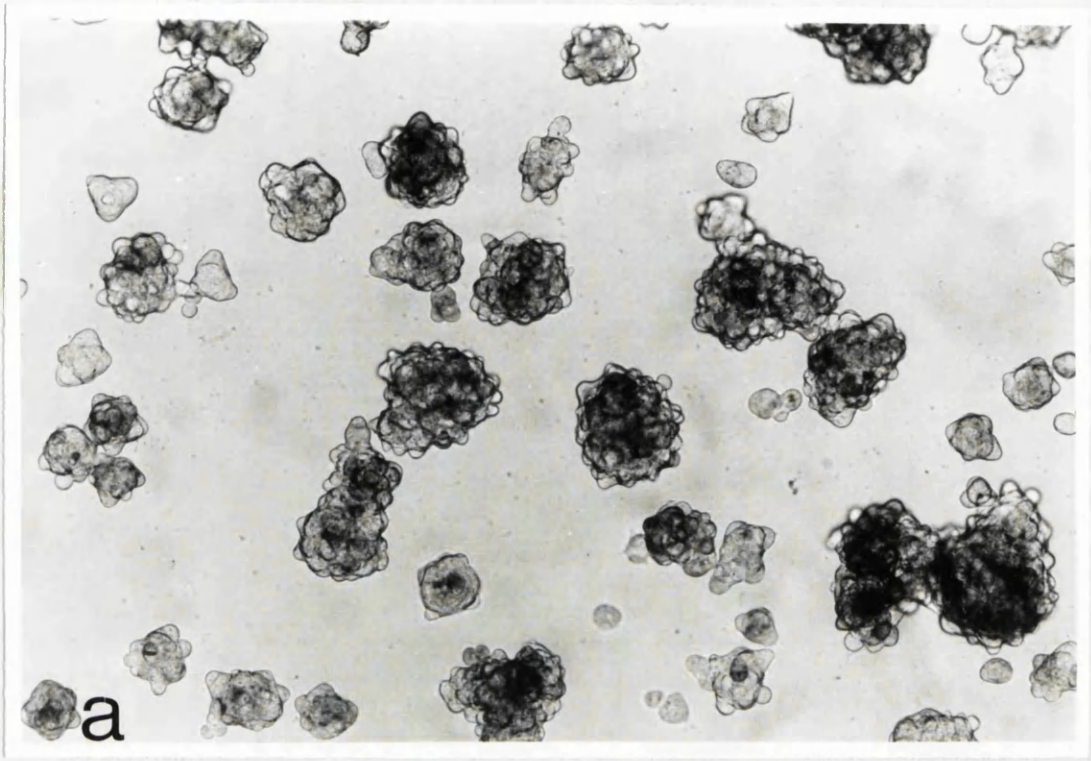
3:4. EFFECT OF RETINOIC ACID ON THE DEGRADATION OF THE EXTRACELLULAR MATRIX.

1. Degradation of ^{35}S-Labelled Extracellular Matrix.

B16F1M2 cells were treated with a concentration range of retinoic acid (10^{-10} - 10^{-5}M), seeded onto intact subendothelial ECM radiolabelled with $^{35}SO_4$, and the release of radiolabelled degradation products monitored. As the major sulphated molecules in the ECM are the sulphated glycosaminoglycans, it was assumed that the degradation products labelled with $^{35}SO_4$ are breakdown products of these molecules. The results are shown in figure 26. Over the lower concentrations of retinoic acid there is a steady decline in the number of radioactive counts released into the medium. This continued until a concentration of 10^{-8}M was reached. At concentrations higher than 10^{-8}M, the number of counts in the medium increased slightly although the significance of this rise is doubtful as the standard errors obtained in this set of experiments were quite high. However, at 10^{-5}M retinoic acid there is again a reduction in the amount of $^{35}SO_4$ detected in the medium. As

Figure 25. Effect of Retinoic Acid on The Formation of Cell-Cell Aggregates.

B16F1M2 Control Cells (a) and Grown in The Presence of 10^{-6}M Retinoic Acid (b).


a

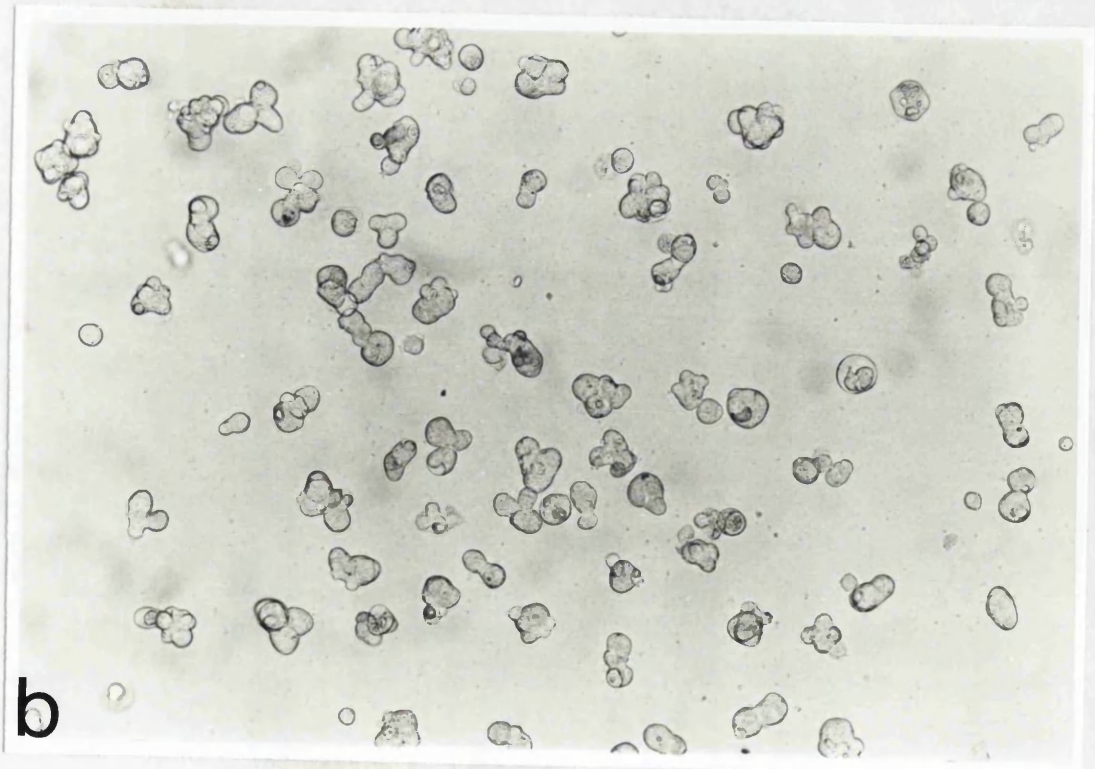

b

Figure 26. Degradation of 35-S Radiolabelled Extracellular Matrix.

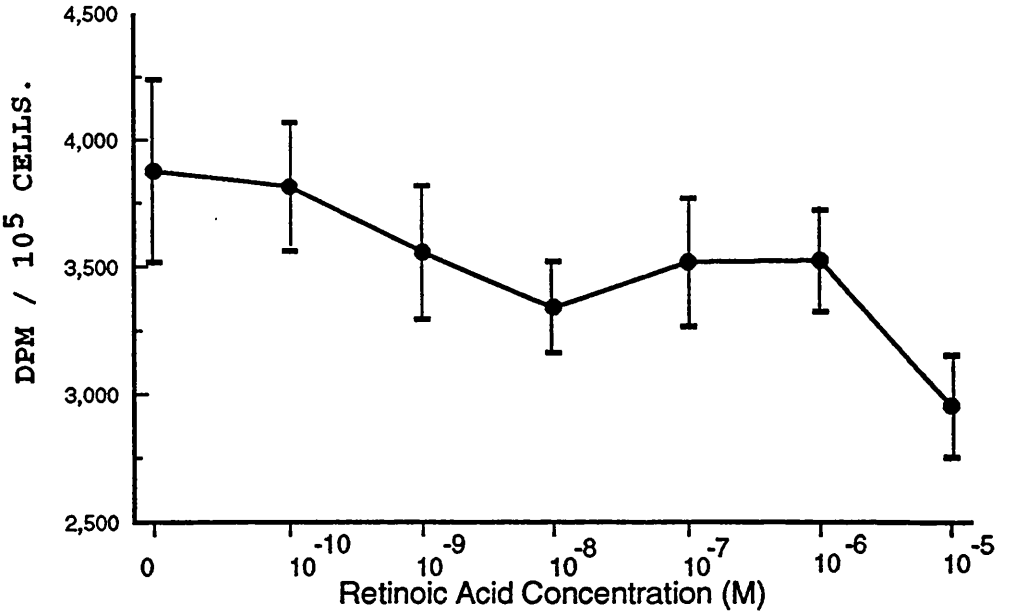
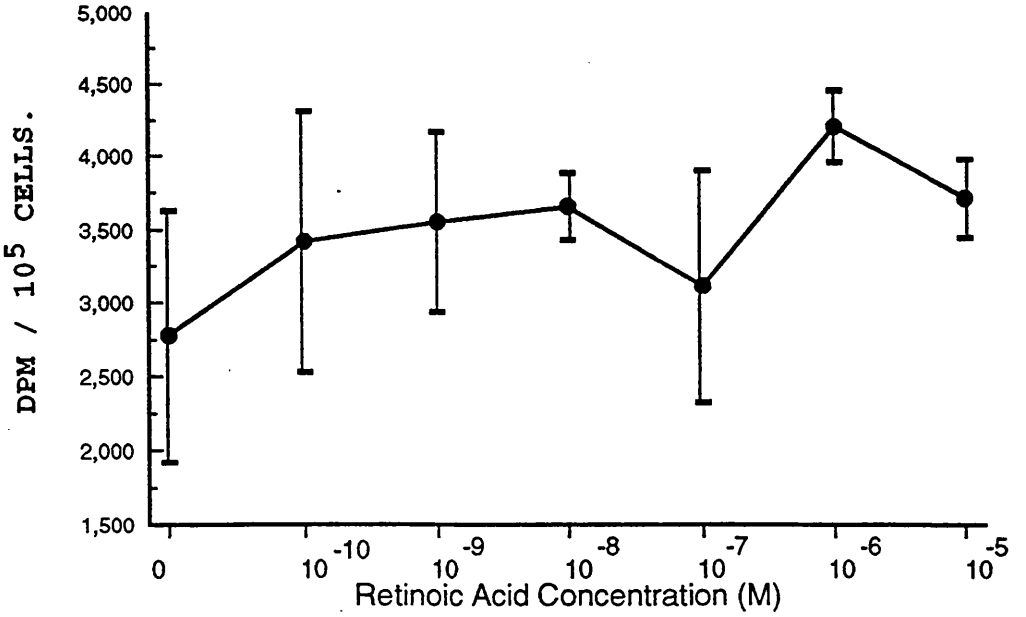


Figure 27. Degradation of 3-H Proline Labelled Extracellular Matrix.



previously stated, at 10^{-5}M ,retinoic acid is probably cytotoxic to the cells and as such the decrease at this concentration should probably be disregarded. Although the amount of $^{35}SO_4$ detected in the medium increased at both 10^{-7}M and 10^{-6}M retinoic acid, the values observed were still lower than control values. As stated earlier, the true significance of the results is unclear. However, it would appear that the general trend is that retinoic acid reduces the amount of breakdown products labelled with [^{35}S] which are released into the medium.

2.Degradation of [^{3}H]Proline-Labelled Extracellular Matrix.

Melanoma cells (B16F1M2) were treated with retinoic acid (10^{-10} - 10^{-5}M) and then seeded onto intact subendothelial ECM labelled with [^{3}H]proline. The major amino acid in the collagens is glycine, but there is also a high percentage of proline and hydroxyproline present in the collagens, and as this is almost exclusive to the collagens, it is assumed that most of the molecules in the matrix labelled with the radioactive proline will be collagens. The results are shown in figure 27. There appeared to be no general trend as the values obtained were inconsistent. The amount of radioactivity increased until a concentration of 10^{-8}M was reached, then it appeared to decrease at 10^{-7}M before increasing again at 10^{-6}M and then dropping again at 10^{-5}M. The amount of ^{3}H detected at all of the tested concentrations was greater than the control value and as such it could be said that the amount of released [^{3}H]proline-labelled compounds was increased with retinoic acid treatment. However, on closer examination it can be seen that the standard errors for each point in the experiment are quite large and as such the validity of the results may be questioned. Thus the real suggestion is that retinoic acid has little or no effect on the release of [^{3}H]proline-labelled degradation products in this system.

3.Effect of Retinoic Acid on Glycosidase Production.

The degradation of glycosaminoglycans in the ECM is brought about, in part, by the action of glycosidase. To determine the effect of retinoic acid on the breakdown of GAGs, the effect of retinoid on the production of these enzymes was

investigated. B16F1M2 cells were grown in the presence of retinoic acid (10^{-10} - 10^{-5}M) and the intracellular and extracellular levels of glycosidase determined. At all tested concentrations, retinoic acid reduced the amount of intracellular glycosidase activity detected in this assay (fig.28a). Between the control value and that obtained with 10^{-10}M retinoic acid there is a decrease of about 50%. This decrease continues until the highest test concentration (10^{-5}M) is reached, and at which point the amount of enzyme activity is almost undetectable.

When the extracellular enzyme levels were examined, a different picture was observed (fig.28b). There was an immediate increase in the detected enzyme activity at the lowest test concentration (10^{-10}M) of about 400%. This value increased slightly but the value remained relatively constant until a concentration of 10^{-6}M when it dropped to just over double the control value. The activity detected at 10^{-5}M retinoic acid was very high and as such was disregarded.

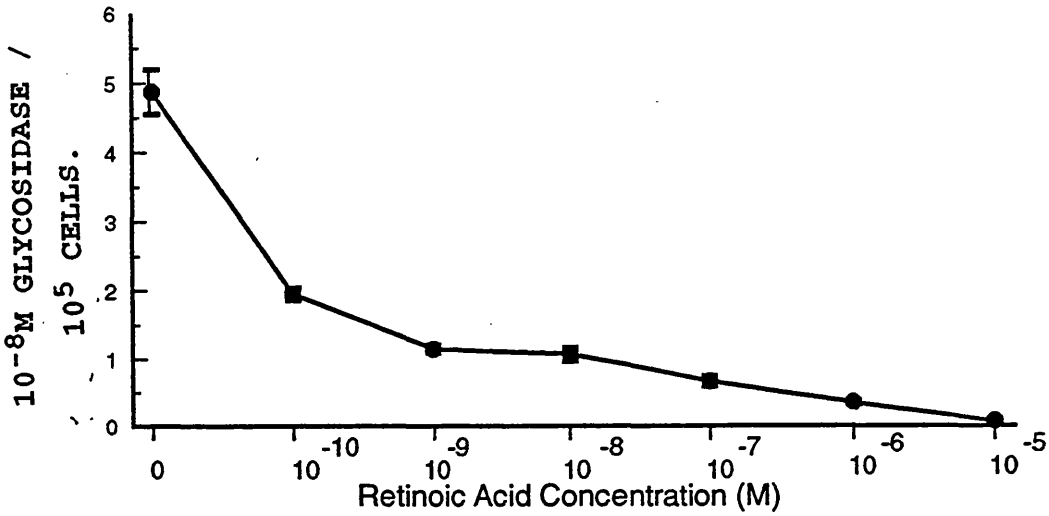
Both sets of experiments were repeated three times with similar trends achieved on all three occasions. The intracellular results are in agreement with the earlier work on the release of $^{35}SO_4$ labelled degradation products from the ECM by cells treated with retinoic acid.

4.Effect of Retinoic Acid on Collagenase Production.

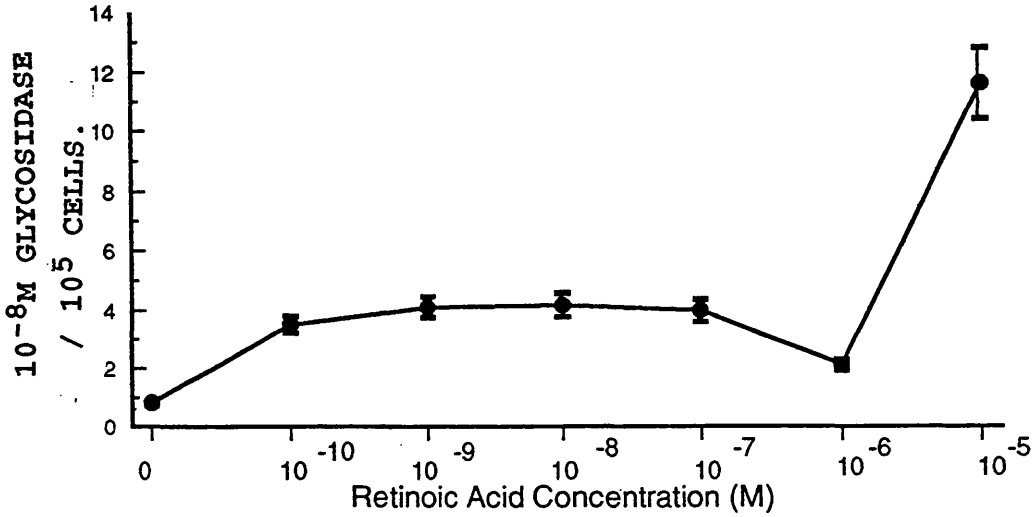
In an attempt to investigate the effect of retinoic acid on the degradation of collagens in the ECM, collagens type I and IV were isolated and radiolabelled with either ^{3}H or ^{14}C and the ability of B16F1M2 cells treated with retinoic acid to degrade these substrates investigated. Using the assay systems outlined in the materials and methods, production of neither type I collagenase nor type IV collagenase could be detected. As such, the effect of retinoic acid on these enzymes could not be determined. Several attempts were made using each assay system and each time no activity was detected.

Figure 28. Effect of Retinoic Acid on Glycosidase Activity.

a/Intracellular.



b/Extracellular.



5.Effect of Retinoic Acid on Plasminogen Activator Activity.

Plasminogen activators activate the conversion of plasminogen to plasmin which has a wide substrate specificity, and can directly degrade matrix proteins as well as activate the collagenases. The effects of retinoic acid on both intracellular and extracellular plasminogen activator activity of B16F1M2 cells are shown in Figure 29. Intracellular plasminogen activator activity is increased at all tested concentrations of retinoic acid (10^{-10} - 10^{-5}M). There is a steady increase in detected activity until a concentration of 10^{-8}M is achieved - the value at 10^{-8}M is almost triple control levels. The amount of activity detected at 10^{-7}M is reduced as compared to the 10^{-8}M activity, but it is still well above control levels. The experiment was repeated on three separate occasions and each time a drop in activity at 10^{-7}M was detected. At concentrations of 10^{-6}M and 10^{-5}M retinoic acid , the detected plasminogen activator activity was increased, with the maximum effect being observed at 10^{-5}M.

Extracellular plasminogen activator activity was also examined (fig.29). As with intracellular levels, extracellular plasminogen activator activity was increased at all tested concentrations of retinoic acid (10^{-10} - 10^{-5}M). Again, there was a steady increase in detected activity until 10^{-8}M retinoic acid - plasminogen activator activity detected after cells were pretreated with 10^{-8}M retinoic acid was almost double that detected in control cells - with a decrease in detected activity at a retinoic acid concentration of 10^{-7}M. The detected activity again increased, but at 10^{-6}M this increase was only very slight and the size of the error bars at this point would suggest that the levels remained the same at both 10^{-7}M and 10^{-6}M whereas the increase at 10^{-5}M is very large. The dramatic increase at 10^{-5}M would perhaps suggest that the effect is due to the overall effect of retinoic acid at this concentration on the cells rather than its effects on the production of plasminogen activator activity. As with the measurements on intracellular levels of activity, the extracellular plasminogen activator activity would appear to increase on treatment of the cells with retinoic acid.

Figure 29. Effect of Retinoic Acid on Plasminogen Activator Activity.

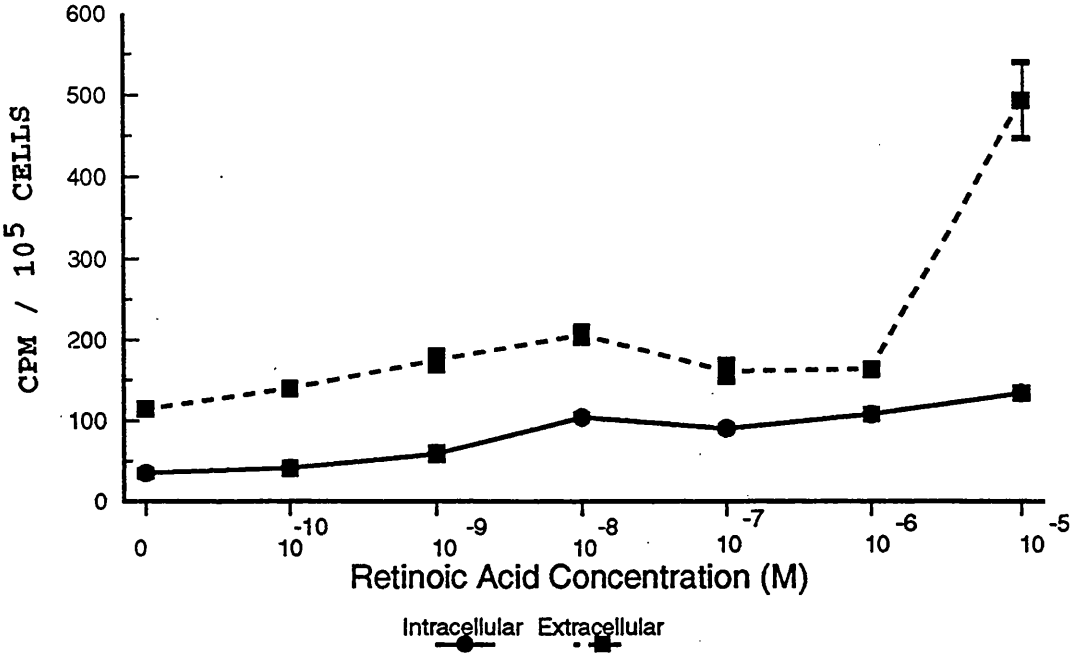
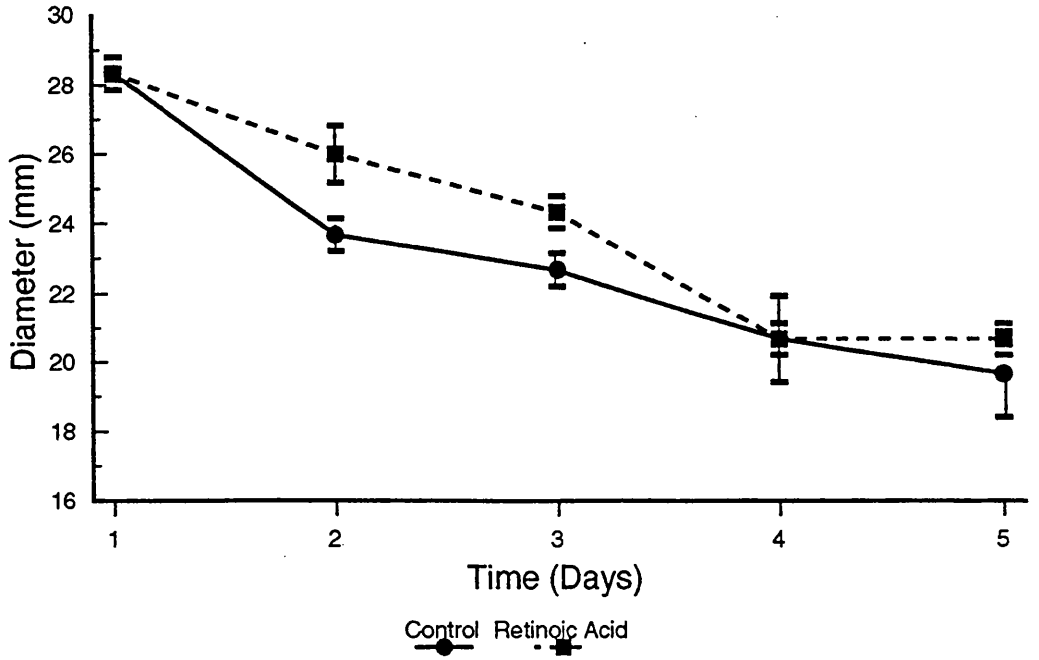


Figure 30. Effect of Retinoic Acid on Collagen Gel Contraction.



6.Effect of Retinoic Acid on the Contraction of Collagen Lattices.

The effect of retinoic acid on the contraction of collagen lattices prepared using type I collagen and adult forearm fibroblasts was determined and the results shown in Figure 30. As can be seen, retinoic acid at a concentration of 10^{-6}M had no effect on the contraction of collagen gels. Although the initial contraction of the gels may have been slightly slower in the presence of the retinoid, by the sixth day of the experiment both sets of gels were equally contracted.

7.Effect of B16F1M2 Serum-Free Conditioned-Medium on Fibroblast-Mediated Collagen Gel Contraction.

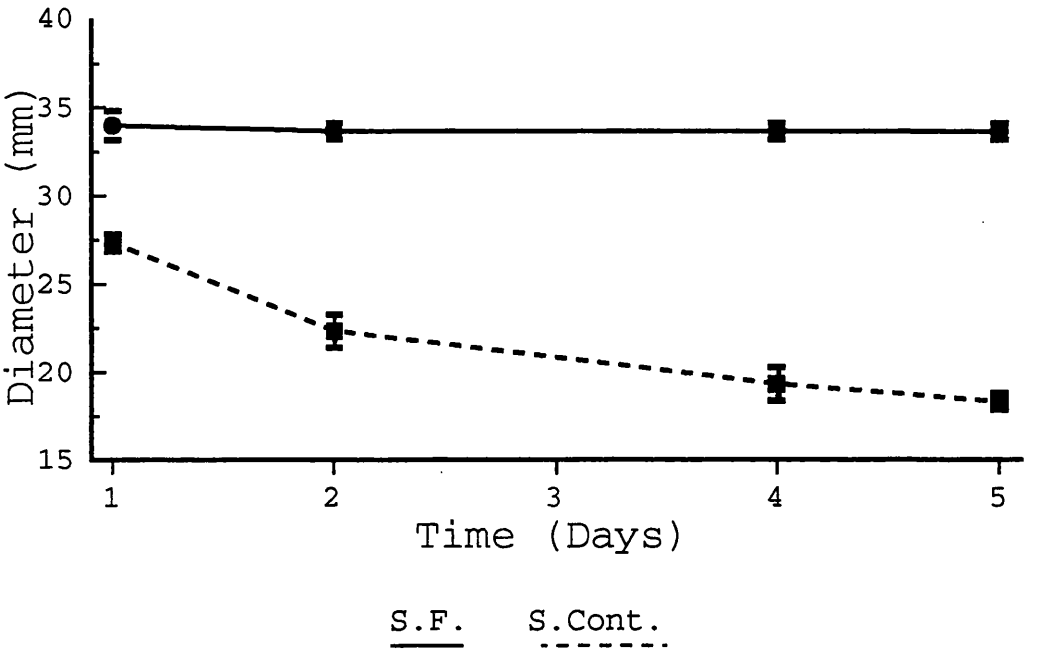
Serum-free conditioned medium from B16F1M2 cells grown in the presence and absence of 10^{-6}M retinoic acid was tested for its ability to affect collagen gel contraction (fig.31). In the presence of 10% foetal calf serum, the lattices contracted rapidly between day 1 and 2 (fig.30), with further contraction, at a slower rate, over the remainder of the incubation period (5 days). If no serum was present, there was only minimal contraction between day 1 and day 2 after which there was no further contraction.

In the presence of B16F1M2 serum-free conditioned medium, the contraction of the lattices followed a similar pattern to that observed in the presence of serum, however, the rate of contraction was slower - in the presence of serum the lattices contracted 5mm between day 1 and day 2, but with the conditioned media the reduction was only 3.3mm. In the serum-containing gels, contraction was still observed on day 5, whereas in the presence of conditioned media, no contraction was measured after day 4.

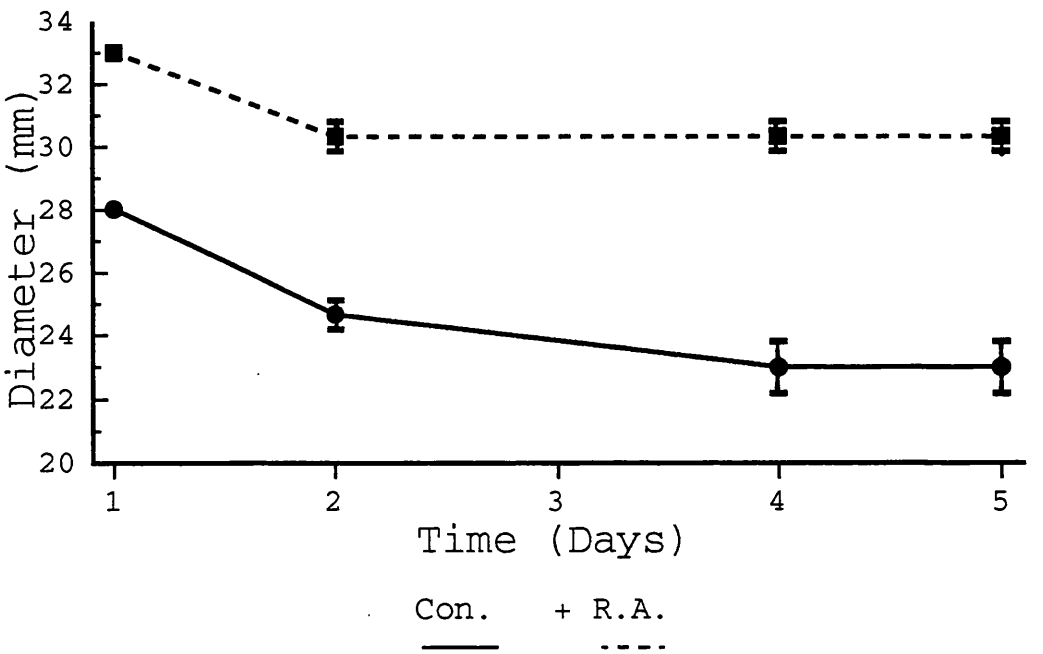
When the medium was conditioned in the presence of retinoic acid, an inhibition of contraction was observed. Although the lattices contracted between day 1 and day 2 (2.67mm), no further contraction was measured on subsequent days. This was similar to the observations with serum-free gels. When retinoic acid was added directly to serum-free medium, there was no noticable contraction of the lattices (results not shown).

Figure 31. Effect of B16F1M2 Conditioned Medium +/- Retinoic Acid on Collagen Gel Contraction.

a/ Control



b/Conditioned Media



3:5. Glycosaminoglycan Analysis.

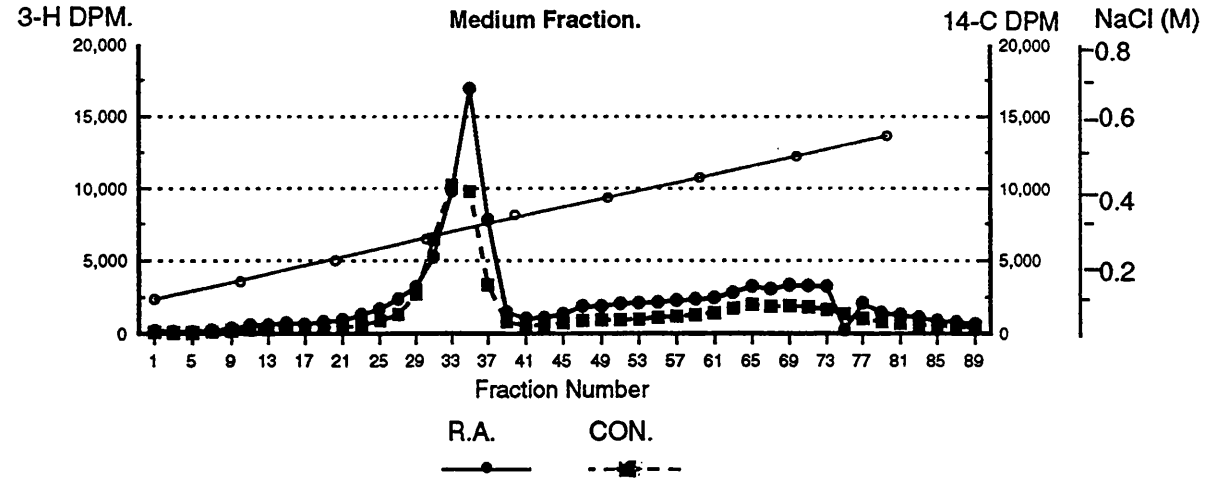
1.Qualitative Analysis.

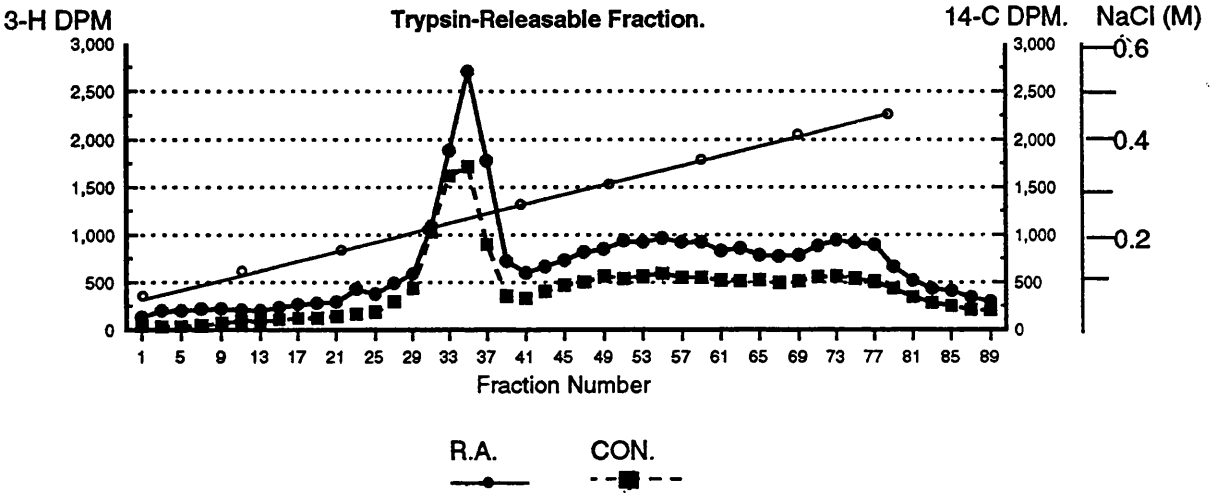
The glycosaminoglycan elution profiles from DEAE-cellulose columns of subconfluent control (^{14}C) /retinoic acid (^{3}H) and subconfluent control (^{14}C)/low density control cultures (^{3}H) of B16F1M2 are shown in Figure 32 a & b respectively. After separation by DEAE ion-exchange chromatography, the sulphated GAGs from subconfluent control/retinoic acid (CON/RA) cultures eluted at a salt concentration of about 0.30M following application of a NaCl gradient (0.05M-0.65M). However, under the same conditions, subconfluent control/low density control (CON/LD) sulphated GAGs eluted slightly earlier at about 0.28M.

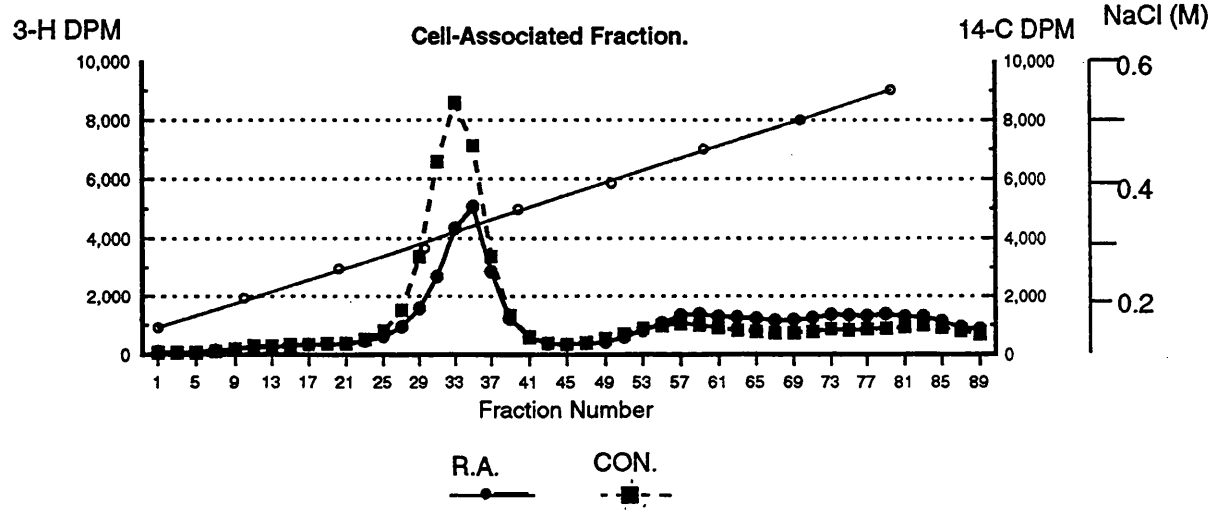
There would appear to be no real difference in the elution profiles of control and retinoic acid-treated sulphated GAGs. In the trypsin-releasable and cell-associated fractions, there is a slight indication that the control sulphated GAGs may elute as two peaks, but the change in the profile is not distinct enough to confirm the suggestion. However, the profiles from CON/LD cultures suggest that there is a difference in the sulphated GAGs. Medium fractions from CON/LD cultures show that the control GAGs do elute as two distinct peaks, whereas those of the low-density control elute as only one peak. Additionally, the low-density GAGs elute at a lower salt concentration than do those of the control (0.48M) as compared to 0.53M for the control GAGs). Similarly, in the trypsin fraction control sulphated GAGs elute as two peaks, however, there is a slight suggestion that the low density sulphated GAGs may also elute as two peaks. Unlike those of the medium fraction, the trypsin-releasable GAGs elute at a similar salt concentration to those of control cells. The elution profiles from the cell-associated fractions are indistinct and as such revealed very little, although there would appear to be a difference in the salt concentration at which they elute.

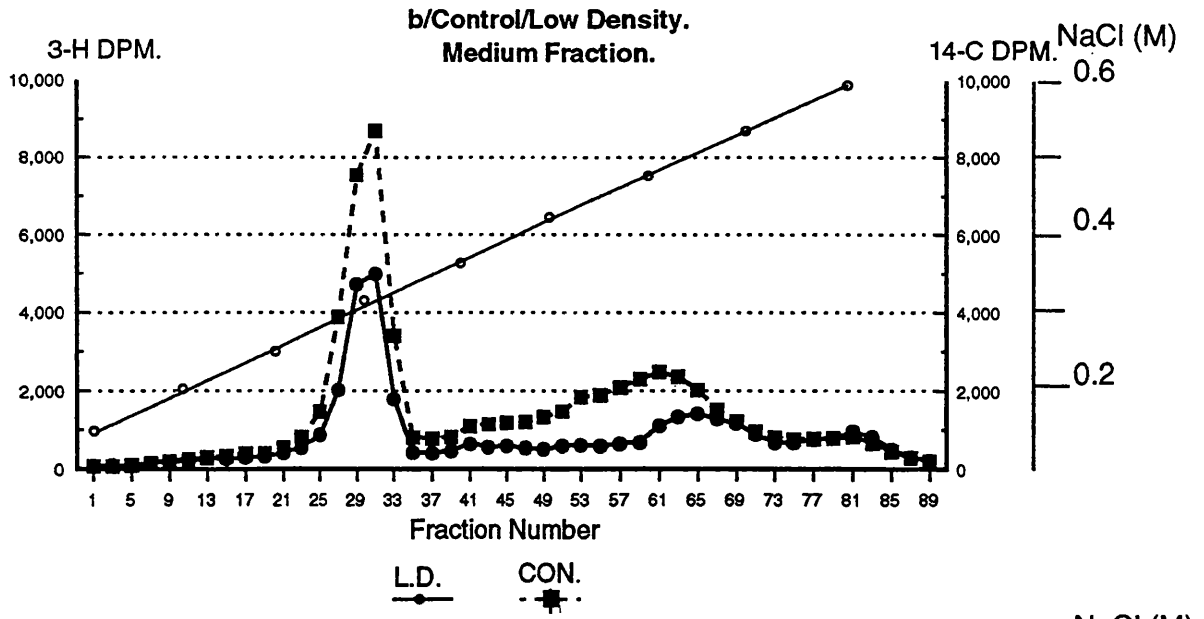
The sulphated GAGs were pooled, digested enzymatically and rechromatographed as previously. The gel filtration profiles obtained after treatment with chondroitinase ABC are shown in Figure 33. The void volume peaks were pooled and treated with nitrous acid to demonstrate the presence of heparan sulphate

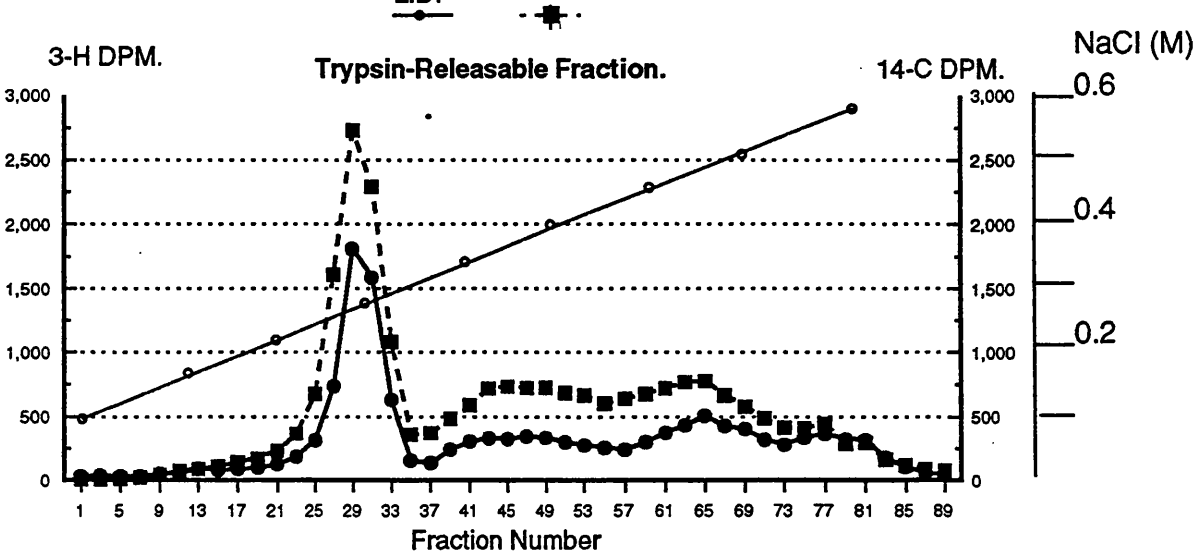
Figure 32. DEAE Anion Exchange Profiles.
a/Control/Retinoic Acid.








b/Control/Low Density.
Medium Fraction.
3-H DPM.
14-C DPM.
NaCl (M)
10,000
8,000
6,000
4,000
2,000
0
0.6
0.4
0.2
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89
Fraction Number
L.D.
CON.


3-H DPM.
Trypsin-Releasable Fraction.
14-C DPM.
NaCl (M)
3,000
2,500
2,000
1,500
1,000
500
0
0.6
0.4
0.2
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89
Fraction Number
L.D.
CON.

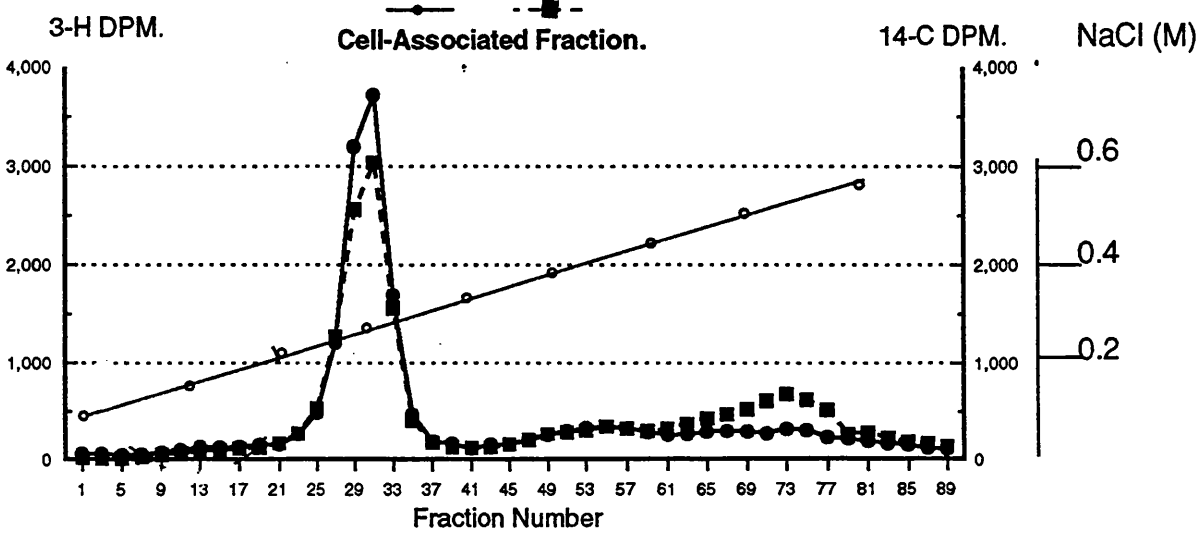
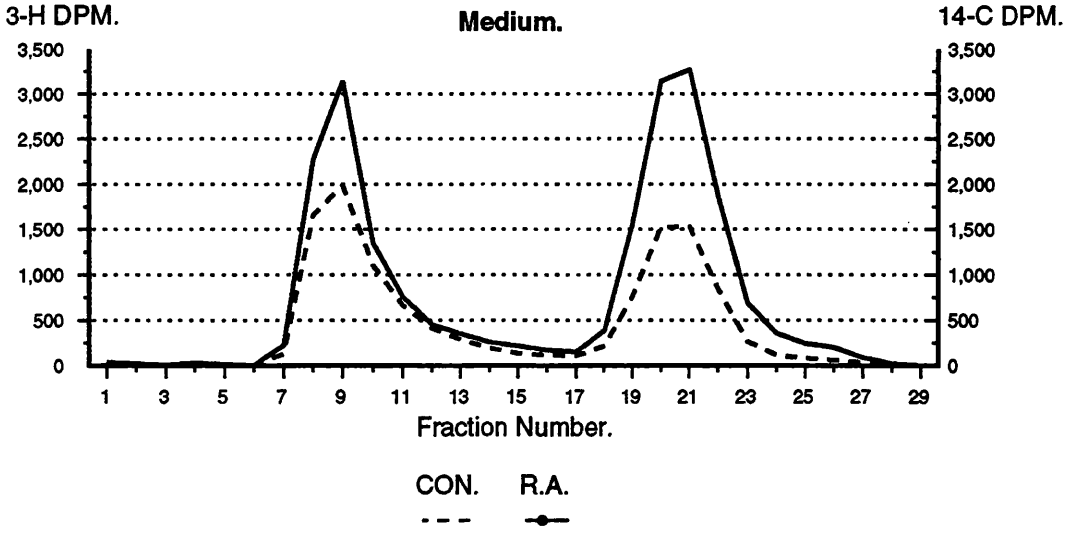
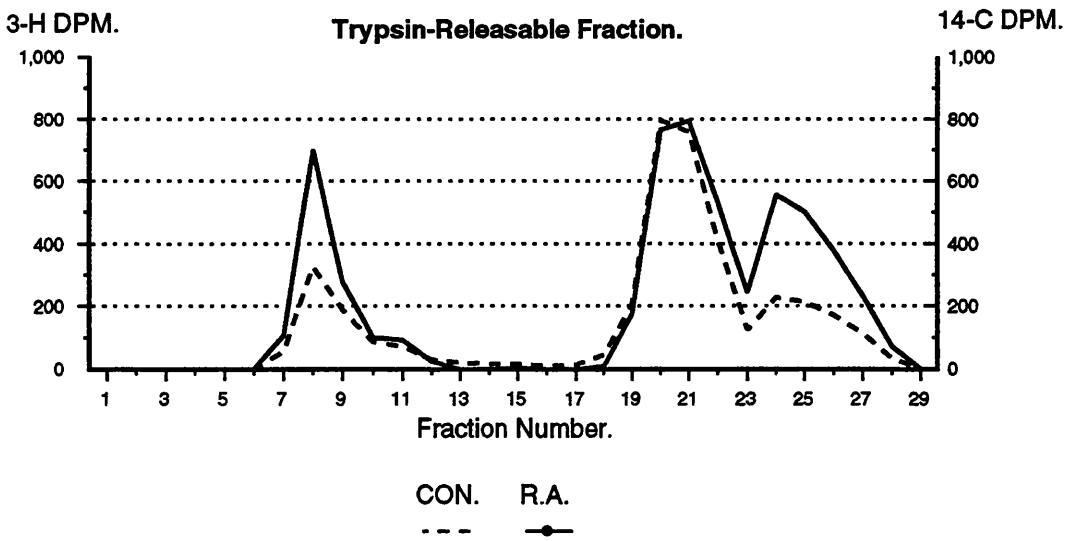
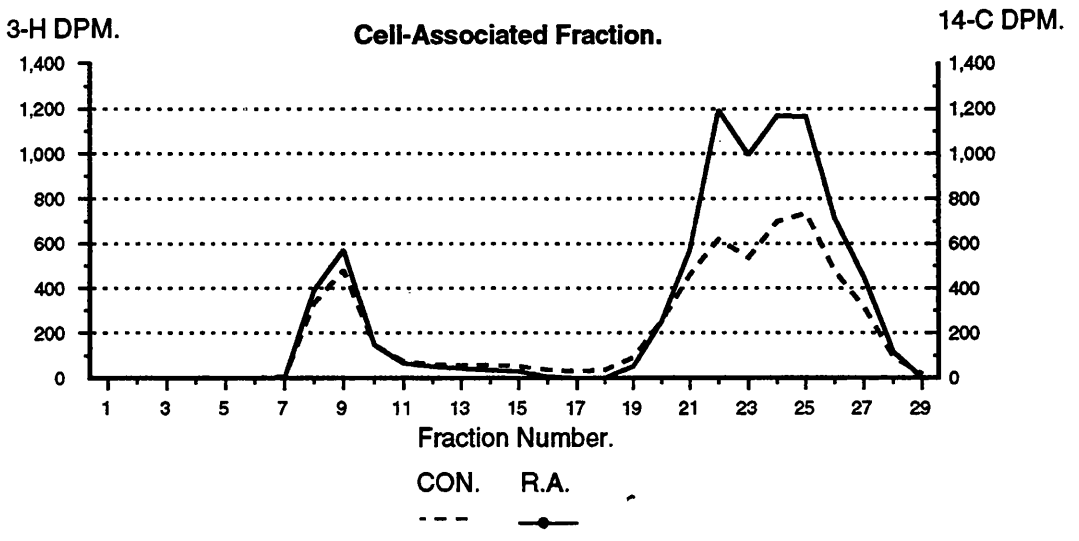

3-H DPM.
Cell-Associated Fraction.
14-C DPM.
NaCl (M)
4,000
3,000
2,000
1,000
0
0.6
0.4
0.2
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89
Fraction Number
L.D.
CON.

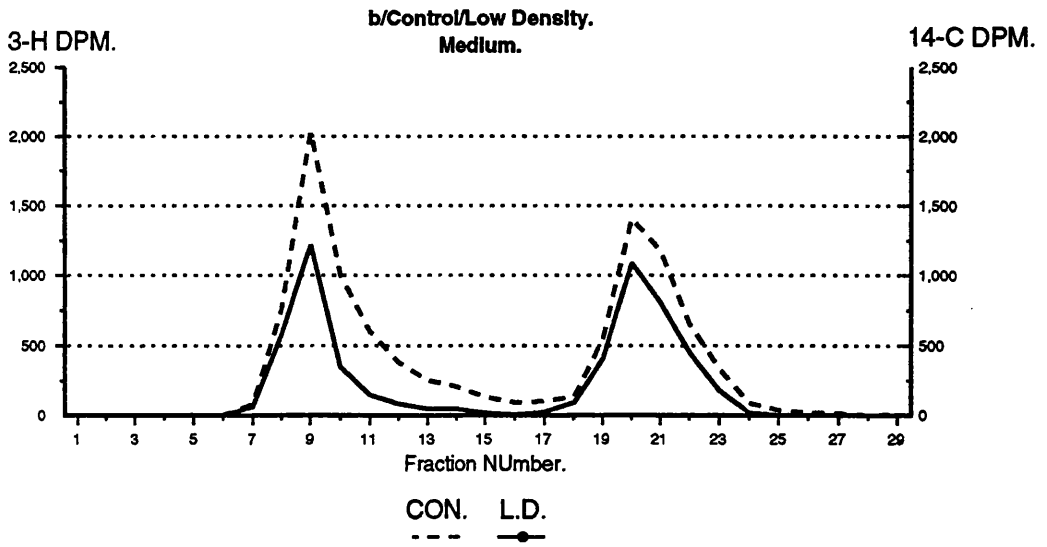
Figure 33. Sulphated-GAGs G-50 Profiles.

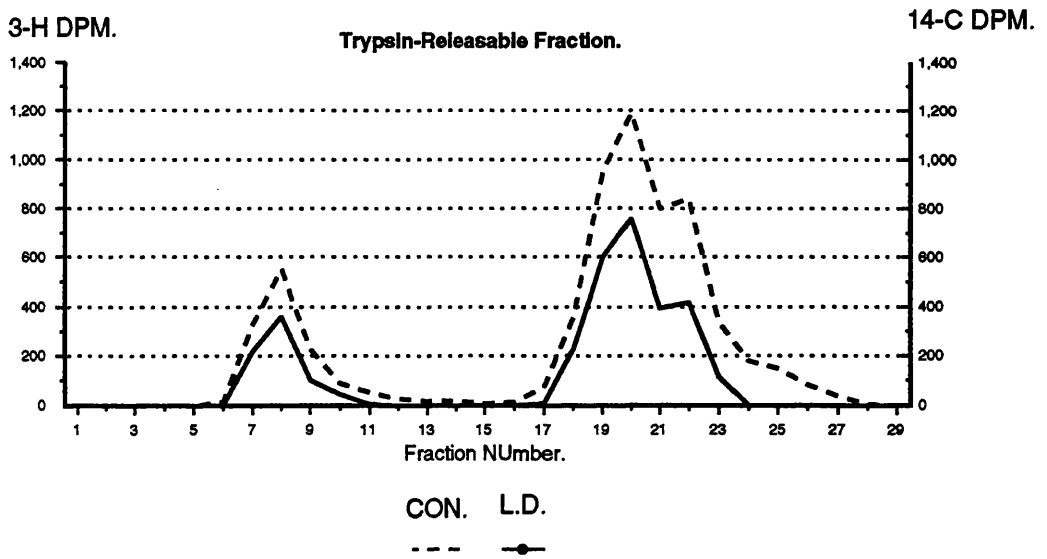
a/ Control/Retinoic Acid.

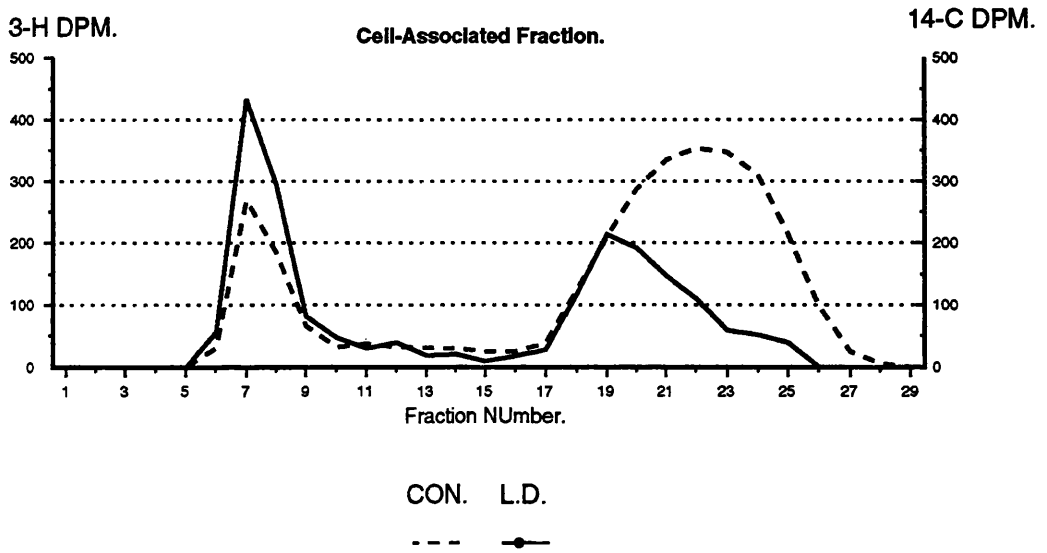







b/Control/Low Density.
Medium.
3-H DPM.
14-C DPM.
2,500
2,000
1,500
1,000
500
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
Fraction NUmber.
CON.
L.D.


3-H DPM.
Trypsin-Releasable Fraction.
14-C DPM.
1,400
1,200
1,000
800
600
400
200
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
Fraction NUmber.
CON.
L.D.


3-H DPM.
Cell-Associated Fraction.
14-C DPM.
500
400
300
200
100
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
Fraction NUmber.
CON.
L.D.

breakdown products in the samples. The profiles of the resulting heparan sulphate breakdown products are shown in Figure 34. In the CON/RA cultures, there would appear to be very little difference in the elution of the chondroitin sulphates. In the medium fraction chondroitin sulphates from both the controls and retinoic acid-pretreated cells eluted with a similar pattern, but the profiles suggest that those of the retinoic acid-pretreated cells may elute slightly later than those of the control. In contrast to this, elution of control and retinoic acid pretreated chondroitin sulphates from the trypsin-releasable fraction was identical. However, the profile of those from the cell-associated fraction presents a different picture. The control heparan sulphates elute as a broad peak with a small initial peak before the shoulder is reached. Pretreatment of cells with retinoic acid results in a change in profile, with small peaks present at each shoulder of the peak.

Eluted chondroitin sulphates from the medium fraction of control and low-density cells have very similar profiles. This was also the case for the trypsin-releasable fraction, however, cell associated chondroitin sulphates demonstrated a change in the elution profile. As with those found in the corresponding retinoic acid-treated fraction, the low-density chondroitin sulphates eluted as a broad peak with a smaller peak at either shoulder.

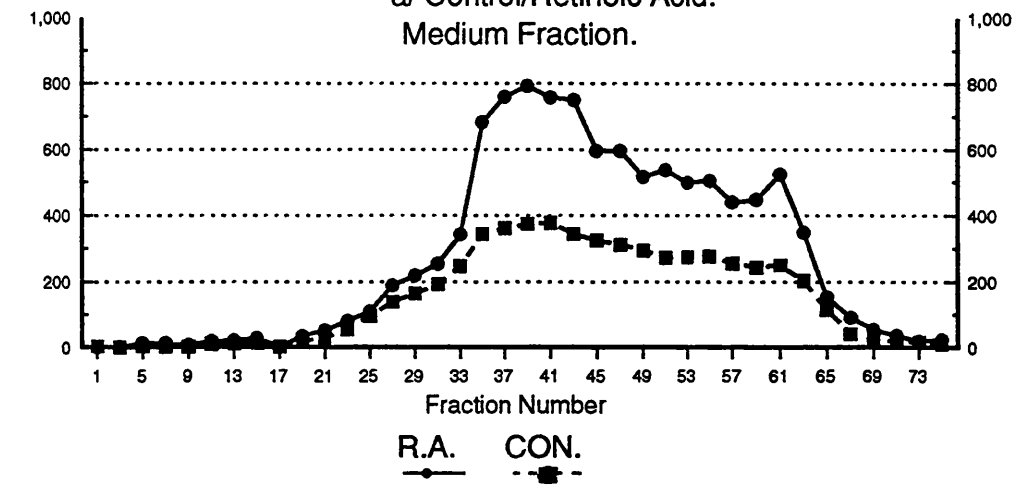
2.Quantitative Analysis.

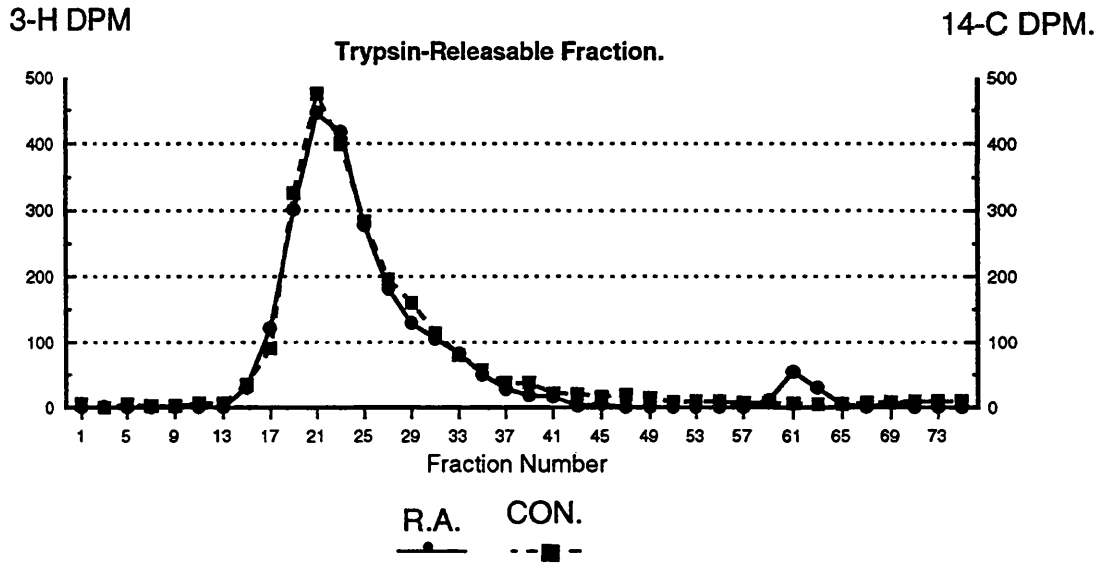
A quantitative analysis of glycosaminoglycans labelled with [^{3}H]glucosamine and $^{35}SO_4$, from subconfluent control, retinoic acid-treated and low-density control cells was carried out. Isolation of the glycosaminoglycans by ion-exchange chromatography was undertaken and the elution profiles are shown in Figure 35. As can be seen, there is little difference in the elution profiles obtained by this separation. There is a slight indication that the sulphated GAGs from the subconfluent control cultures may elute slightly earlier than those of the retinoic acid-treated or low-density control cultures, but the difference is too small to be conclusive.

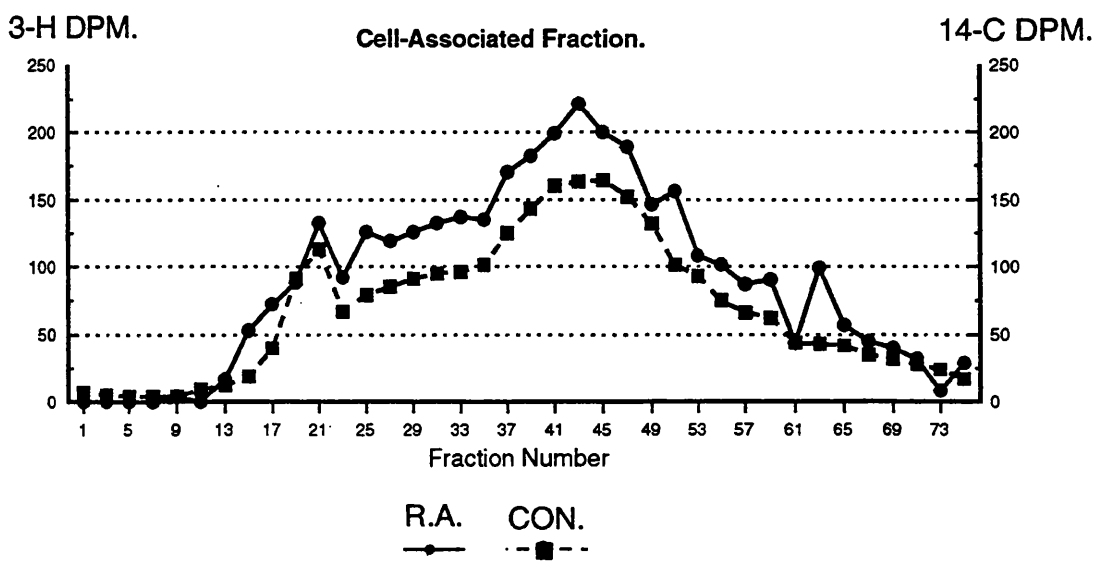
Figure 34. Isolated Chondroitin Sulphates.

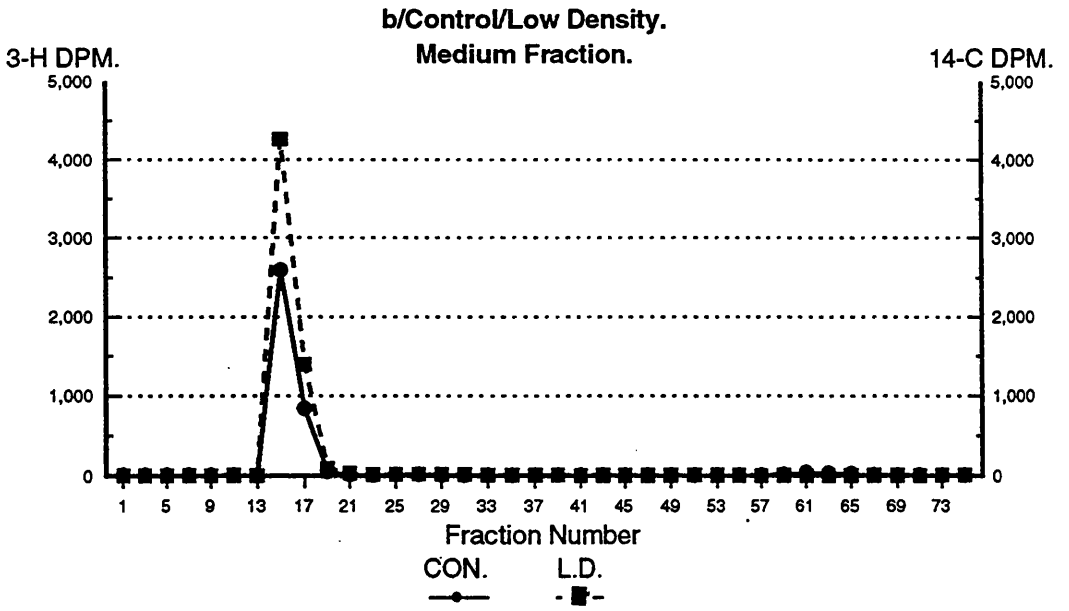
a/ Control/Retinoic Acid.

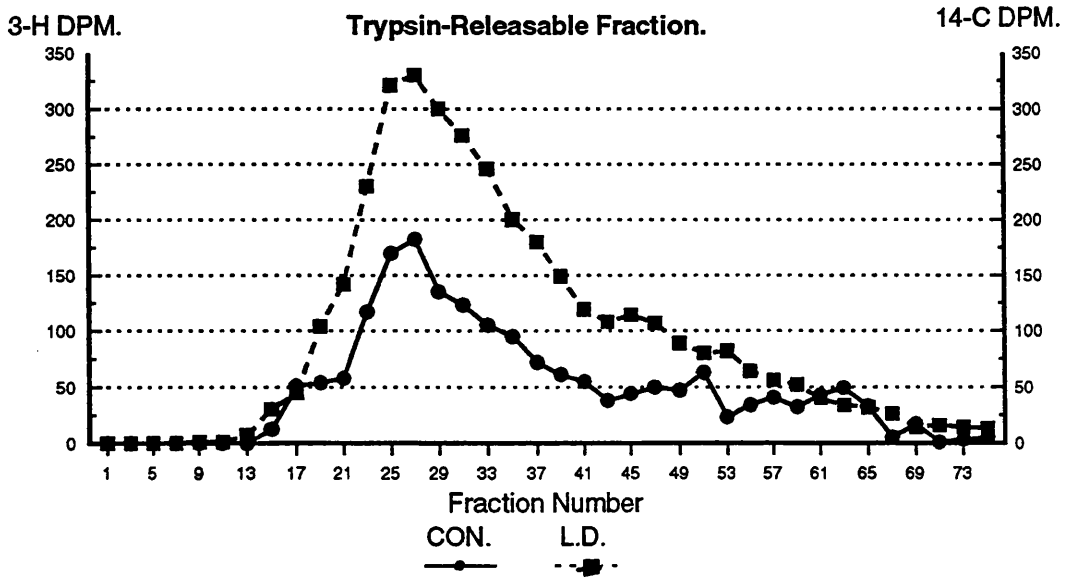
Medium Fraction.








b/Control/Low Density.
Medium Fraction.
3-H DPM.
14-C DPM.
5,000
4,000
3,000
2,000
1,000
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73
Fraction Number
CON.
L.D.


3-H DPM.
Trypsin-Releasable Fraction.
14-C DPM.
350
300
250
200
150
100
50
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73
Fraction Number
CON.
L.D.

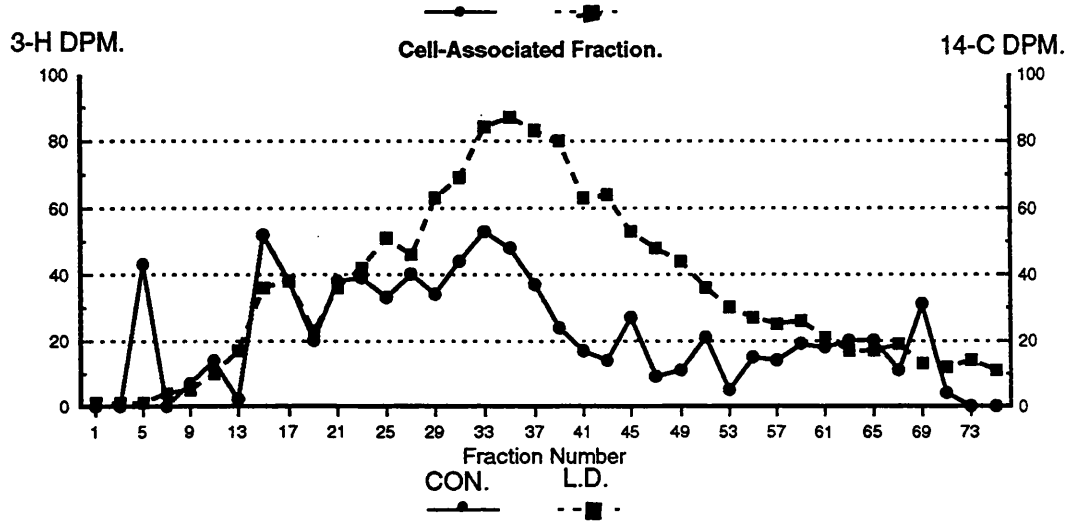
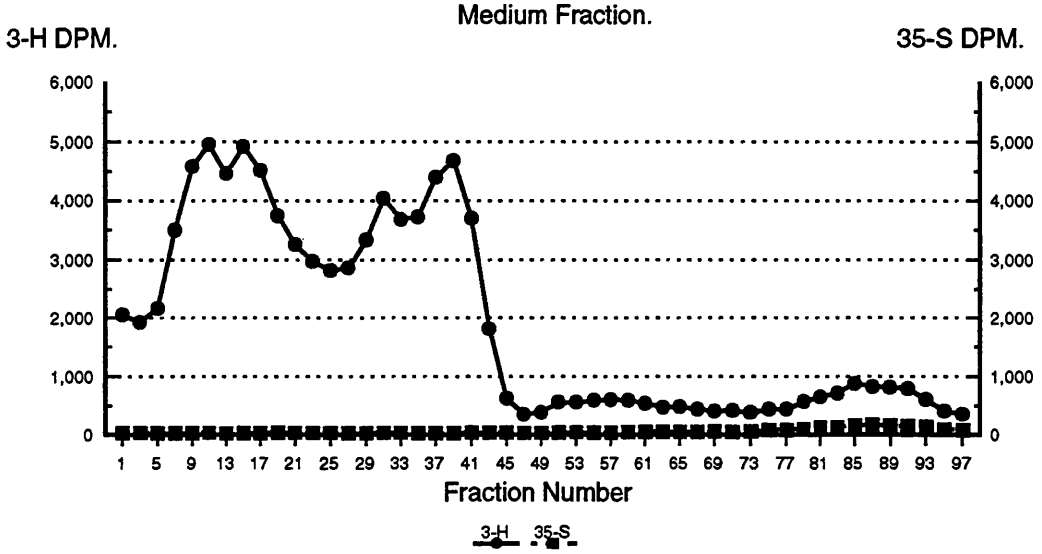
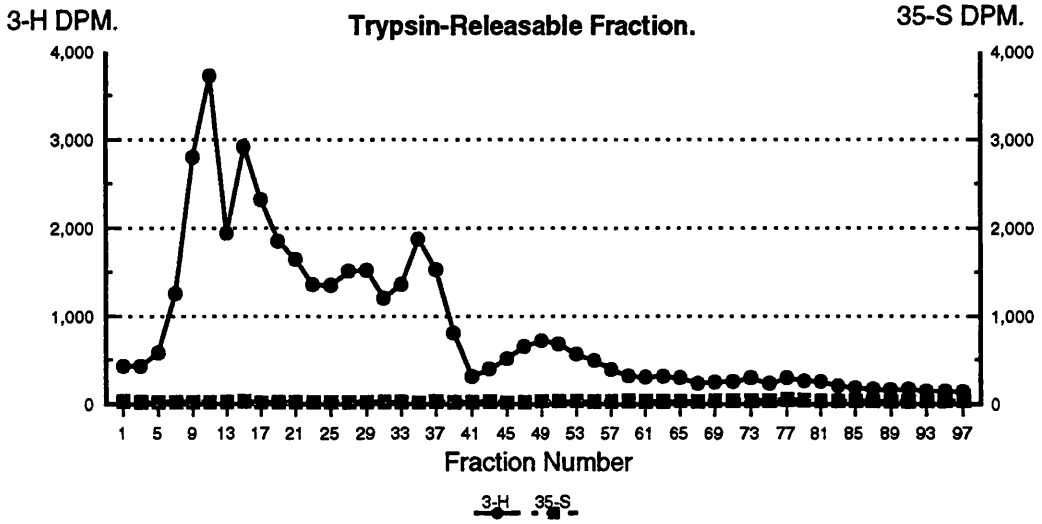
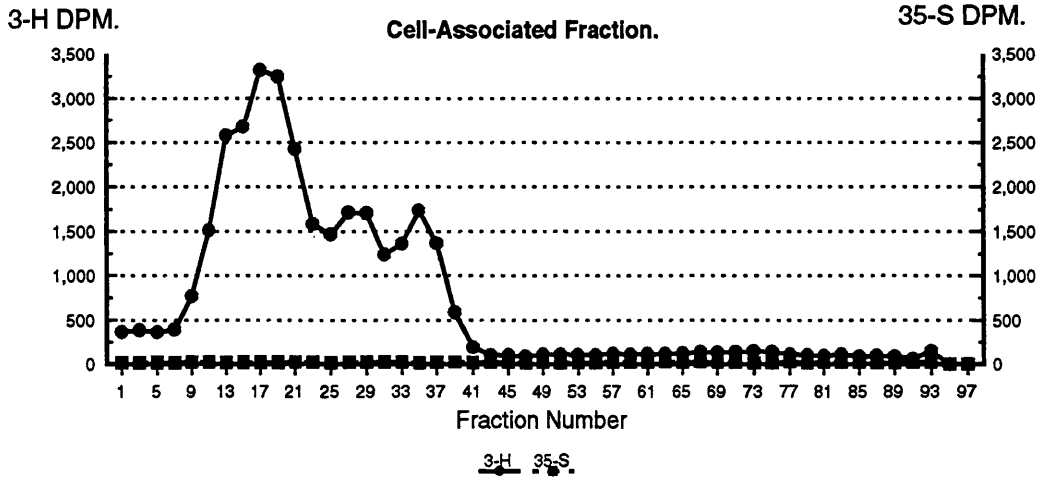

3-H DPM.
Cell-Associated Fraction.
14-C DPM.
100
80
60
40
20
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73
Fraction Number
CON.
L.D.

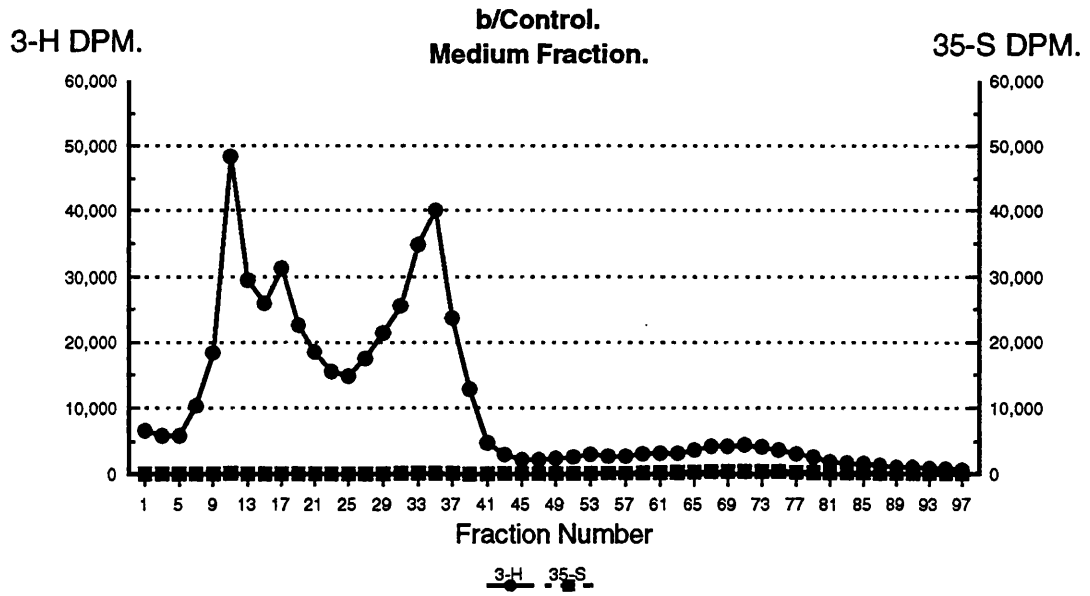
Figure 35. DEAE Anion Exchange Profiles.
a/Low Density

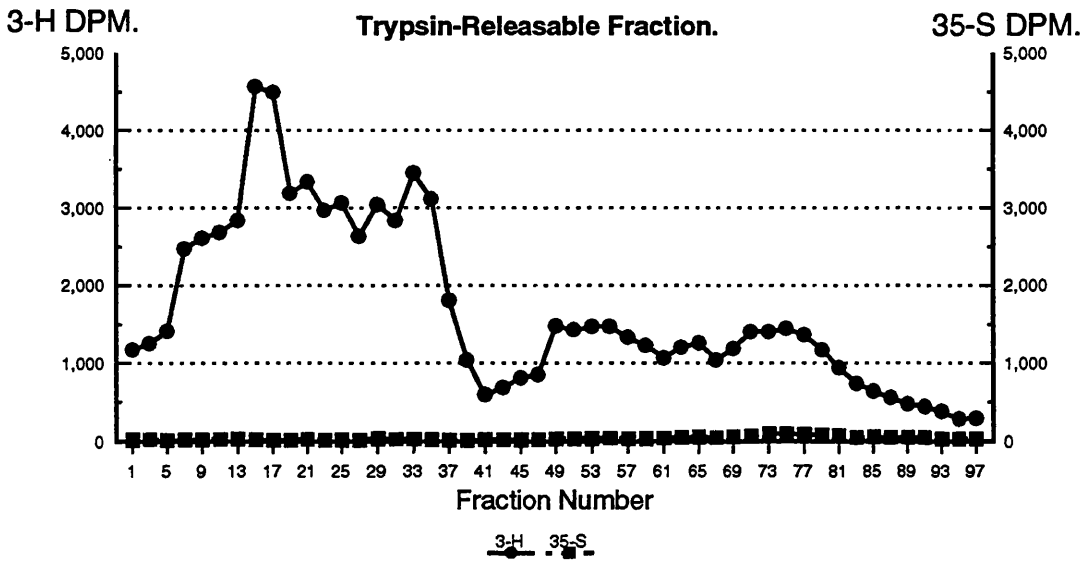
Medium Fraction.

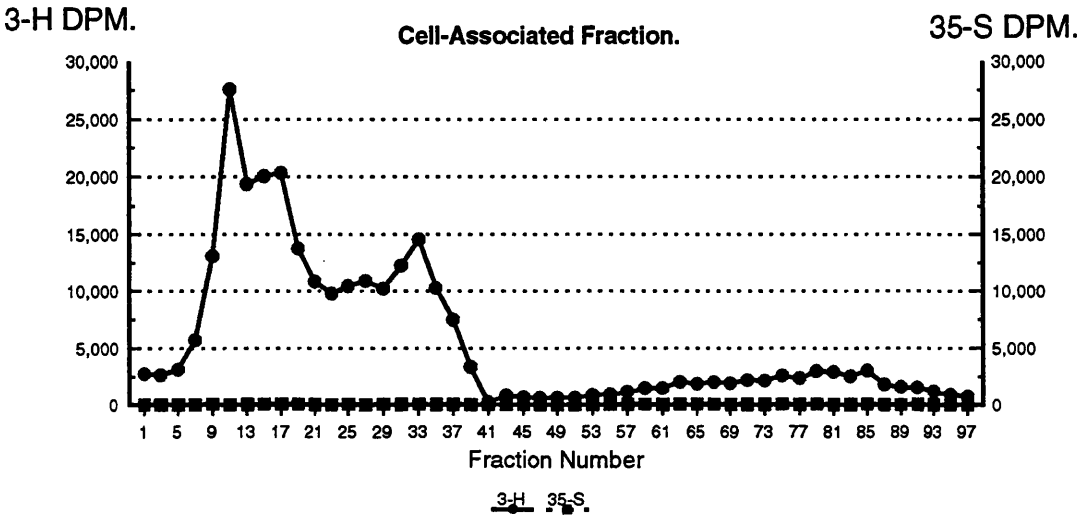


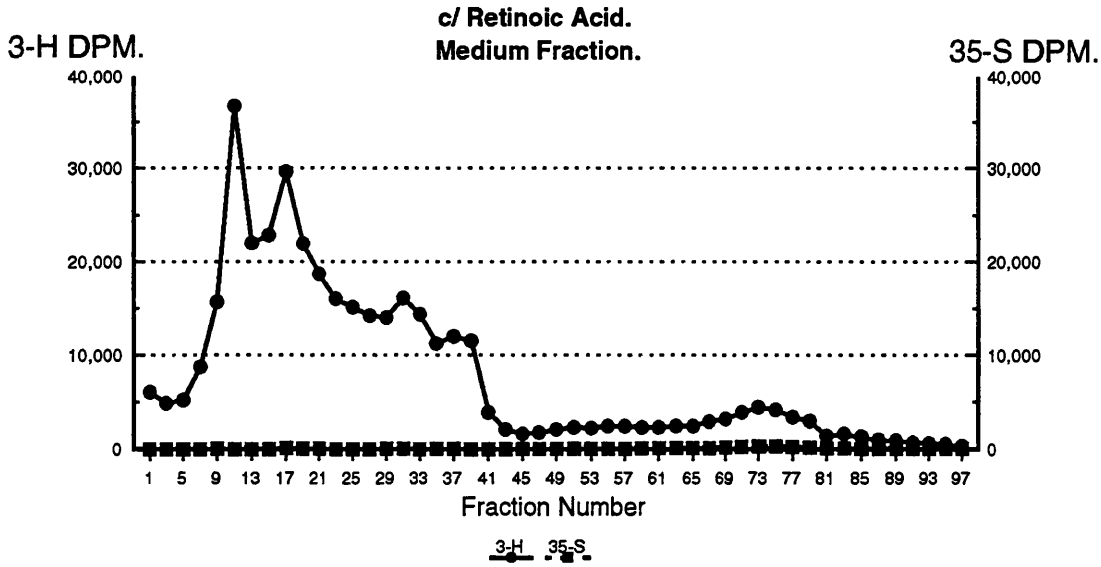


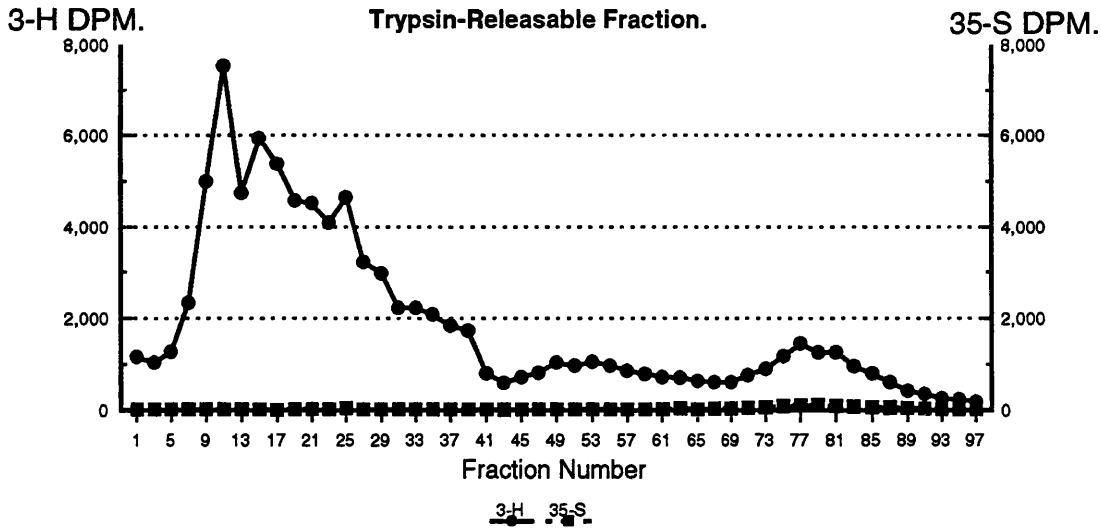


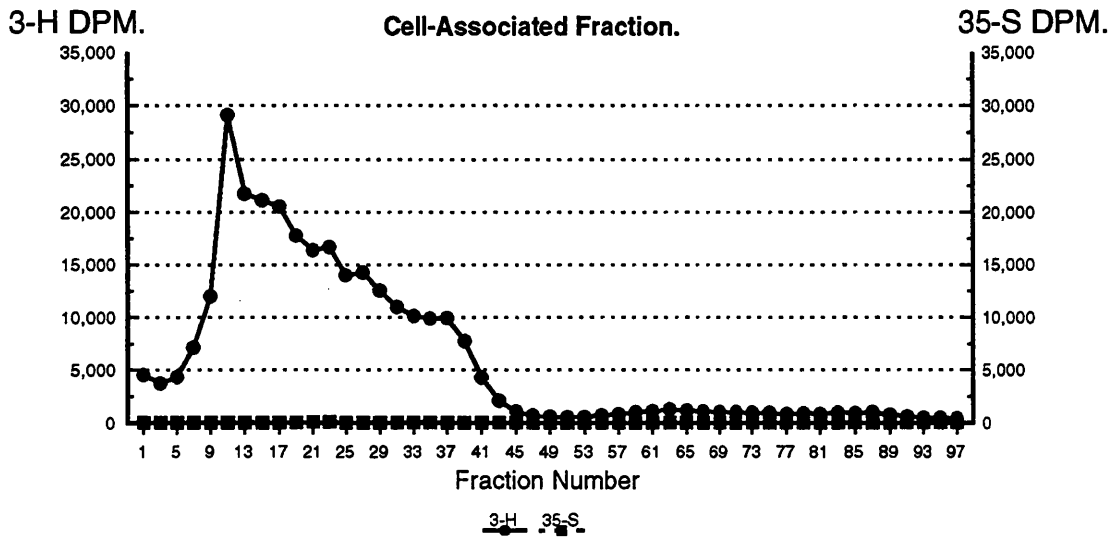

b/Control.
Medium Fraction.
3-H DPM.
35-S DPM.
60,000
50,000
40,000
30,000
20,000
10,000
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89 93 97
Fraction Number
3-H
35-S


Trypsin-Releasable Fraction.
3-H DPM.
35-S DPM.
5,000
4,000
3,000
2,000
1,000
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89 93 97
Fraction Number
3-H
35-S


Cell-Associated Fraction.
3-H DPM.
35-S DPM.
30,000
25,000
20,000
15,000
10,000
5,000
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89 93 97
Fraction Number
3-H
35-S


c/ Retinoic Acid.
Medium Fraction.
3-H DPM.
35-S DPM.
40,000
30,000
20,000
10,000
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89 93 97
Fraction Number
3-H
35-S


Trypsin-Releasable Fraction.
3-H DPM.
35-S DPM.
8,000
6,000
4,000
2,000
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89 93 97
Fraction Number
3-H
35-S


Cell-Associated Fraction.
3-H DPM.
35-S DPM.
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89 93 97
Fraction Number
3-H
35-S

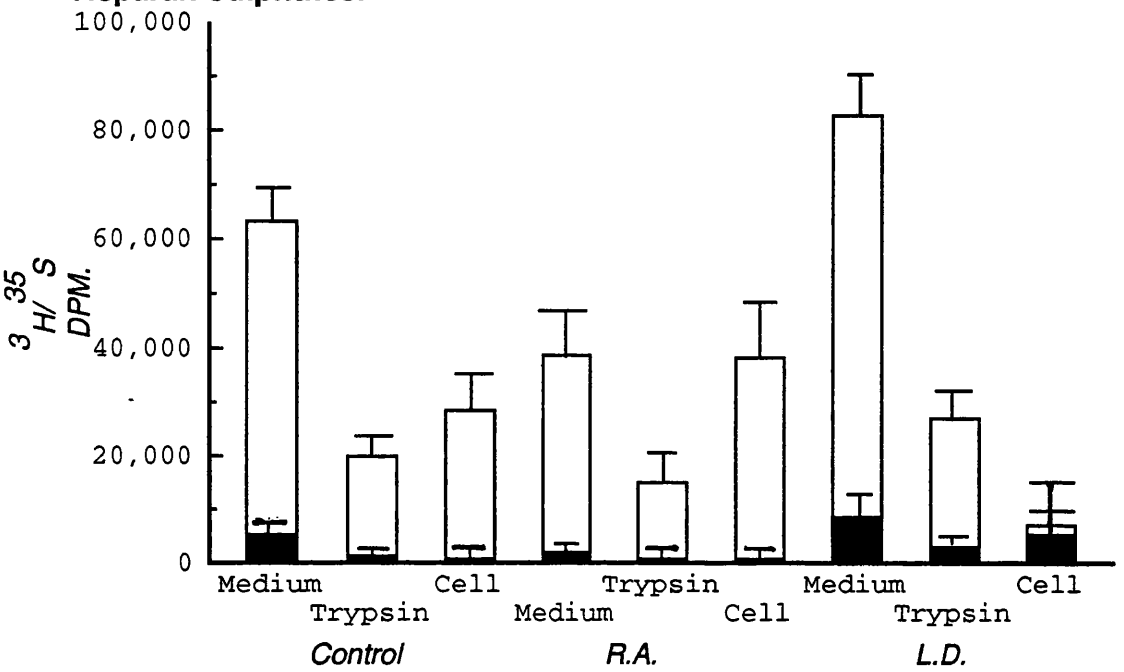
The sulphated GAGs were pooled, and then sequentially digested with chondroitinase ABC and nitrous acid. The distribution of the individual sulphated GAGs produced by subconfluent control, retinoic acid-treated and low-density control cells is shown in Figure 36. Treatment of cells with retinoic acid resulted in a decrease in the incorporation of $^{35}SO_4$ into the heparan sulphates. However, there was no difference in [^{3}H] glucosamine incorporation (for all three control fractions, the incorporation of [^{3}H]glucosamine was approximately 38% of that incorporated into all the GAGs, and this figure was the same after retinoic acid treatment). When low-density cell culture-derived heparan sulphates were examined, overall there was a slight increase in $^{35}SO_4$ incorporation when compared to subconfluent control-derived heparan sulphates but, again, the incorporation of [^{3}H] glucosamine remained the same.

If the individual fractions of each set of results are examined, then a different picture is obtained. In the medium fractions, retinoic acid-treatment reduces the incorporation of both [^{3}H] glucosamine and $^{35}SO_4$ into the heparan sulphates (36% and 61% respectively). This is again the case in the trypsin-releasable fractions, although the percentage reductions are smaller (23% for [^{3}H] glucosamine and 43% for $^{35}SO_4$ incorporation). However, in the cell-associated fractions, there is a marked increase in the incorporation of [^{3}H] glucosamine into the heparan sulphates from retinoic acid-treated cells (35%), and this is accompanied by a small increase in $^{35}SO_4$ incorporation (2.3%). When the low-density cell culture heparan sulphate fractions were examined, it was found that in the medium fractions, there was an increase in [^{3}H] glucosamine and $^{35}SO_4$ incorporation (28% and 40% respectively). A similar result was obtained with the heparan sulphates from the trypsin-releasable fraction. However, on examination of the cell-associated fractions, it was found that the incorporation of [^{3}H] glucosamine was reduced by 81% whereas $^{35}SO_4$ was increased by 66%.

The incorporation of [^{3}H] glucosamine and $^{35}SO_4$ into the chondroitin sulphates was also considered. [^{3}H] glucosamine incorporation was reduced slightly after retinoic acid treatment, but $^{35}SO_4$ incorporation was increased. Chondroitin

Figure 36. Distribution of Sulphated GAGs.

Heparan Sulphates.



Chondroitin Sulphates.

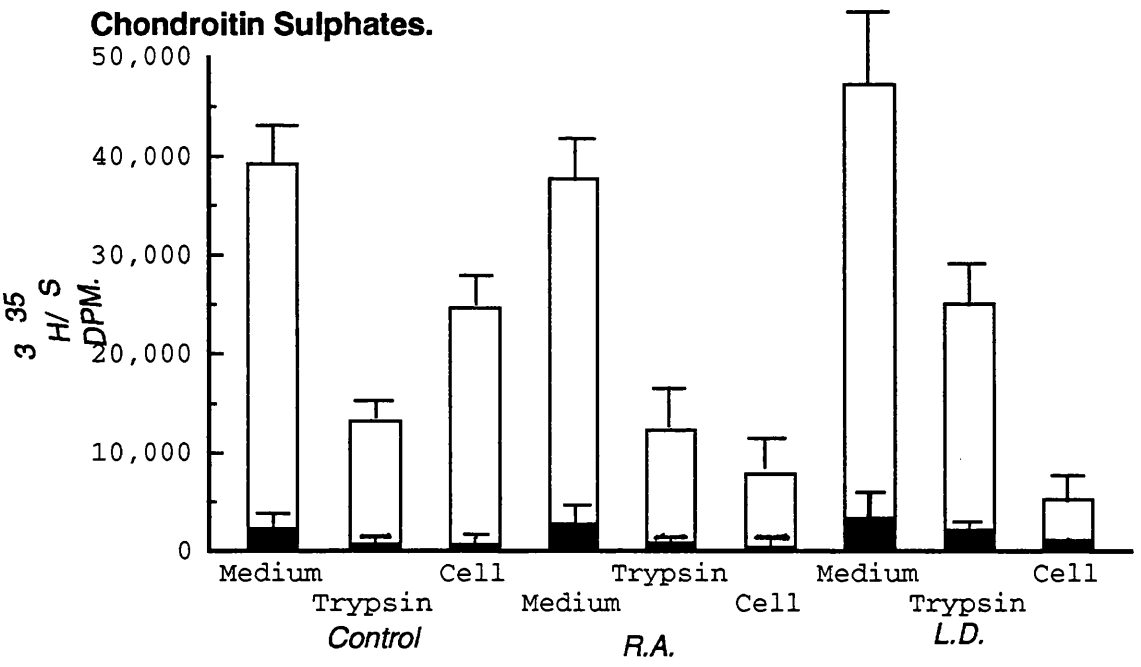


Table 2. 3-H/35-S Ratios

	Heparan Sulphate	Chondroitin Sulphate
Low Density	7.86	10.71
Control	14.95	19.69
Retinoic Acid	26.66	13.59

sulphates from low-density cell cultures exhibited a slight increase in incorporation of both isotopes. On examination of the individual fractions, [^{3}H] glucosamine incorporation into the chondroitin sulphates in the medium fraction of retinoic acid-treated cells was similar to that of control chondroitin sulphates, however there was a slight increase in $^{35}SO_4$ incorporation (15%). There was little or no change in incorporation into the chondroitin sulphates of the trypsin-releasable fraction, but in the cell associated fraction, [^{3}H] glucosamine incorporation was decreased by 69% and $^{35}SO_4$ by 39%.

Examination of the chondroitin sulphates from low-density cells revealed that in the medium fraction, the incorporation of both isotopes was increased - [^{3}H] glucosamine by 16% and $^{35}SO_4$ by 32%. This was also the case for the chondroitin sulphates in the trypsin-releasable fraction. However, in the cell-associated fraction, the incorporation of [^{3}H] glucosamine was decreased by 83% whereas $^{35}SO_4$ incorporation was increased by 37%.

The $^3H/^{35}SO_4$ ratios were also determined and are shown in Table 2. Retinoic acid treatment increases the ^3H:$^{35}SO_4$ ratio in the heparan sulphates but decreases it in the chondroitin sulphates. However, in the low-density cultures, the ratios are reduced for both sets of sulphated GAGs. The changes in ratios indicates a possible change in the degree of sulphation in the molecules and thus the charge densities may also be affected.

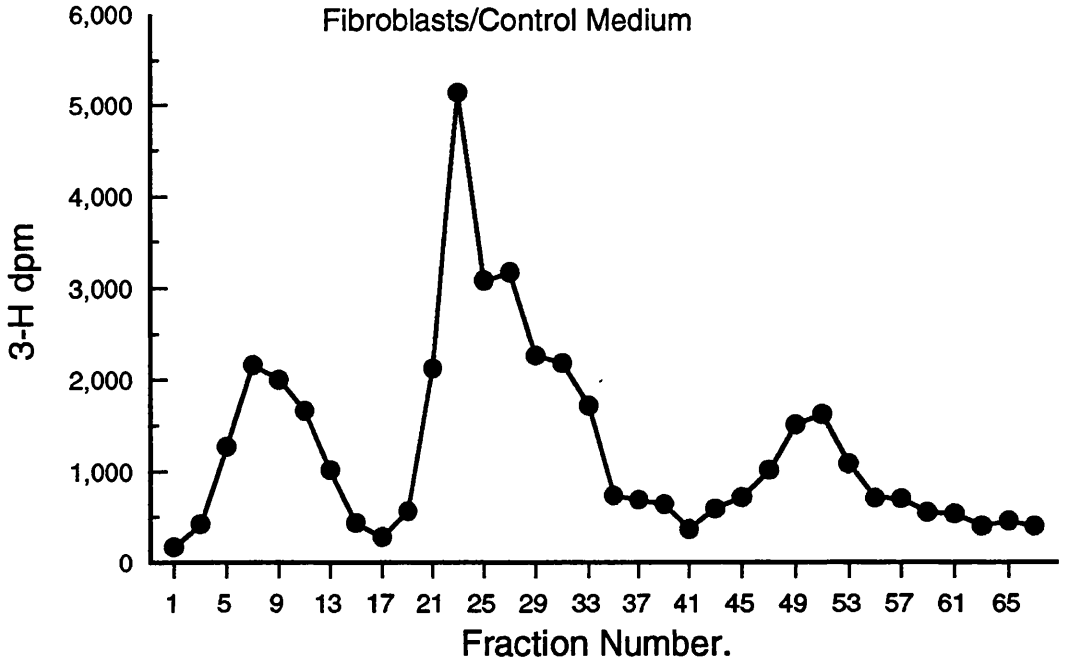
3.Effect of B16F1M2-Conditioned Medium on Fibroblast Glycosaminoglycan Production.

The ability of B16F1M2 melanoma cell-conditioned medium to alter the production of fibroblast glycosaminoglycans was examined. The DEAE ion-exchange profiles are shown in Figure 37. From these profiles it would appear that the tumour-conditioned medium has an effect on the production of the GAGs. The shape of the hyaluronate peak is also altered in as much as the presence of

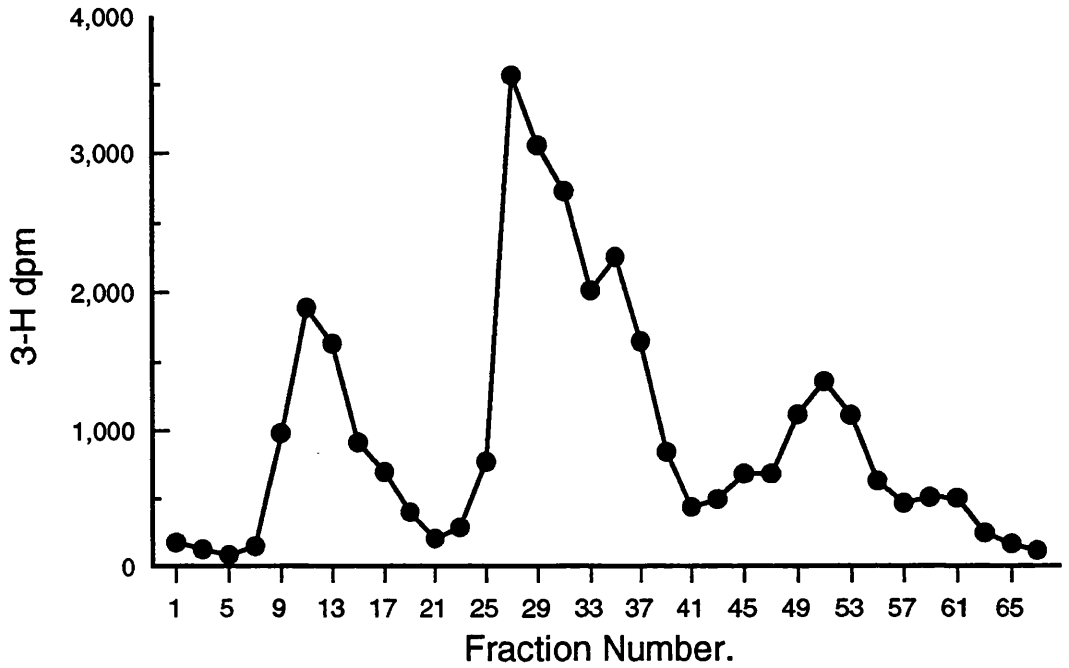
Figure 37. DEAE Anion Exchange Profiles

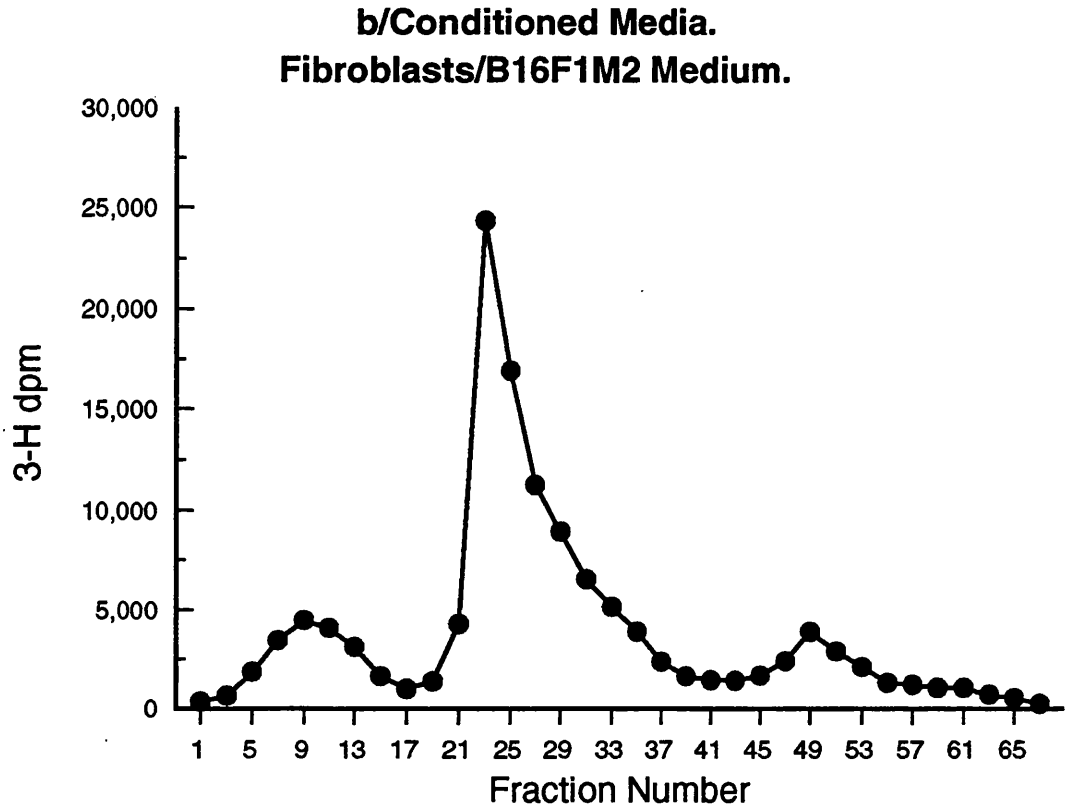
a/Controls

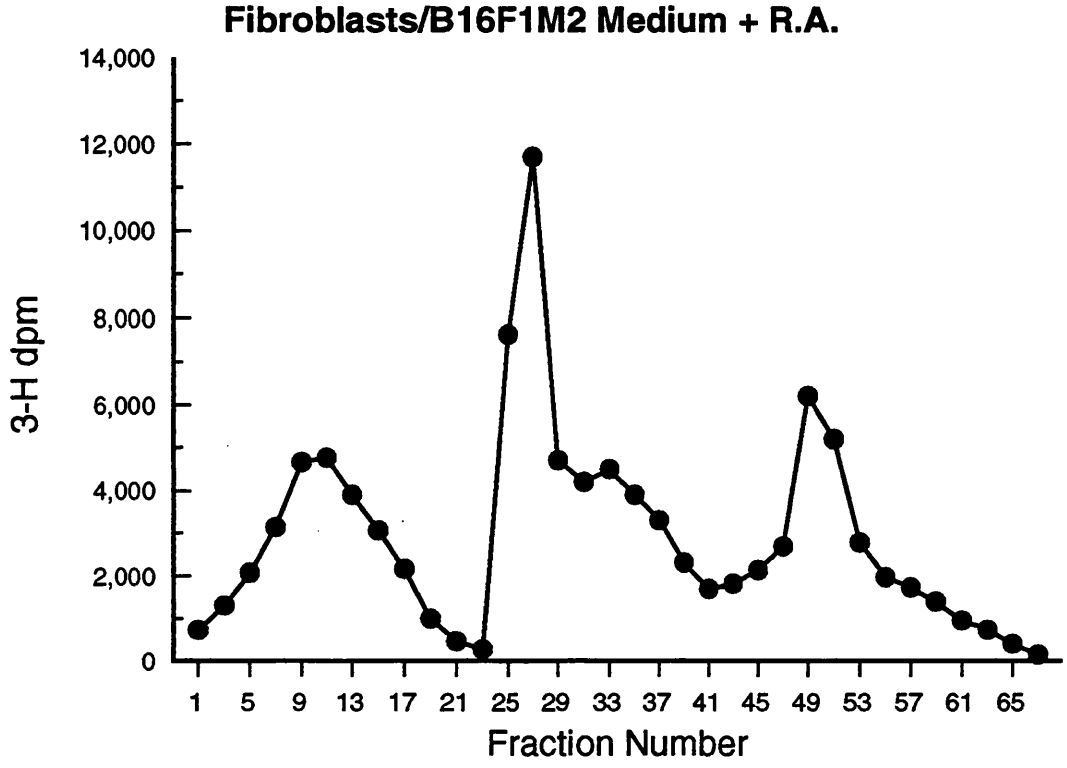
Fibroblasts/Control Medium



Fibroblasts/Medium + Retinoic Acid.




b/Conditioned Media.
Fibroblasts/B16F1M2 Medium.
30,000
25,000
20,000
15,000
10,000
5,000
0
3-H dpm
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65
Fraction Number

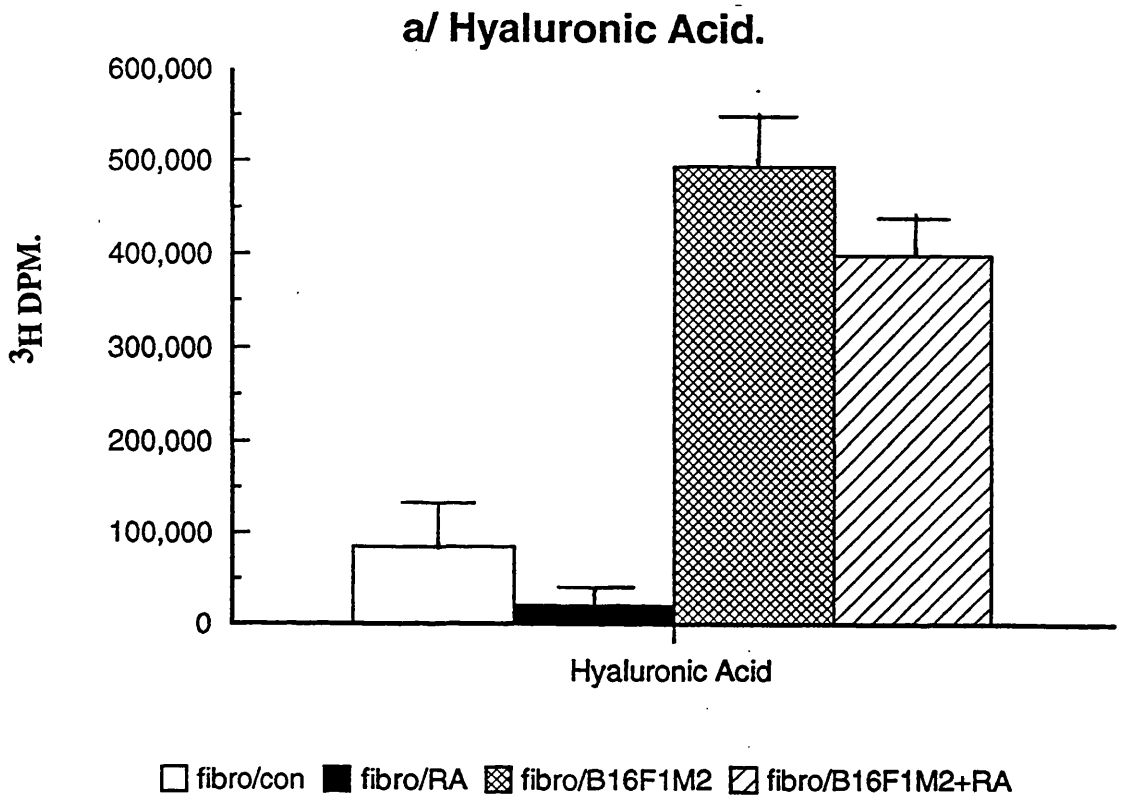

Fibroblasts/B16F1M2 Medium + R.A.
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
3-H dpm
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65
Fraction Number

tumour-conditioned medium removes the shoulder from the peak. If the medium is conditioned in the presence of 10^{-6}M retinoic acid GAG production is also affected but the shape of the hyaluronic acid peak is unaffected.

The distribution of radioactivity between the hyaluronate and the sulphated GAGs was also investigated (fig.38). As can be seen treatment of fibroblasts with tumour-conditioned medium increases the amount of hyaluronate present. Treatment of fibroblasts with melanoma cell-conditioned medium would appear to increase the amount of hyaluronate present by approximately 5 fold. When the sulphated glycosaminoglycans were examined it was found that B16F1M2-conditioned medium reduced the amount of S-GAGs in the cultures by approximately 3 fold. However, in the presence of tumour-conditioned medium prepared in the presence of 10^{-6}M retinoic acid the sulphated glycosaminoglycans were found to be increased by 8 fold. To determine the composition of the sulphated GAG peak, the samples were treated with chondroitinase ABC, and the void volume peak obtained after gel chromatography treated with nitrous acid. The distribution of the heparan sulphates and chondroitin sulphates is shown in Figure 39.

As can be seen B16F1M2 tumour-conditioned medium reduces the amount of both heparan sulphate and chondroitin sulphate in the cultures. Treatment of the fibroblasts with control medium incubated in the presence of retinoic acid also induces reduction in both heparan and chondroitin sulphate. However, if the tumour-conditioned medium is prepared in the presence of retinoic acid there is an increase in the amount of heparan sulphate as compared to both controls - i.e fibroblasts treated with control medium and control medium incubated with 10^{-6}M retinoic acid. In the chondroitin sulphates, medium conditioned in the presence of B16F1M2 cells and retinoic acid caused a slight increase in the amount of chondroitin sulphate when compared to fibroblasts treated with control medium with retinoic acid, but not when compared to cells incubated only with control medium.

Figure 38. Distribution of Fibroblast Glycosaminoglycans.



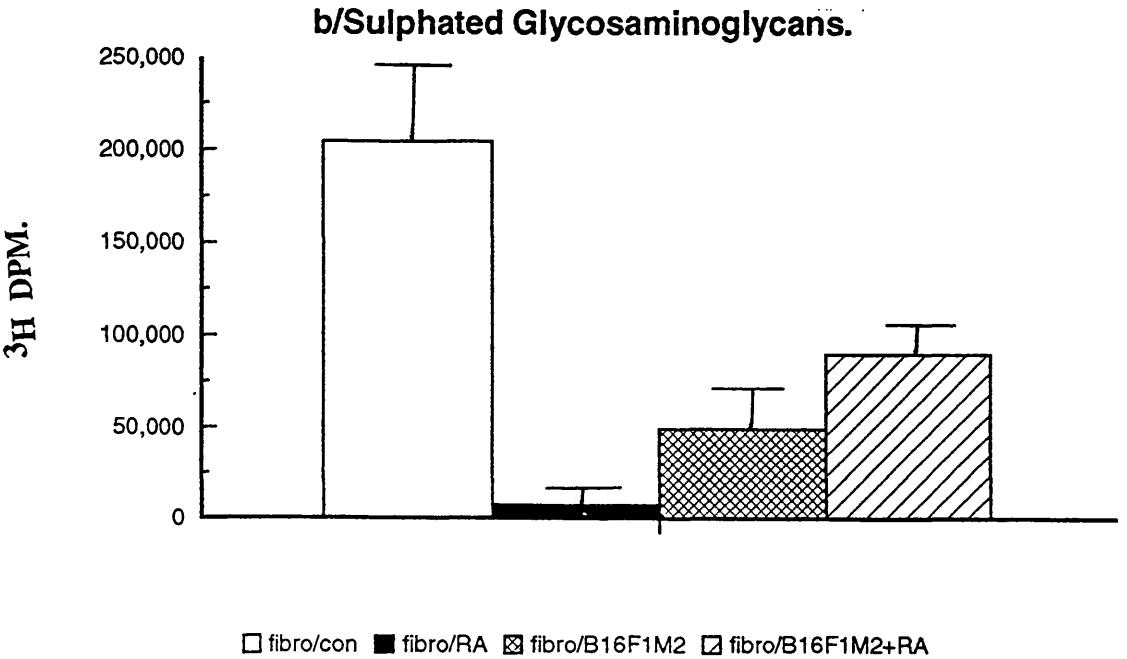
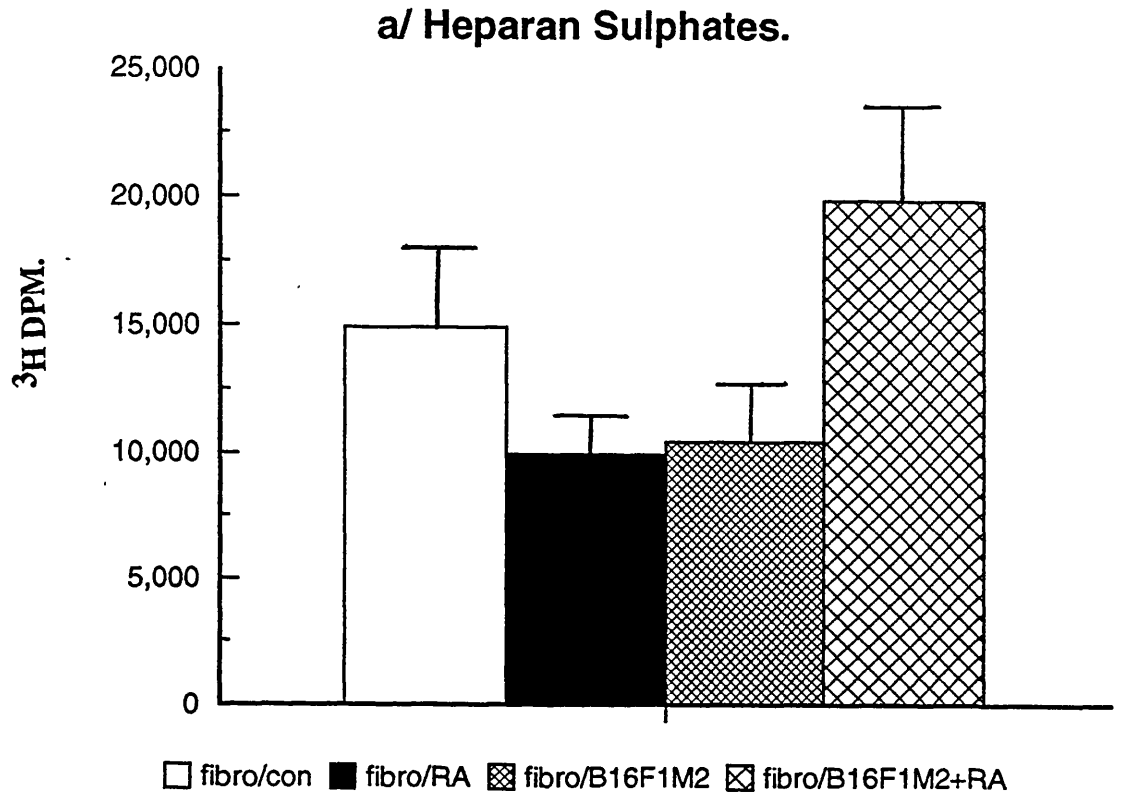
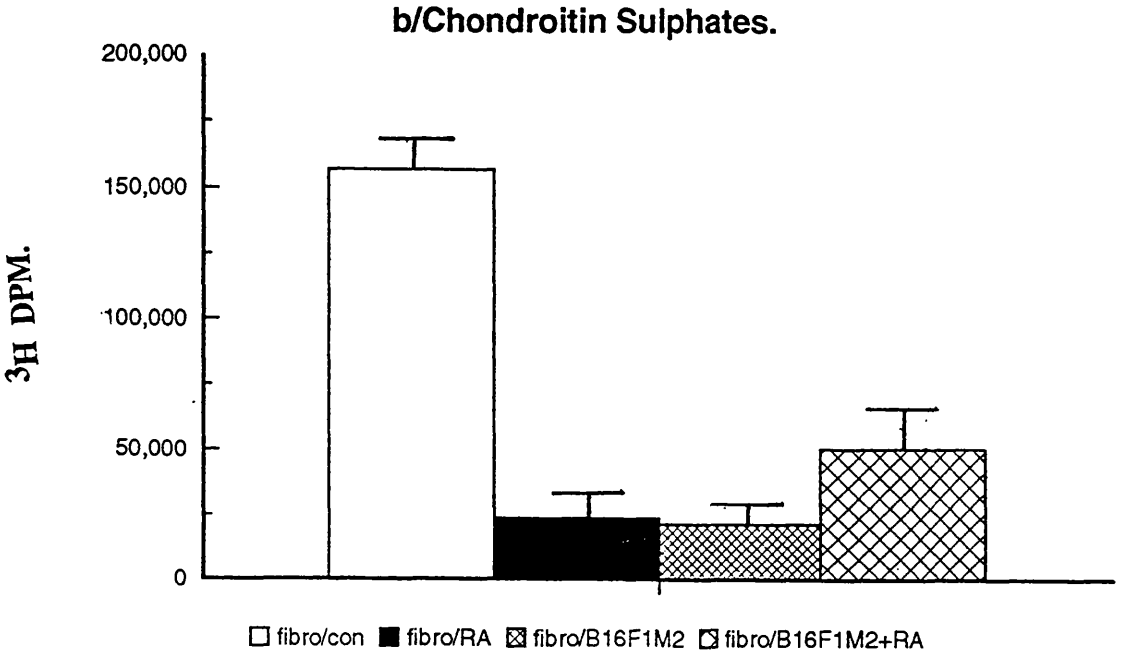


Figure 39. Distribution of Fibroblast Sulphated Glycosaminoglycans.





B16F1M2 cells do not synthesise hyaluronic acid as can be seen from the profiles shown for their glycosaminoglycan analysis. However, hyaluronic acid is thought to be important in the movement of cells in the metastatic process and as such the elicited increase by the B16F1M2 medium factors on fibroblast hyaluronate may be of importance.

DISCUSSION.

The metastatic process involves numerous complex steps, and the fact that retinoic acid has a wide variety of effects on tumour cells in culture would suggest that its inhibitory effects may be accomplished by several mechanisms. Using the murine metastatic melanoma cell line B16F1M2 cultured on plastic, retinoic acid has been shown to have an inhibitory effect on cell growth, with cell morphology also affected - cells pretreated with retinoic acid appear more elongated and their dendritic processes more complex and extensive. Under similar conditions these cells also exhibited an enhanced expression of melanogenesis and when injected into the tail veins of syngeneic mice, retinoic acid pretreatment brought about a decrease in the number of metastatic lung nodules formed.

The inhibitory effect of retinoic acid on cell growth has been shown to be accompanied by an increase in cAMP-dependent protein kinase activity (protein kinase A) (Ludwig et al, 1980). Another possible candidate for a role in the growth inhibitory effects of retinoic acid is protein kinase C, a phospholipid and Ca^{2+}-dependent kinase which phosphorylates its substrate proteins on serine and threonine residues. It can be activated by several growth factors, hormones and neurotransmitters, and is the major receptor for the phorbol ester tumour promotors (Nishizuka, 1984; Nishizuka, 1986; Ashendel, 1984). Protein kinase C also phosphorylates the epidermal growth factor receptor, the insulin receptor and the protein product of the ras oncogene (Niles, 1987), which would suggest a role for this enzyme in the regulation of cell proliferation.

Niles and Loewy (1989) demonstrated that the growth inhibitory effects of retinoic acid on B16 melanoma cells are accompanied by a substantial increase in protein kinase C, and that it would appear that the enhancement was due to an increase in the number of protein kinase C molecules present within the cells. In the promyelocytic cell line HL-60, retinoic acid can induce differentiation which is accompanied by an increase in protein kinase C activity (Durham et al, 1985), and as such the increase is used as a marker of differentiation. However, in the system utilising melanoma cells, it is not clear whether the increase in protein kinase C is due

to differentiation of the cells or to the inhibition of cell proliferation which accompanies the process. In B16 melanoma cells, retinoic acid specifically blocks the progression of the cells through the G_1 phase of the cell cycle and inhibits their growth on soft agar (Ludwig et al, 1980). Melanocyte stimulating hormone (MSH) can also arrest B16 cells in the G_1 phase, however this is not accompanied by an increase in protein kinase C, thus in these cells the increase in protein kinase C is thought to be due to an increase in the number of enzyme molecules in the cell which would suggest that retinoic acid may act at the level of transcription (Niles, 1987). In the study reported here no attempt was made to measure protein kinase C or cAMP levels.

4:1. ADHESION TO BASEMENT MEMBRANE COMPONENTS.

When adhesion of B16F1M2 cells to basement membrane components was examined, it was found that adhesion and spreading of the cells on laminin/nidogen and type IV collagen was reduced by pretreatment of the cells with retinoic acid, while adhesion to fibronectin was virtually unaffected. Adhesion of the cells to intact subendothelial extracellular matrix was also reduced when the cells were pretreated with 10^{-6}M retinoic acid.

The interaction of cells with components of the extracellular matrix has been shown to play a critical role in the regulation of cell morphology, growth, differentiation and in the morphogenetic processes during development (Yamada, 1983). Alterations in cell-matrix interactions have also been implicated in the pathogenesis of cancer (Poste & Fidler, 1980; Liotta, 1986; Nicolson, 1984), as interactions between normal cells and the matrix may be altered in neoplasia, and this may influence tumour proliferation and invasion (Liotta et al, 1983).

General and widespread changes occur in the distribution and quantity of the epithelial basement membrane during the transition from benign to undifferentiated invasive carcinomas (Barsky et al, 1983; Burtin et al, 1982). Benign pathological disorders with epithelial disorganisation or proliferation are usually

characterised by a continuous basement membrane separating the epithelium from the stroma. The basement membrane is also defective around tumour cells in lymph nodes and organ metastases (Burtin et al, 1982).

Both normal and neoplastic cells are capable of binding to laminin, either by specific high affinity cell surface receptors (Terranova et al, 1982; Rao et al, 1983; Rao et al, 1985), or with lower affinity by utilising membrane glycolipids. The sites of biological activity on the laminin molecule have been defined using sequence data for the B chains. Synthetic peptides prepared from these chains showed no activity in themselves, but antibodies against a specific sequence in domain III of the B_1 chain inhibited cell attachment. Additional peptides were prepared from neighbouring sequences and a nonapeptide Cys-Asp-Pro-Gly-Try-Ile-Gly-Ser-Arg (CDPGYIGSR) was found to support cell attachment, to be chemotactic and to interact with a variety of cells. From the nonapeptide the primary sequence of YIGSR has been defined as that responsible for binding (Graf et al, 1987).

Examination of sequences neighbouring YIGSR revealed a second site, Pro-Asp-Ser-Gly-Arg (PDSGR), which can also support cell attachment and it competes with the intact laminin molecule in this respect (Kleinman et al, 1989). The PDSGR sequence resembles the RGDS (Arg-Gly-Asp-Ser) cell attachment sequence found in fibronectin (McCarthy & Furcht, 1984), but shows no competition with fibronectin in either cell attachment or migration (Kleinman et al, 1989). Quantitatively, PDSGR was found to be less active than YIGSR in assays measuring cell adhesion and migration and in reducing experimental metastases in melanoma cells (Kleinman et al, 1989).

A third cell adhesion receptor has been identified on the laminin molecule (Charnois et al, 1988). A sequence designated peptide F-9 has been derived from the inner globular domain of the lateral short arm and has been demonstrated to have specific binding for heparin (Charnois et al, 1988). Although free heparin may not exist as a basement membrane component, there are many heparin-like domains present in the vicinity of laminin. These include the glycosaminoglycan chains of basement membrane proteoglycans (Fujiwara et al, 1984; Kleinman et al, 1982; Stow

& Farquhar, 1987), and of cell surface-associated proteoglycans of various cell types associated with basement membranes (Gallagher et al, 1986; Lindahl & Hook, 1978; Stow & Farquhar, 1987). Recent work has shown that an endothelial cell-derived heparan sulphate proteoglycan may contain heparin-like stretches in regions of its side chains (Nader et al, 1987). Thus, heparin-binding sites on laminin may be important not only in the structural arrangement of the basement membrane, but also in the regulation of cell adhesion to laminin.

The positioning of this region on the laminin molecule is relatively close to the YIGSR cell-binding site (Graf et al, 1987), and may also be close to the binding sites for nidogen (Paulsson et al, 1987) and type IV collagen (Terranova et al, 1983). This close arrangement of interactive sites for basement membrane molecules and cell surface components may be important in the structural diversity of basement membranes (Charnois et al, 1986b).

Pretreatment with retinoic acid also had an effect on the adhesion of the melanoma cells to type IV collagen. Although it is generally thought that epithelial cells do not attach directly to type IV collagen but utilise laminin as an attachment factor, a native type IV collagen binding protein, colligin, has been isolated from the cell membranes of a variety of cell lines (Kurkinen et al, 1984). It has been identified as a glycoprotein which may either be involved in the assembly of type IV collagen into a network, or be an intercellular component involved in the biosynthetic pathway that reaches the cell surface as a result of fusion of secretory vesicles from the Golgi with the cell membrane. Some cells do attach readily and directly to type IV collagen, for example hepatocytes (Rubin et al, 1981) and metastatic melanoma cells (Dennis et al, 1982). Asparagine linked oligosaccharides on the cell surface have been implicated in this attachment (Dennis et al, 1984).

In this study it was found that the B16F1M2 cells adhered to type IV collagen, and that adhesion was reduced by pretreatment with retinoic acid. In cell systems in which laminin is known to mediate attachment to type IV collagen, factors that alter the expression or affinity of laminin receptors would be expected to affect cellular adhesion to both laminin and type IV collagen. This was found not to be the

case, as the B16 melanoma cells adhered readily to type IV collagen when laminin was not present which would suggest a direct interaction with type IV collagen, probably due to specific receptors. Pretreatment of type IV collagen-coated dishes with laminin/nidogen did increase both the rate of cell adhesion and the number of adherent cells, suggesting a possible synergistic effect.

A study using the highly metastatic murine melanoma cell line K1735 M4 indicated an active role for type IV collagen in promoting the adhesion, spreading and migration of malignant melanoma cells (Chelberg et al, 1989), thus supporting the findings reported here. They also demonstrated the involvement of multiple nonoverlapping domains of type IV collagen in promoting tumour cell adhesion, with de novo protein synthesis required for cell adhesion to the major noncollagenous domain of the molecule, while adhesion to the helical domain was less dependent on de novo protein synthesis. When they examined the RGD-related peptides, they found that the peptide GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) had virtually no effect on melanoma cell adhesion to the substrate, but in contrast to this, when threonine was substituted for serine (GRGDTP), cell adhesion to type IV collagen was significantly reduced (Chelberg et al, 1989).

The effect of retinoic acid on the adhesion of B16F1M2 cells to fibronectin was also investigated, and it was found that retinoic acid pretreatment had little or no effect on cell attachment, spreading and migration of a variety of cell types including tumour cells (Ruoslahti, 1988b; Ruoslahti & Pierschbacker, 1987). Fibronectin is known to have two distinct cell binding domains - one located in the centre of the molecule and the other in the type III connecting segment (IIICS) (Yamada, 1988). The central binding domain is recognised by a variety of cell types including fibroblasts, endothelial cells, myoblasts and tumour cells (Yamada, 1988). To date only cells derived from the embryonic neural crest - neural crest precursor cells, peripheral neurones, corneal fibroblasts and melanoma cells have been shown to recognise the IIICS binding domain (Humphries et al, 1989). The cell type specificity

of this domain suggests that it may be involved in adhesive events during development and differentiation of the neural crest and its derivatives (Humphries et al, 1988).

The IIICS domain is located between the COOH-terminal heparin and fibrin binding domains, and its active sites have been identified using synthetic peptides. Two peptides, CS1 and CS5, have been found to promote melanoma cell adhesion directly, and to inhibit the adhesion of these cells to intact fibronectin (Humphries et al, 1986; Humphries et al, 1987). The active site within the CS5 peptide has been identified as Arg-Glu-Asp-Val (REDV), a sequence which resembles the RGDS sequence of the central binding domain. Synthetic REDV was found to be inhibitory to melanoma cell adhesion to fibronectin, but had no effect on fibroblast adhesion (Humphries et al, 1986).

In the central binding domain of the molecule, one crucial attachment site has been localised to a specific tetrapeptide sequence Arg-Gly-Asp-Ser (RGDS) (Pierschbacker & Ruoslahti, 1984; Yamada, 1988). This sequence, on its own, can promote fibroblast adhesion and it can block the interaction of fibroblasts with intact fibronectin (Yamada & Kennedy, 1984). RGDS containing peptides can competitively inhibit the fibronectin receptor and other matrix-receptor interactions (Ruoslahti & Pierschbacker, 1986; Pytela et al, 1985; Yamada et al, 1985;).

The attachment of cells to fibronectin is, in part, mediated by the interaction of the RGDS sequence with a specific cell surface fibronectin receptor which is a member of the integrin family (Ruoslahti, 1988b). The integrins are a family of membrane glycoproteins which consist of two subunits, α and β . Both subunits have a large extracellular domain, a transmembrane domain, and a short cytoplasmic domain (Buck & Horwitz, 1987; Hynes, 1987). The subunits are noncovalently bound to one another by an association promoted by divalent cations (Ginsberg et al, 1988). The integrin responsible for binding to the RGDS sequence of fibronectin has been identified as integrin $\alpha_5\beta_1$, and it is commonly expressed by cultured cell lines (Ruoslahti, 1988). However, as previously outlined, for melanoma cells adhesion to fibronectin also occurs via a binding domain found in the IIICS

region of the molecule. Recent work using the human metastatic melanoma cell line A375-M has identified an integrin receptor for this region - integrin $\alpha_4\beta_1$ (VLA-4) (Mould et al, 1990). However in these human melanoma cells, adhesion to fibronectin occurs via both binding domains. This is in contrast to the findings using B16 murine melanoma cells where attachment to fibronectin occurs almost exclusively via the IIICS binding domain (Humphries et al, 1989).

Cell adhesion to laminin and type IV collagen can also be mediated by integrins (Ramos et al, 1990; Kramer et al, 1989). Using the B16 mouse melanoma system Ramos and co-workers (Ramos et al, 1990) identified integrin-like complexes specific for laminin and type IV collagen. Neither receptor could be eluted with RGD peptides which would indicate that unlike, for example, the fibronectin receptor, the adhesion of these receptors to their substrates is not mediated through this sequence. Additionally YIGSR peptides were not effective at eluting the laminin-binding complex, which would suggest that the integrin receptor binds to a different site on the laminin molecule. Antibody work revealed that the laminin integrin receptor is composed of an α_6 and a β_1 subunit and is similar to the human laminin-binding integrin complex VLA-6 (Kramer et al, 1989b). A VLA-6 monoclonal antibody can substantially inhibit B16 adhesion to laminin, but inhibition is not always complete which would imply that other receptors are important in adhesion to laminin. In addition to the laminin-integrin complex and the receptor which recognises the YIGSR peptide, some work has indicated that B16 cells may express a surface galactosyltransferase capable of binding laminin oligosaccharides and promoting cell spreading, but not essential for the initial attachment of the cells (Runyan et al, 1988).

The type IV collagen receptor has been shown to be similar to the VLA-1 receptor complex found in humans (Kramer & Marks, 1989) and has been identified as integrin $\alpha_1\beta_1$. Additional activity has also been demonstrated for integrin $\alpha_2\beta_1$ (VLA-2) in the human system (Kramer & Marks, 1989). The collagen binding sequence could not be eluted with RGDS-containing peptides which would suggest that other sequences are involved in the interaction (Ramos et al, 1990). The collagen-binding complex has been shown to bind preferentially to type IV collagen

and only weakly to interstitial collagens suggesting a substrate specificity (Ramos et al, 1990). This collagen receptor has also been shown to bind to laminin immobilised on Sepharose columns, thus it has some affinity for the molecule, although it is thought that the collagen receptor is unable to initiate adhesion to laminin for which the presence of the $\alpha_6\beta_1$ receptor is required (Ramos et al, 1990).

Working with three pairs of normal and transformed cells Plantefaber and Hynes (1989) have shown that transformed cells express different levels of β_1 integrins from their normal counterparts. Transformed cells were observed to express reduced levels of $\alpha_5\beta_1$ fibronectin receptor, but higher levels of the $\alpha_3\beta_1$ integrin which can also mediate adhesion to fibronectin (Takada et al, 1988; Gehlsen et al, 1989). Unlike $\alpha_5\beta_1$, the $\alpha_3\beta_1$ integrin does not bind to intact fibronectin or its cell-adhesive fragment at physiological salt concentrations (Takada et al, 1988; Plantefaber & Hynes, 1989), but it does bind to fibronectin in radioreceptor assays (Gehlsen et al, 1989). Thus $\alpha_3\beta_1$ may have a lower affinity for fibronectin than $\alpha_5\beta_1$ and thus may preclude its participation in matrix assembly without affecting its activity as an adhesion receptor.

Functional studies suggest that integrins are important in the metastatic process. Synthetic peptides containing the RGD sequence can inhibit the movement of human melanoma cells through an amniotic basement membrane (Gehlsen et al, 1988). These peptides are also able to reduce the number of lung colonies formed in mice after injection of B16F10 murine metastatic melanoma cells (Humphries et al, 1986; Saiki et al, 1988; Humphries et al, 1988). Consistent with this is the observation that antibodies against the β subfamily of integrins prevent the establishment of tumours when human melanoma cells are implanted into nude mice (Boukerche et al, 1989).

The deposition of matrix components other than fibronectin is also disrupted in malignant cells (Ruoslahti & Pierschbacher, 1987). As integrins can also serve as receptors for collagen and laminin (Takada et al, 1988; Gehlsen et al, 1989), it is possible that changes in integrin expression may, in part, be responsible for the defective matrix formation. Consistent with this suggestion is the observation that

integrins other than $\alpha_5\beta_1$ are also expressed at reduced levels after transformation (Plantefaber & Hynes, 1989). These changes need not affect the ability of cells to attach to collagen or laminin, as the $\alpha_3\beta_1$ integrin, which can serve as a collagen and laminin receptor, has been shown to increase in transformed cells (Takada et al, 1988; Gehlsen et al, 1989).

Modulation of the binding affinities of the integrins for their ligands may also play a part in defective matrix deposition by transformed cells. The affinity of integrins for their ligands can be modulated experimentally, by replacing Ca^{2+} and Mg^{2+} cations with Mn^{2+}. This greatly enhances the affinity of several ligands including that of the fibronectin receptor (Ruoslahti & Pierschbacher, 1987). Also, the fact that the $\alpha_2\beta_1$ integrin is a laminin and collagen receptor in some cells (Languino et al, 1989), whereas in other cells it is only a collagen receptor (Takada et al, 1988; Santoro et al, 1989) would indicate that integrin affinities are subject to regulation.

The results presented here indicate that although retinoic acid has an effect on cell adhesion, the process is not totally inhibited. As shown, retinoic acid pretreatment has no significant effect on B16F1M2 adhesion to fibronectin. This would suggest that only some of the mechanisms of adhesion are subject to alteration by retinoic acid treatment. Unlike other cell lines, the main binding domain for melanoma cells to fibronectin is located in the IIICS region of the molecule. It may be that retinoids act, in part, by altering the expression of integrin receptors for the main binding domain situated in the central region of the molecule. This could also account, in part, for the inhibition of adhesion to type IV collagen and laminin. The ability of retinoids to influence the adhesion of cells to basement membrane components is not surprising when the wide variety of effects these molecules can ellicit are considered. How exactly the retinoids induce their effects is, as yet, unknown. The ability of tumour cells to attach to laminin has been shown to affect a number of cellular processes including differentiation, invasion and metastasis (Liotta et al, 1986b; Barsky et al, 1984). The interaction of laminin with tumour cells is mediated by laminin cell surface receptors, and studies have shown that more laminin is associated with cells of a high metastatic potential than with those of lower

metastatic potential, which would suggest a correlation between the number of cell surface laminin receptors and metastatic potential (Varani et al, 1983; Albini et al, 1989).

In this study, the adhesion of B16F1M2 melanoma cells to laminin/nidogen complex was inhibited by pretreatment with retinoic acid. As one of the possible effects of retinoic acid is to induce cell differentiation as demonstrated in HL-60 promyelocytic cells (Durham et al, 1985), it is possible that the observed reduction in cell adhesion may be related to a lowering of the metastatic potential and as such, possibly making the cells more "normal". In partial support of this is the fact that the study also demonstrated that on laminin/nidogen, the cells exhibited a morphology more consistent with normal melanocytes ie. the cells spread out more and had an increased number of dendritic processes. Also, retinoic acid pretreatment of the cells reduced the number of lung metastases formed after tail vein injection into mice and, in vitro, retinoic acid modulates melanogenesis. Melanogenesis is a marker of differentiation and enhancement of its expression would suggest a move towards a more "normal" cell type. However, in contrast to this, there have been reports that in a variety of melanoma cells of human origin, retinoic acid can increase the number of cells that express the laminin receptor (Hendrix et al, 1990). This would suggest that the role of the laminin receptor does not correlate with cell attachment and invasion in the human system.

Work on the effect of retinoic acid on fibroblasts demonstrated an increase in cell adhesion to laminin following retinoic acid pretreatment, with the suggestion that the effect was due to an increase in the number of laminin receptors (Kato & DeLuca, 1987). It is possible that this is further evidence of the bifunctional activity of retinoic acid. However, another study on cells derived from a murine adenocarcinoma demonstrated no correlation between the ability of cells to invade a reconstituted basement membrane and the presence of laminin and laminin-binding proteins (Penno et al, 1989), with another cell surface glycoprotein implicated in invasion.

The adhesion of B16F1M2 cells to basement membrane components laminin/nidogen and type IV collagen were, in this study, considered important in the process of metastasis. The reduction in this adhesion elicited by retinoic acid was thought to be associated with the observed reduction in lung colony formation by the cells. However, the evidence on the role of laminin in the process of invasion must also be considered. No attempt was made to measure the expression of laminin receptors by the cells nor was the effect of retinoic acid on the expression quantitated. It is possible that inhibition of attachment was achieved by modulation of some binding mechanisms other than the integrins.

The finding that adhesion of retinoic acid pretreated cells to laminin/nidogen and type IV collagen had not reached a maximum after an incubation period of 90 min would suggest that adhesion required the synthesis of new cell surface molecules and that retinoic acid may inhibit this synthesis. This would again suggest that retinoic acid may act at the level of transcription. Various cell surface molecules have been identified as possible receptors for basement membrane components, and there have been reports of retinoids exerting effects on some of these. One such family of molecules are the cell surface proteoglycans.

Several extracellular matrix molecules and growth factor peptides have been demonstrated to bind to heparin (Ofosu et al, 1989). Although heparin is found extracellularly when mast cells and basophils degranulate, the binding, in vitro, is thought to represent the in vivo interactions of the heparin-like glycosaminoglycan heparan sulphate (Bernfield & Sanderson, 1990). Heparan sulphate proteoglycan is a ubiquitous component of the surfaces of all adherent vertebrate cells. It predominantly contains a core protein which spans the cell membrane, however, peripheral cell surface components exist as do heparan sulphate proteoglycans that can be released from the cell surface by phosphatidyl inositol-specific phospholipase C, for example glypicans (Carey & Evans, 1989). One of the most widely studied cell surface proteoglycans is syndecan, which contains both heparan sulphate and chondroitin sulphate chains (Rapraeger et al, 1985). It binds to several extracellular matrix

components via its heparan sulphate chains - it binds with high affinity and specificity to the interstitial collagens (Koda et al, 1985), to fibronectin (Saunders & Bernfield, 1988) and to thrombospondin (Sun et al, 1989).

Although syndecan can bind cells to a variety of components of the extracellular matrix, there is insufficient evidence to categorise it as a physiologically relevant matrix receptor. However, syndecan does show several biological properties that are consistent with its possible role as a matrix receptor, with most of this evidence coming from work carried out on cultured mammary epithelial (NMUMG) cells. Before these cells reach confluence, they are surrounded by syndecan and its ectodomain is shed as a non-lipophilic proteoglycan into the culture medium (Jalkanen et al, 1987). Once the cells reach confluence, the shedding of syndecan decreases rapidly and it becomes localised at the basolateral cell surfaces. In vivo, simple epithelial cells also show this localisation of syndecan at their basolateral surfaces (Hayashi et al, 1987). This localisation would be an appropriate site for a matrix receptor. Studies on a variety of cell types have indicated that the level of cell surface syndecan correlates with cell type (Hinkes et al, 1988), but at a molecular level the variation in expression is not always accompanied by a concurrent variation in the amount of syndecan mRNA, which would suggest there is substantial post transcriptional regulation of syndecan expression (Bernfield & Sanderson, 1990).

Retinoids have been shown to induce changes in cell surface glycoconjugates which include glycosaminoglycans such as heparan sulphate (Lotan et al, 1983). Indeed, changes in the synthesis and charge density of B16 glycosaminoglycans after pretreatment with retinoic acid have been reported (Edward & MacKie, 1989). This evidence would suggest that these macromolecules may possibly have a role in the retinoic acid-induced changes in cell adhesion.

When B16F1M2 cells were pretreated with retinoic acid, their adhesion to intact subendothelial extracellular matrix was also reduced. Presumably the mechanisms involved in the inhibition were similar to those involved for the individual components. No analysis of the matrix was carried out, but it was assumed

to be composed mainly of laminin/nidogen and collagen, with the amount of type IV collagen present being increased by feeding the endothelial cells with ascorbate prior to isolation of the substratum.

4:2. CELL-CELL ADHESION: HOMOTYPIC AND HETEROTYPIC.

The inhibition of cell-cell adhesion, both homotypic and heterotypic, was also observed after B16F1M2 cells were pretreated with retinoic acid. In metastasis, both types of adhesion are thought to be important in determining the success of the process at several steps (Nicolson, 1982; Nicolson & Poste, 1983). Studies with homotypically aggregated B16F1 cells have shown that the frequency of lung colonies per cell number, after intravenous injection of tumour cells, was significantly higher than with a single cell suspension inoculum of B16F1 cells (Fidler, 1973b). Also, several B16 sublines have been selected, both in vivo and in vitro, for various properties and they show correlations between homotypic or heterotypic adhesiveness and experimental metastatic potential (Lotan & Raz, 1983; Nicolson & Winkelhake, 1975). The selection of a subline of B16F1 with a reduced homotypic aggregation resulted in a lower lung colonizing potential than that observed with the parental line (Lotan & Raz, 1983).

Of importance in cell-cell interactions are the cell adhesion molecules (CAMs). Down regulation of N-CAM in migrating cells led to the suggestion that CAM expression might be altered in transformed cells (Brackenbury et al, 1984). In order to test this hypothesis, neural retinal cells were transformed with temperature-sensitive mutants of Rous sarcoma virus. These cells normally express N-CAM and aggregate by an N-CAM-dependent mechanism. After transformation, the cells behaved in this way at the non-permissive temperature for the virus, however, at the permissive temperature, the cells failed to aggregate to the same extent, and decreased amounts of N-CAM RNA and cell surface N-CAM were observed, with the change being reversible on return to the non-permissive

temperature. From this it was suggested that CAM expression might be inversely correlated with the transformed state of certain cells and possibly with their metastatic potential (Brackenbury et al, 1984).

Similarly, work on MDCK cells transformed with either Harvey or Moloney sarcoma virus has demonstrated that the calcium-dependent CAM uvomorulin (E-cadherin) can be affected by transformation (Beherens et al, 1989). MDCK is an epithelial cell which normally expresses uvomorulin at high levels. Transformed cells were selected on the basis that they either retained both their epithelial character and high levels of surface uvomorulin, or that they had lost both of these characteristics. The ability of the cells to invade collagen gels was examined, and this was found to correlate with the expression of uvomorulin ie. cells which expressed uvomorulin were non-invasive (Behrens et al, 1989).

The loss of CAM expression in cell lines can be correlated with decreased aggregation, increased motility and increased invasiveness. However, this may not always be the case as observations have indicated that different CAMs are expressed differently in a variety of tumours (Behrens et al, 1989).

As retinoids are thought to act at the level of transcription, mediation of the observed effects on cell-cell adhesion may, in part, be due to altered expression of CAMs. Loss of aggregation in the spheroid model is thought to be an indication of a decrease in the potential of a tumour to invade, and this may correlate with a decrease in the expression of some CAMs, or an increase in the expression of others such as uvomorulin.

One of the factors that can influence aggregation is the presence of serum in the culture system. When the ability of B16F1M2 cells to adhere to monolayers of the same cells was examined, both cell populations were washed extensively to remove as much of the residual serum as possible before initiation of the experiment. However, when cell-cell aggregation was examined by culturing the cells on soft agar, the cells were continually in the presence of serum. In both cases, retinoic acid had an inhibitory effect on cell-cell adhesion, although this could not be compared quantitatively as the effect on cell-cell aggregation was not measured only observed.

There have been reports that the presence of serum can stabilise cell aggregates, with the effect being greatest in sublines of B16 melanoma which have the highest proteolytic activities, suggesting that serum proteinase inhibitors may act in the process (Updyke & Nicolson, 1986).

The ability of tumour cells to form cell-cell aggregates is important in the metastatic process for a number of reasons. Firstly, the formation of aggregates may give the cells some protection against the defence mechanisms of the host. Cells at the centre of aggregates may be protected from the action of, for example, NK cells or from the actions of various antitumour substances such as retinoic acid. In this study, the effects of retinoic acid were only examined in the context of aggregate formation and not on preformed aggregates. Thus any protective effects aggregation may have against retinoic acid treatment were not determined in the course of the study.

Aggregation is just one of the methods by which tumour cells protect themselves in the circulation. One of the other important factors in this survival is the mechanical properties of the cells themselves. Tumour cells which survive in the microvasculature must be resistant to the shear stresses arising in the vascular bed (Brooks, 1984), the frictional forces arising between their peripheries and the vessel walls (Weiss & Dimitrov, 1984), and be able to traverse capillaries which generally are rigid and smaller in diameter than the tumour cells (Sato & Suzuki, 1976; Weiss & Dimitrov, 1984). Depending on the type of tumour, the deformability of tumour cells may play an important role in their ability to form neoplastic foci. Using the B16 mouse melanoma, work has been undertaken to determine if there is a correlation between deformability and metastatic potential (Ochalek et al, 1988). Cell deformability was measured as the percentage of cells crossing a 10μm Nucleopore filter membrane at a constant pressure as a function of time. They found that cells with the lowest metastatic potential required the most time to cross the membrane, while those with the highest metastatic rate required the least time (Ochalek et al, 1988). Physiochemical factors, such as changes in the cytoskeleton, may play an important role in metastasis.

The heterotypic adhesion of tumour cells to host cells, such as those of the endothelium, is also important in metastasis. Adhesion of circulating tumour cells to specific organ microvessel endothelial cells is probably one of the most important events in determining organ-specific metastasis (Alby & Auerbach, 1986; Auerbach et al, 1987; Nicolson, 1988). An example of this is the finding that brain-colonizing B16 melanoma cells will adhere to brain-derived endothelial cells at a higher rate than lung colonizing B16 cells (Tohgo et al, 1986). Also, it has been demonstrated that the adhesive interactions of malignant cells with organ-derived microvessel, but not large vessel, endothelial cells correlated with their origin, metastatic properties and the preferential organ of metastasis (Alby & Auerbach, 1986; Auerbach et al, 1987).

In the system reported here, the endothelial cells were derived from bovine pulmonary artery. However, the purpose of the study was not to look at organ specificity, but to look at cell-cell interactions of these cells and B16F1M2 melanoma cells, and the effect of retinoic acid on these interactions. In addition, it may be that adhesion of malignant cells to endothelium is only important in as much as it is the initial step in the attachment of the metastasising cells to the underlying extracellular matrix.

Heterotypic cell-cell adhesion may be mediated, in part, through cell adhesion molecules. Monoclonal antibodies have been used to identify and characterise three cytokine-inducible endothelial cell adhesion molecules - intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1), and vascular cell adhesion molecule 1 (VCAM-1) (Bevilacqua et al, 1989; Rothlein et al, 1986; Osborn et al, 1989). As well as playing an important role in leukocyte-vascular endothelial cell adhesion (Bevilacqua et al, 1989; Osborn et al, 1989; Dustin & Springer, 1988), recent studies have demonstrated that activation of large-vessel endothelial cells by cytokines can alter the adhesion of human melanoma cells and carcinoma cells in vitro (Dejana et al, 1988; Rice et al, 1988; Rice & Bevilacqua, 1989). The expression of ELAM-1 and a protein which is closely related

to VCAM-1, inducible cell adhesion molecule 110 (INCAM-110) on large-vessel endothelium appears to correlate with the ability of specific tumour cells to adhere to human umbilical vein endothelial cells (Rice & Bevilacqua, 1989).

Another study examined the mechanisms by which human melanoma cells attached to microvascular endothelial cells derived from human dermis (Lee et al, 1992). They found that cytokine or protein kinase C agonist stimulation of the dermal endothelial cells increased human melanocyte cell binding in vitro. Some of the effects appear to be specific for microvascular endothelial cells, and they suggest that it is extremely unlikely that the increases in binding can be completely explained by pathways involving ICAM-1, VCAM-1 or ELAM-1. This suggests that melanoma cell adhesion to these endothelial cells may be mediated by novel microvascular endothelial-specific adherence molecules (Lee et al, 1992). The inhibitory effects of retinoic acid on cell-cell adhesion could be mediated through several systems. In the formation of aggregates, retinoic acid may act upon the proteolytic enzyme/proteinase inhibitor system. Support for this comes from the observation that, in the spheroid model system, cells in the middle of the spheroid tend to be necrotic. The enzymes produced in this state would be capable of preventing aggregation, however spheroids can grow to a reasonable size before they fall apart, usually due to lack of nutrients and oxygen reaching the centre. An alternative to this is the possibility that retinoic acid acts directly on the binding site(s), altering their expression and either blocking them or, as is more likely, inducing changes. As retinoic acid is thought to act at the level of transcription, it is possible that it alters the expression of cell adhesion molecules at this level.

The cell morphology of retinoic acid-treated cells was also affected, with retinoic acid-pretreated B16F1M2 cells remaining rounded on CPAE monolayers. It is possible that the retinoid treatment has affected the cytoskeleton of the cell and thus the deformability making them less invasive. The adherence of the B16F1M2 cells to endothelial cells is important, as retraction of the endothelial cells allows access to the

underlying extracellular matrix. It may be that the observed inhibition of cell spreading may reduce the endothelial cell retraction, thus preventing exposure of the extracellular matrix.

In the homotypic cell-cell adhesion system retinoic acid had an inhibitory effect. When retinoic acid was added to both populations of cells as opposed to only the seeding cell suspension, there was little difference in the reduction of adhesion. Possibly once the cells have been attached for a period of time (the monolayers were incubated for 4 days prior to use), they are capable of synthesising new proteins that can counteract the effect of the retinoid. Alternatively, it may be that retinoic acid affects only some of the cell-cell adhesion mechanisms, thus allowing some cell-cell interaction. No attempt was made to determine how strong the cell-cell interactions were as the strength of such interactions may be important in the process.

The effect of seeding density on the adhesion of B16F1M2 cells was also examined. Cells seeded at the routine seeding densities and incubated for 2 days exhibited a decreased adhesion to all tested substrates - fibronectin, type IV collagen and laminin/nidogen. If the melanoma cells were seeded at very low numbers and this adhesion examined there was also a reduction in adhesion. However, this was not as pronounced as that obtained for the cells incubated for 2 days. This would perhaps suggest that the microenvironment of the cells has an effect on their ability to adhere to the substrates. The cells which were seeded very thinly and incubated for 4 days produced a number of small colonies containing a reasonable number of cells and the cells within these colonies may have produced a microenvironment similar to that found in confluent control cultures. However, in the cultures seeded at the standard seeding densities and incubated for 2 days, the cells were thinly spread over the surface of the dish and as such did not form concentrated colonies, thus having a different microenvironment.

In summary, retinoic acid pretreatment of B16F1M2 melanoma cells was shown to inhibit their attachment to laminin/nidogen, type IV collagen and to an intact subendothelial extracellular matrix. Retinoic acid pretreatment also had an inhibitory effect on cell-cell adhesion, - both homotypic and heterotypic. Adhesion to

fibronectin remained unaffected, with the melanoma cells adhering readily to this substrate both in the presence and absence of the retinoid. Cell morphology was also affected by retinoic acid treatment, with the cells becoming more elongated and having more dendritic processes when grown on plastic, and this was accompanied by an enhancement of melanogenesis. On laminin/nidogen, type IV collagen, the intact matrix and on the monolayer of endothelial cells, retinoic acid pretreated B16F1M2 cells remained rounded, suggesting that retinoic acid also had an inhibitory effect on cell spreading.

4:3. DEGRADATION OF THE EXTRACELLULAR MATRIX.

The ability of invading tumour cells to degrade the underlying extracellular matrix is a crucial step in the process of metastasis. The invasive step is mediated, at least in part, by the production of certain degradative enzymes. The second part of the study reported here examined the effect of retinoic acid on the production of these enzymes.

The first set of results relate to the ability of B16F1M2 cells to degrade the sulphated components of the extracellular matrix. As proteoglycans, and in particular heparan sulphate, are the most highly sulphated components of the matrix, it was assumed that the majority of the $^{35}SO_4$ was incorporated into these molecules (Robinson & Gospodarowicz, 1983). The results indicate that, in general, retinoic acid-pretreatment of the cells reduced their ability to degrade the sulphated components of the matrix.

Melanoma cells, and in particular the murine B16 melanoma cell's ability to degrade the heparan sulphate components of extracellular matrix is well documented (Nakajima et al, 1983; Nakajima et al, 1984; Kramer & Vogel, 1984). The enzyme produced by these cells has been identified as an endo-β-glucuronidase (heparanase), which is capable of cleaving heparan sulphate at specific intrachain sites (Nakajima et al, 1984). The production of this enzyme has been correlated to

metastatic potential, with highly metastatic cells being capable of solubilizing the extracellular matrix heparan sulphate proteoglycans at a higher rate than cells of low metastatic potential (Nakajima et al, 1983).

Another set of enzymes thought to be involved in the degradation of the extracellular matrix are the glycosidases which can mediate the hydrolysis of proteoglycans. Glycosidases, particularly hexosaminidases, have been identified at elevated levels in a variety of solid tumours and/or their interstitial fluid (Bosmann, 1969; Whitehead et al, 1979; Whitehurst et al, 1982). Studies using ovarian tumour cells have demonstrated a relationship between extracellular -N-acetylglucosaminidase activity and the ability of these cells to morphologically and biochemically degrade the extracellular matrix (Niedbala et al, 1987). Additionally, the rate of enzyme accumulation by the tumour cells was progressive with time, and closely paralleled tumour cell-mediated release of [^{3}H]-N-acetylglucosamine-containing fragments from the extracellular matrix (Niedbala et al, 1987).

In the study reported here, the ability of B16F1M2 cells to produce N-acetyl-glucosaminidase was investigated. Intracellular levels of the enzyme decreased as the retinoic acid concentration increased. Examination of intracellular enzyme levels revealed an increase in activity at all tested concentrations. The results may at first seem contradictory, however it may be that although the retinoic acid has decreased the amount of intracellular enzyme, perhaps at the level of transcription, the expression of extracellular enzyme may require a longer period of treatment before being inhibited. However, that does not explain the extracellular enzyme levels. It is possible that elevated extracellular levels are required to release factors held in the matrix which, in turn, inhibit the extracellular production ie. a feedback loop system. The results reported were obtained with B16F1M2 cells grown on plastic, cells grown on extracellular matrix may produce different results.

The other major components of the extracellular matrix examined were the collagens, and degradation of these molecules was monitored by the release of [^{3}H] from the matrices labelled with [^{3}H]-proline. At all tested concentrations of

retinoic acid, there was an increase in the release of [^{3}H] from the extracellular matrix. However, the curve produced from the results is somewhat erratic with some points having large standard errors, and as such the results may not be totally reliable. There have, however, been reports that when added to an intact subendothelial extracellular matrix, B16 melanoma cells are not able to solubilize [^{3}H]-proline labelled material (Kramer & Vogel, 1984). This is an unexpected finding as the ability of highly metastatic cells to produce collagenase is well documented (Salo et al, 1982; Eisenbach et al, 1985; Starkey et al, 1984). Indeed, as with the action of heparanase, type IV collagenase activity in the cell culture media of B16 melanoma cells has been shown to correlate with metastatic potential (Poste & Fidler, 1980).

It is possible that the proteins found in the subendothelial extracellular matrix are resistant to proteinase digestion because they are arranged in a complex structure in which many molecular interactions exist. Laminin is known to bind tightly to both type IV collagen (Kleinman et al, 1981) and heparan sulphate (Sakashita et al, 1980), and these interactions could result in the masking or protecting of specific macromolecules from digestion by degradative enzymes. Also, it may be that the presence of glycoproteins delay the digestion of elastin and collagen by tumour cells (Jones & deClerck, 1980), and work with isolated collagens has demonstrated that the presence of contaminating proteoglycans can protect the collagen from enzymatic degradation (Etherington, 1977).

The susceptibility of collagens to enzymatic action can be modified in the presence of inter molecular cross-links which can stabilise the collagen fibres, thus inhibiting degradation (Fessler & Fessler, 1982; Etherington, 1977). It is possible that if such a network of cross-linked proteins was present in the CPAE extracellular matrix, then insoluble homopolymers and heteropolymers may form, which could provide some resistance to degradation. Alternatively, the effect of the retinoids on the action of the enzymes may be hampered by the orientation of the enzymes in such structures. Although the enzymes may be able to cause some breakdown, the fragments produced may be immobilised on the matrix superstructure by cross-links or noncovalent interactions. Any degradation deeper in the structure might then be

hindered by inaccessibility. Another consideration may be the presence of endogenous proteinase inhibitors, either in the matrix itself or synthesised by the cells seeded onto it. Retinoic acid may be able to reduce the synthesis or secretion of these inhibitors as well as the collagenases, thus any inhibitory effects on degradation may be countered by the effects on the inhibitors.

An attempt was made to investigate the ability if B16F1M2 cells to degrade purified collagen substrates - type I and type IV - but no activity was detected. This may be due to a variety of reasons. It is possible that collagenases were produced, but in a latent form. Experimental conditions may not have been appropriate for activation to occur. Another possibility is that impurities in the substrate are required for the release of collagen breakdown products by enzymatic degradation.

In contrast to the work presented here, a study utilising human melanoma cell lines has demonstrated anti-invasive effects for retinoic acid (Hendrix et al, 1990; Wood et al, 1990). Similar results have also been obtained using rat mammary adenocarcinoma cells (Nakajima et al, 1989). Using a reconstructed basement membrane gel (Matrigel), retinoic acid treatment of cells was found to inhibit invasion in a dose and time-dependent manner (Hendrix et al, 1990). Further investigation revealed that retinoic acid-treated cells secreted lower levels of collagenolytic enzymes, which was measured by the degradation of [^{3}H]-proline-labelled type IV collagen substrate and by the reduction in the activity of the secreted type IV collagenolytic enzymes. The cells also expressed lower levels of human type IV collagenase mRNA and exhibited decreased amounts of tissue-type plasminogen activator activity. A decreased adhesion to Matrigel was also observed, and this was related to an increase in the high affinity metastasis-associated cell surface laminin receptor, and a decrease in the expression of a cell surface receptor for motility factor (Hendrix et al, 1990). From these studies they concluded that retinoic acid inhibited tumour cell invasion through a reconstituted basement

membrane by inhibiting matrix degradation by suppression of type IV collagenolytic activity and plasminogen activator activity, and by alteration of cell surface receptors (Hendrix et al, 1990; Woods et al, 1990).

Other workers have examined the effects of retinoids on type IV collagenolytic activity produced by human melanoma cell lines using type IV collagen substrate labelled with [^{3}H]-proline (Oikarinen & Salo, 1986). They found that retinoids, at concentrations of up to 10^{-6}M, did not significantly affect type IV collagenolytic activity. It is possible, therefore, that the effects of retinoids on tumour cell production of type IV collagenolytic activity may be inconsistent, or may be dependent upon the type of cell line being studied.

The ability of retinoic acid to influence the production of proteinase inhibitors is also a consideration. There have been reports that in normal skin fibroblasts, retinoids can increase the production of TIMP. Clark and co-workers (Clark et al, 1987) found that in monolayer cultures, retinoid treatment could increase the production of TIMP by 2-3 fold and this was accompanied by a simultaneous decrease in collagenase activity. The effects were reversible on the removal of the retinoid, and the effect was found to be mediated by an increase in the biosynthesis of new inhibitor protein. Increased levels of TIMP mRNA were accompanied by a decrease in the amount of collagenase mRNA, which would suggest that the retinoid acts on transcriptional control. From this they concluded that the retinoid co-regulated the expression of collagenase and TIMP in an inverse manner (Clark et al, 1987). It is possible that the effect arises due to the action of another factor stimulated by retinoid treatment. In some cells, such as cultured keratinocytes, retinoic acid induces TGF-β. Modulation of TGF-β activity can inhibit the expression of various proteinases including collagenases, while stimulating the synthesis of various proteinase inhibitors. This would also explain the observations made with the fibroblast system. In the study reported here, the effect of retinoic acid on the production of proteinase inhibitors was not examined. The method of investigation used was to seed a cell suspension onto a substrate, thus a monolayer was not formed. It is possible that reports implicating the production of TIMP and the inhibition of invasion by retinoic

acid were performed in such a way that the conditions were conducive to the production of inhibitors, thus retinoids would then be able to activate the required biosynthetic pathways. Alternatively, it may be a feature of the particular cell line being studied.

The fact that malignant tumour tissues usually contain high proteinase activities does not prove decisively that the enzymes are of tumour cell origin. It should be remembered that tumour tissue usually contains host tissue cells such as fibroblasts, macrophages and monocytes in addition to the tumour cells, and that all the host cell types are reported to be able to secrete a variety of proteinases including collagenases (Tryggvason et al, 1987). Therefore, it may be that, in some cases, the host cells play a role in the degradation of the extracellular matrix during invasion. Human basal cell carcinoma extracts have been shown to contain greater levels of immunoactive interstitial collagenase than normal skin extracts, and immunostaining has shown that the enzyme is present only around the stromal elements surrounding the tumour islands (Bauer et al, 1979). The implication from this is that host cells may have been stimulated to increase the secretion of collagenase. It is unlikely that the B16F1M2 cells used in this study would not produce sufficient collagenase to allow invasion to occur, and indeed the production of type IV collagenase by these cells has been correlated to their metastatic potential (Liotta et al, 1980).

One of the other important enzymes associated with the metastatic phenotype is plasminogen activator, a serine proteinase capable of activating plasminogen by converting it to plasmin which can, in turn, degrade fibrin and laminin (Quigley et al, 1986; Liotta et al, 1981; Saksela & Rifkin, 1988). It has been suggested that the breakdown of extracellular matrix components may occur by plasminogen activator activation, with the plasmin formed activating procollagenases (Tryggvason et al, 1987; Saksela & Rifkin, 1988; Reich et al, 1988). Additionally, a role for the plasminogen activator-plasmin-collagenase activation pathway has been demonstrated in an invasive model for B16 melanoma cells utilising human amniotic basement membrane (Mignatti et al, 1986).

Immobilisation of plasminogen activators on the surface of proteolytically active cells may direct proteolysis to specific sites of activity, and in mobile cells such as macrophages, fibroblasts and several types of malignant cells which express u-PA receptors, the bound enzyme may be localised to sites where the activity is required (Saksela & Rifkin, 1988). Immunofluorescence and immunoelectron microscopy have demonstrated a co-localization of u-PA with vinculin, but not with extracellular matrix components such as fibronectin (Pollanen et al, 1988; Herbert & Baker, 1988).

When the effect of retinoic acid on the activation of urokinase plasminogen activator was examined, it was found that treatment of the cells increased plasminogen activator activity both intracellularly and extracellularly. This is in direct contrast to work carried out using human melanoma cell lines in which retinoic acid treatment produced a reduction in plasminogen activator activity. However, in that study the plasminogen activator activity assayed was primarily that of tPA (Wood et al, 1990), whereas in the study reported here u-PA activity was assayed.

In other systems it has been demonstrated that low concentrations of retinoic acid can stimulate plasminogen activator activity. In chick embryo fibroblasts and chick embryo muscle cells both the induction of the enzyme and its reduction by the removal of retinoic acid required mRNA synthesis (Wilson & Reich, 1978; Miskin et al, 1978). However, the effect of retinoids on plasminogen activator activity has been examined in a variety of human cultured cells lines of both neoplastic and normal origin (Wilson & Dowdle, 1980), and in murine tumour cells (Lotan et al, 1983). Results from these studies conclusively show that retinoids have no consistent effects on plasminogen activator activity.

Although correlations between plasminogen activator secretion and the metastatic potential of tumour cells have been made, they are not as consistent as the relationship of type IV collagenase to metastatic potential (Quigley et al, 1986; Saksela & Rifkin, 1988; Reich et al, 1988; Mignatti et al, 1986). Migration of tumour cells through the basement membrane has been shown to be dependent on a proteolytic cascade in which collagenase is activated by plasminogen-activated

plasmin (Saksela & Rifkin, 1988; Reich et al, 1988; Mignatti et al, 1986; Yagel et al, 1989). In support of this, direct genetic evidence for a causal role of plasminogen activators in invasion and metastasis has been produced by transfecting H-ras-transformed NIH 3T3 cells with human plasminogen activator cDNA of both types. The cells were then monitored for their ability to invade a basement membrane and to form experimental lung metastases, both of which were increased (Axelrod et al, 1989).

The formation of plasmin can be controlled by either inhibition of plasmin or plasminogen activator activities by proteinase activities. Proteinase inhibitors such as α_2-macroglobulin, α_1-proteinase inhibitor and antithrombin III may inhibit a wide range of proteinases, whereas some may act against only one. Proteinase inhibitors have an important function in the control of excessive proteolytic reactions in blood coagulation, fibrinolysis, tissue remodelling and inflammatory processes. Plasminogen activator inhibitors belong to the serpin family of inhibitors and are classified as arginine-serpins according to the location of arginine at the reactive centre (Carrell & Travis, 1985; Hill & Hastie, 1987). Enzyme activity is inhibited by the formation of 1:1 covalent complexes between the enzyme and the inhibitor. Three different effective and functionally rapid plasminogen activator inhibitors have been characterised - PAI-1, PAI-2 and the protease nexins. PAI-1 and PAI-2 have a more limited target specificity than the protease nexins, which primarily inhibit the action of thrombin, but also that of both types of plasminogen activator and trypsin (Knauer & Cunningham, 1984).

Some hormones and hormone-like substances have been implicated in the regulation of the proteolytic activity of cultured cells. The enhancement of plasminogen activator activity by such factors has, in some cases, been connected with the malignant behaviour of these cells, but enhanced plasminogen activator activity also seems to be required for several developmental and reparative processes (Dano et al, 1985; Saksela, 1985). Growth factors which affect various growth parameters of cells may also mediate plasminogen activator activity, but the effects may vary depending upon the cell line under investigation, with different cells

responding by either a stimulation or inhibition of the secretion of plasminogen activator. It may be that if bound plasminogen activator is present at the surface of the melanoma cells, the extracellular levels of enzyme activity are increased by the release of the binding complex. This would, however, have little effect on intracellular levels unless cell attachment required a u-PA receptor-bound complex at the surface, and continuing amounts of plasminogen activator were produced in order to fulfill this requirement. Alternatively, a growth factor could regulate the expression of plasminogen activators or their inhibitors.

The stimulation of plasminogen activator activity by retinoic acid may be brought about either by de novo synthesis of the enzyme, or by the inhibition of plasminogen activator inhibitors. It is also possible that retinoic acid may act in a similar manner to the growth factors, with responses depending on the cell line under investigation. Further work is required to investigate the mode of action in the system reported here.

In summary, retinoic acid appears to have very little effect on the extracellular matrix degradative processes relating to the B16F1M2 melanoma cell line. Although the results observed for type IV collagenolytic activity and plasminogen activator activity are in contrast to recent reports (Hendrix et al, 1990; Wood et al, 1990), this could be due to the different assay systems used or to differences between the murine and human melanoma systems. Previous work has shown no consistent effect of retinoids on plasminogen activator synthesis by various tumour cell lines (Lotan et al, 1982). Similarly, there have been conflicting reports on the effects of retinoids on type IV collagenolytic activity, again suggesting a dependency on the assay system used and the cell line under investigation.

4:4. EFFECT OF RETINOIC ACID ON THE ACTION OF TUMOUR CELL-DERIVED FACTORS.

If fibroblasts are suspended in type I collagen gels in the presence of serum and the gels allowed to set, contraction of the gel occurs over a few days. If serum is omitted from the system, there is no contraction. In the presence of serum

retinoic acid had no effect on gel contraction. There have been reports that tumour cells can alter the production of various macromolecules by fibroblasts - fibronectin and collagen (Noel et al, 1992), hyaluronate (Knudson et al, 1984) and glycosaminoglycans (Merrilees & Finlay, 1985). When serum-free B16F1M2-conditioned medium was tested, gel contraction occurred at a rate similar to that obtained with serum. If the conditioned medium was prepared in the presence of retinoic acid, the rate of contraction was reduced. This would suggest that a factor or factors produced by the B16F1M2 cells were capable of stimulating the fibroblasts to reorganise the gel, and mediate contraction. Retinoic acid would appear to be able to partially inhibit either the production of these factors or affect their mode of action. To ensure that the observed effects were due to the action of retinoic acid on the melanoma cells as opposed to on the fibroblasts, the conditioned media were U.V.-irradiated to destroy any residual retinoic acid before being introduced into the system.

Tumour-conditioned medium can alter fibroblast glycosaminoglycan synthesis. Thus the effect of B16F1M2-conditioned medium on fibroblast glycosaminoglycan production was examined, with the fibroblasts grown on plastic. The presence of conditioned medium would appear to increase the production of hyaluronate by the fibroblasts even in the presence of retinoic acid. The sulphated glycosaminoglycans were also affected by the presence of the conditioned media - the second sulphated GAG peak is composed of two distinct peaks in control experiments, but becomes one solid peak after exposure to B16F1M2-conditioned medium. Medium conditioned in the presence of retinoic acid caused this second peak to become more like that observed in control cultures.

The effect of tumour-conditioned medium on the sulphated glycosaminoglycans was to ellicit a decrease in their overall production. The reverse of this was found to be the case if the medium was prepared in the presence of retinoic acid. When the individual sulphated glycosaminoglycans were examined it was found that both heparan sulphate and chondroitin sulphate were reduced by the conditioned medium, with the decrease in chondroitin sulphate the more dramatic.

However, tumour-conditioned medium prepared in the presence of 10^{-6}M retinoic acid produced an increase in the amount of heparan sulphate detected but a decrease in the amount of chondroitin sulphate. This is the opposite to the effect of retinoic acid on sulphated GAG production in B16F1M2 melanoma cells - retinoic acid increases the chondroitin sulphates by a small amount while eliciting a decrease in the heparan sulphates. This may be a cell line-specific response or, alternatively, it may reflect the different responses induced by retinoic acid in normal and malignant cells.

The increase in the hyaluronate produced by the fibroblasts is, perhaps, the more important effect caused by the conditioned medium. Recent evidence has suggested a direct role for cell surface hyaluronic acid in the regulation of cell migration. Hyaluronic acid-binding protein has been demonstrated to concentrate in the ruffling lamellae of actively locomoting cells (Turley & Auersperg, 1989). If hyaluronic acid and hyaluronic acid-binding protein are added to fibroblasts cell movement is stimulated (Turley et al, 1985). A 70kDa factor associated with foetal fibroblasts and fibroblasts derived from cancer patients has been shown to stimulate confluent cells to penetrate collagen gels by a mechanism which is dependent on hyaluronic acid. This effect can be inhibited if the cells are treated with a hyaluronic acid-specific hyaluronidase (Schor et al, 1989).

A cell surface proteoglycan, CD44, which is involved in lymphocyte activation and adhesion, and homing of circulating lymphocytes has also been identified as a hyaluronic acid receptor (Toole, 1990). CD44 has been demonstrated in a number of tumour cell lines including human melanoma cell lines (Birch et al, 1991). Work on human melanoma cells has suggested a relationship between the expression of CD44 and the metastatic potential of the cell line (Birch et al, 1991). Thus it may be that in vivo, melanoma cells produce factor(s) which induce host fibroblasts to produce more hyaluronic acid, allowing migration of these cells along the hyaluronate tracks. In the glycosaminoglycan analysis of the conditioned medium-treated fibroblasts, retinoic acid pretreatment appeared to have little effect on this increase in hyaluronate which would suggest that it has limited effects on cell

locomotion by this method. Interestingly, a glycosaminoglycan analysis of B16F1M2 cells revealed that they did not express hyaluronic acid, a fact which will be discussed later.

4:5. ANALYSIS OF CELL-SURFACE GLYCOSAMINOGLYCANS.

Proteoglycans are intrinsic constituents of the cell surface, extracellular matrix and basement membrane, three logistically and functionally important structures involved in most cellular interactions. They can influence the behaviour of normal and malignant cells by virtue of their expanded configuration, polyanionic nature and their ability to interact with a variety of cellular products. Thus they have been implicated in a number of biological processes including proliferation, recognition, adhesion and migration. They can serve as links between the extracellular and intracellular environment thus transducing key biological signals. In addition to this, they can act as receptors for matrix proteins thereby contributing to the organisation of the pericellular matrix. During neoplastic development, there is a profound structural arrangement of these macromolecules at both the plasma membrane and the pericellular level. Qualitative and quantitative abnormalities in proteoglycan metabolism may contribute to some aspects of neoplasia such as lack of cohesiveness, abnormal assembly of the extracellular matrix, abnormal growth and invasion.

Both proteoglycans and their carbohydrate side chains, the glycosaminoglycans, have been implicated in the regulation of cell division, with indirect evidence for this concept coming from studies of glycosaminoglycans in neoplastic tissues (Dietrich et al, 1977; Chiarugi et al, 1978; Iozzo et al, 1981). High amounts of chondroitin sulphates were detected in a variety of mesenchymal and epithelial neoplasms as compared to their normal counterparts. A positive role in tumour cell proliferation has been demonstrated for chondroitin sulphates on mammary carcinoma cells in vitro (Ozzello et al, 1960), and Ehrlich ascites tumour in

vivo (Takeuchi, 1972a). Furthermore, enzymes capable of degrading chondroitin sulphates can inhibit or retard the growth of Ehrlich ascites tumour cells (Takeuchi, 1972b).

In contrast to this, heparan sulphates have been implicated in the negative control of cell proliferation (Kraemer, 1971; Kraemer & Smith, 1974). Cell surface heparan sulphate is released during premitosis and the cyclic exposure of this glycosaminoglycan to the cell surface has been proposed to negatively affect the transport of nutrients and ions across the plasma membrane, resulting in a negative effect on cell growth (Kraemer & Tobey, 1972; Chiarugi & Vannucchi, 1976). Heparan sulphate may exert its effects either locally or distally following its release from the cell surface. It has been demonstrated that glutaraldehyde-fixed confluent cultures of fibroblasts, which are enriched in cell surface heparan sulphate proteoglycan, inhibit the growth of freshly seeded cells, and this effect can be abolished by pretreating the fibroblasts with heparitinase (Culp et al, 1978). Heparan sulphate isolated from liver cells or from their plasma membrane inhibits the growth of hepatoma cells in vitro (Ohnishi et al, 1975; Kawakami & Terayama, 1981). Also, it has been shown that endothelial cells release a heparin-like molecule, or a highly sulphated heparan sulphate proteoglycan which can inhibit the growth of smooth muscle cells in vitro (Castellot et al, 1981), with the active molecules apparently released from the cell surface by a platelet endoglycosidase found in serum (Castellot et al, 1982).

There have been several reports describing profound changes in the amounts and types of glycosaminoglycans and proteoglycans associated with neoplasms. One of the first demonstrations of this was the elevated levels of hyaluronic acid detected in the pleural effusion of mesotheliomas. However, in tissue extracts from mesotheliomas, elevated amounts of chondroitin sulphate have been detected and this suggests the existence of biochemical variants in this neoplasm (Iozzo et al, 1981).

Alterations in heparan sulphate proteoglycan have been linked to the neoplastic phenotype. One of the most consistent qualitative abnormalities in the structure of the macromolecule that has been proposed to be directly related to the transformed phenotype is a reduced degree of sulphation in the heparan sulphate glycosaminoglycan side chains. SV40-transformed 3T3 cells have been demonstrated to synthesise undersulphated heparan sulphate chains (Underhill & Keller, 1975). This has also been shown to be the case for human hepatoma (Nakamura & Kojima, 1981), ascites hepatoma (Hurst et al, 1981) and myeloma (Stamatoglou & Keller, 1983). Also, spontaneously transformed mouse mammary cells synthesise undersulphated basement membrane heparan sulphate proteoglycan (David & Van Den Berghe, 1983).

There are several possible mechanisms and resulting functional consequences for the observed changes. It is possible that transformation has an effect on the enzyme required in the modification reactions of the molecules. The major difference between normal and transformed 3T3 cells has been shown to be the degree of O-sulphation (Keller et al, 1980), whereas in hepatoma cells the difference occurs primarily at the level of N-sulphation of the glucosamine residues caused by a defect in the deacetylase (Robinson et al, 1984), which is the key enzyme involved in the modification of the unsulphated heparan, and the extent of the initial deacetylation appears to regulate the subsequent polymer modification reactions (Riensenfeld et al, 1982). It is also possible that transformation may generate some inhibitor of sulphotransferase, or it may directly alter the levels of the major sulphate donor, 3'-phosphoadenosine-5'-phosphosulphate (PAPS). The availability of the sulphate donor plays a fundamental role in the sulphation of glycosaminoglycans (Sugahara & Schwartz, 1979).

The undersulphation of glycosaminoglycans could exert a multiplicity of effects. It is possible that undersulphation could lower the affinity of the molecules for laminin, collagen and fibronectin, all of which can contribute to the abnormal assembly of the extracellular matrix and a decrease in adhesiveness, two properties of malignant cells (Iozzo, 1988). In myeloma and hepatoma cells, undersulphation of

heparan sulphate has been detected, and both of these lack a detectable pericellular matrix and show little or no affinity for fibronectin (Stamatoglou & Keller, 1983; Robinson et al, 1984). This is in contrast to the B16F1M2 murine melanoma cell line which has been shown to adhere readily to fibronectin (Edward et al, 1989). Another possible consequence of undersulphation would be a decrease in overall cell surface charge, which could favour the entrance of cells into the mitotic cycle (Chiarugi & Vannucchi, 1976). Also, this structural change may significantly influence the molecular interactions which normally occur during the assembly of the basement membrane, and this may be directly involved in the abnormal or lack of basement formation by neoplastic cells (David & Van Den Berghe, 1983).

The changes observed in heparan sulphate after transformation have been associated with their abnormal self affinity (Fransson et al, 1981). When SV40-transformed cells were examined, it was found that their heparan sulphates had no affinity for agarose-substituted heparan sulphate species (Fransson et al, 1981). The alterations in the structure of the macromolecules may contribute to the abnormal behaviour of the tumour cells by inhibiting self-association and adhesion, alternatively they may interfere with the recognition of a suitable substrata. It may be that the presence of a certain type of microenvironment around the tumour cells is essential for the full expression of the neoplastic phenotype (Gallagher et al, 1986).

Over production of heparan sulphate can also be identified in neoplasia as demonstrated in patients with multiple myeloma who have high levels of circulating heparan sulphate which has anticoagulant activity (Khoory et al, 1980; Palmer et al, 1984). A similar finding has been reported in some cases of acute monoblastic leukaemia (Russell et al, 1984). It is possible that the neoplastic cells produce and release the molecule in large quantities or, alternatively, the circulating proteoglycan may be derived from endothelial cells which have been damaged by chemotherapy. Another possibility is that it may be produced by tumour cells to induce an immunological response towards endothelial cells or platelets. However, it has been shown that cloned endothelial cells synthesise anticoagulant heparan sulphate

proteoglycan (Marcum et al, 1986), and that some patients with heparin-associated thrombocytopenia contain antibodies that react against heparan sulphate synthesised by endothelial cells (Cines et al, 1987).

The changes in cell surface glycosaminoglycans after transformation have been linked to the metastatic potential of the cells. The glycosaminoglycan synthesis of B16 metastatic melanoma variants, both in vivo and in vitro, have been examined. Turley and Tretiak (1985) found both an increase in the deposition of hyaluronic acid around the tumours during the early stages of invasion in vivo, and an increase in the release of hyaluronic acid and heparan sulphate in vitro by the cell lines, and this correlated with enhanced metastatic capabilities. In the study reported here, it was found that B16F1M2 cells did not synthesise hyaluronic acid in vitro. However, it has been shown that B16F1M2-conditioned medium can stimulate hyaluronic acid synthesis by fibroblasts, which in turn could be responsible for the increased amounts of hyaluronic acid detected around tumours in vivo.

The presence of hyaluronic acid, either within a capsule or distributed throughout the tumour tissue adjacent to the stromal-tumour interface has been widely reported (Nigam & Cantero, 1972). It has been demonstrated that the largest amount of hyaluronic acid surrounding each tumour variant coincided with the onset of observable metastasis, suggesting an instructive role for hyaluronic acid during early tumour invasion (Turley & Tretiak, 1985). It was noted that the absolute levels of hyaluronic acid were lower for the highly invasive variants, and it may be that this is related to the increased degradative abilities of these invasive cells (Nicolson, 1982). Although the highly metastatic cells were shown to secrete more hyaluronic acid than the less metastatic cells, the reduction in deposition of the glycosaminoglycan around the tumour in vivo may be related to a higher turnover of the molecule or the absence of an extracellular matrix, and as such other molecules to bind to it (Turley & Tretiak, 1985). In the capsule surrounding the tumour produced by the least metastatic variant B16F1, the presence of hyaluronic acid was shown to have an inhibitory effect on the invasion of cells into the surrounding muscle tissue (Turley & Tretiak, 1985), with invasion occurring only after extensive breakdown of the capsule. The presence of

stromal capsules and basement membranes around other tumours has led to the suggestion that part of their function may be to act as physical barriers restricting extravasation and intravasation (Ingber et al, 1981; Gusterson et al, 1982).

A variety of epithelial and mesenchymal tumours have been shown to express abnormal levels of chondroitin sulphate, and there have been attempts to correlate the production of this glycosaminoglycan with the properties of the tumour cells (Iozzo, 1985b). Chondroitin sulphate is found in only small amounts in most adult noncartilaginous tissue, but is present in raised concentrations in foetal and neoplastic tissue (Chiarugi et al, 1978; Dietrich et al, 1977), with raised amounts being reported in breast (Takeuchi et al, 1982), lung (Horai et al, 1981), liver (Kojima et al, 1975), colon (Iozzo et al, 1982; Iozzo & Wight, 1982) and prostate (DeKlerk et al, 1984) carcinomas. In melanomas, chondroitin sulphate is a major cell surface component which can be identified in cell lines (Bumol et al, 1984) and in biopsy specimens (Harper et al, 1984). Chondroitin sulphate has been shown to stimulate the growth of Ehrlich ascites tumours in vivo (Takeuchi, 1972a) and conversely, enzymes capable of degrading chondroitin sulphate can inhibit the growth of these cells (Takeuchi, 1972b).

Heparan sulphate is the major proteoglycan associated with the basement membrane. Using monoclonal antibodies directed against the glycosaminoglycan side chains, heparan sulphate glycosaminoglycan expression on the surface of B16 murine melanoma cells has been studied (Kure et al, 1987). Sublines of B16 which expressed low amounts of cell surface heparan sulphate were highly metastatic and the parental variant, which was only weakly metastatic, expressed the highest amounts of the glycosaminoglycan. If cells were prepared from the metastatic lung colonies found in mice injected with the parental B16F1 cell line, examination of the expressed heparan sulphate revealed lower levels than seen in the original cells, which suggests that cells with reduced amounts of heparan sulphate are selected in the process of lung colonization. In agreement with this is the finding that in vitro selection of B16 cells with low surface heparan sulphate resulted in the selection of cells with high metastatic potential. The correlation between the expression of cell surface heparan

sulphate and metastatic potential is not linear, thus it is possible that the expression of low levels of this cell surface glycosaminoglycan is either one of many phenotypes which contribute to the metastatic potential of the cells, or a change which is somehow associated with a potentiating phenotype (Kure et al, 1987).

In contrast to this, work on other cell lines such as the Lewis lung carcinoma, has indicated that highly metastatic cells contain a relatively higher amount of heparan sulphate than do cells of lower metastatic potential (Timar et al, 1987). Using xenografts of human melanomas, similar results have been obtained (Timar et al, 1989). The differences in the patterns obtained may be due to the experimental conditions used. Another possibility is that the variations are due to differences in the cell type being studied, as it may be that heparan sulphate expression in the murine system differs from that of the human system. This would seem unlikely as there have been reports describing glycosaminoglycan production in B16 cells, and it has been shown that the more invasive and highly metastatic variants produce higher amounts of heparan sulphate in vitro than did cells which were less invasive and poorly metastatic (Turley & Tretiak, 1985).

The results presented here suggest that retinoic acid has an effect on the production of glycosaminoglycans by B16F1M2 cells. A quantitative analysis of glycosaminoglycan synthesis revealed that incorporation of [^{3}H] glucosamine and $^{35}SO_4$ into the heparan sulphates, in the medium fraction, was reduced by 33% and 63% respectively. Thus it would appear that heparan sulphate production in this fraction is reduced, with an accompanying reduction in the degree of sulphation. In the low density medium fraction, incorporation of the radiolabel into the heparan sulphates showed a different pattern, with a large increase in [^{3}H] glucosamine incorporation (30%) and for $^{35}SO_4$, a 35% increase which indicates an overall increase in the synthesis of this glycosaminoglycan and an increase in the degree of sulphation. This would suggest that the density of the cells can have an effect on the production of this macromolecule. The chondroitin sulphates of retinoic acid-treated cells were also altered, with incorporation of [^{3}H] glucosamine reduced by 9%, while $^{35}SO_4$ incorporation remained essentially unaffected, suggesting a slight decrease in

chondroitin sulphate synthesis accompanied by a small increase in the degree of sulphation. The low density cultures exhibited an increase in chondroitin sulphate and an increase in the degree of sulphation. This finding is obviously related to cell density.

These results are in partial agreement with a previous study using B16BL6 melanoma cells treated with retinoic acid, which showed that there was an overall decrease in glycosaminoglycan synthesis, but an increase in the degree of sulphation of heparan sulphate (Edward & MacKie, 1989). Although they reported a decrease in the amount of chondroitin sulphate, the sulphation was hardly affected which agrees with the findings reported here. The results are, however, in total contrast to other work carried out on the B16F10 murine melanoma treated with retinoic acid which demonstrated an increase in glycosaminoglycan synthesis (Maniglia & Sartorelli, 1981). It is possible that the differences are due to the different methods employed to estimate glycosaminoglycan production or alternatively, it may be that although all three cell lines were derived from the same parental line (B16 melanoma) each may have a different response to retinoic acid treatment. Indeed, this has been shown to be the case with variants of B16 treated with retinoic acid and the melanotic expression estimated (Edward et al, 1988).

Qualitatively, retinoic acid would appear to have little effect on the heparan sulphates or the chondroitin sulphates, although in the initial isolation of the glycosaminoglycans, the sulphated molecules eluted as two peaks as opposed to one peak for the controls. Ion-exchange chromatography revealed elution profiles for retinoic acid-treated cells which were very similar to those of control cells, indicating little or no change in the charge density of the eluted glycosaminoglycans. This is perhaps unexpected as a change in the sulphation of the molecules may have been expected to elicit a change in the overall charge of the structure. The results are in contrast to the B16BL6 melanoma study in which the heparan sulphates of the retinoic acid-treated cells eluted at a lower salt concentration than control cells

suggesting that they have a lower charge density. Similarly, the chondroitin sulphates exhibited a lower charge density after retinoic acid treatment (Edward & MacKie, 1989).

The changes elicited in the synthesis of glycosaminoglycans by retinoic acid can have an effect on a variety of cellular processes. The positioning of glycosaminoglycans on the cell surface make them ideal candidates for receptors, and indeed they have been identified as receptors for matrix components such as fibronectin and laminin. Any change in the structure of these molecules, heparan sulphate in particular, could affect the receptor properties and as such the adhesive capabilities of the cells. Undersulphation of heparan sulphate in myeloma and hepatoma cells has been associated with the lack of affinity these cells have for fibronectin (Stamatoglou & Keller, 1983; Robinson et al, 1984). In this study, a reduction in the sulphation of heparan sulphate was detected, but both control and retinoic acid-treated cells adhered readily to fibronectin. This would suggest that, at least in B16F1M2 melanoma cells, changes in the sulphation and the amount of heparan sulphate have no direct affect on fibronectin binding.

In this system, it is possible that retinoic acid may be important in receptor-mediated interactions. Heparin and heparan sulphates can bind fibroblast growth factors (FGFs), giving them some protection from degradation. This may be one method of holding a reservoir of the growth factor, with changes in the proteoglycans, either by chemical modification or enzymatic degradation, releasing the active growth factor. TGF-β has been shown to stimulate decorin synthesis, thus decorin may act to effect a negative feedback system. Again it is possible that formation of the decorin-TGF complex holds a reservoir of TGF-β in the matrix. Retinoids may modulate the activities of these growth factors by altering the properties of the proteoglycan - changes in sulphation or synthesis may affect the binding properties of the molecules. The reported effects of retinoic acid on the production of enzymes involved in the degradation of the matrix may also modify the action of the growth factors and as such the properties of the metastasizing cell.

Heparan sulphate proteoglycans have self affinity properties, and as such may be involved in the process of cell-cell adhesion. Changes in the structure or sulphation of the molecules may have profound effects on this process. Exactly how this may come about is unknown but it is possible that cell adhesion molecules, some of which have been shown to have proteoglycan structures in their binding regions, may be of importance.

The effects of retinoic acid on glycosaminoglycan synthesis have, in some instances, been shown to be reversible. In a study using HL-60 leukaemia cells, retinoic acid was shown to inhibit the production of chondroitin sulphate, the major glycosaminoglycan produced by these cells. When retinoic acid was removed from the system, production of chondroitin sulphate returned to control levels (Reiss et al, 1985). The study also revealed the presence of a second class of molecules believed to be keratan sulphate, which also exhibited a reduction in production and was undersulphated. Removal of retinoic acid had no effect on the production of this molecule demonstrating the diverse effects retinoids can elicit within one cell type.

As part of the study reported here, the reversibility of retinoic acid on adhesion to basement membrane components was examined. Adhesive properties were reinstated on the removal of retinoic acid from the culture system. It would seem likely, therefore, that if the synthesis of glycosaminoglycans was investigated after removal of retinoic acid from the system, the pattern observed would be similar to that of controls. This would suggest that one of the possible modes of action for retinoic acid would be in blocking some particular stage in the synthetic process or in the inhibition of the synthesis of some proteins or enzymes required for the process.

Some of the evidence for the mode of action of retinoids suggests that they may act at the level of transcription. The presence of specific retinoic acid receptors within both the cytoplasm and nucleus of the cell would perhaps agree with this suggestion. The cellular retinoic acid binding proteins (CRABPs) are found in the cytoplasm and appear to transport their non-covalently bound ligands, either

extracellularly or intracellularly, to sites of action or metabolism, with the ability of these proteins to bind their ligand being associated with biological activity (Jetten & Jetten, 1979).

Binding of retinoic acid to specific nuclear chromatin acceptor sites can be demonstrated by incubating isolated nuclei with preformed RA-CRABP complexes (Barkai & Sherman, 1987; Takase et al, 1986). Two theories for the role of CRABPs have been proposed. The "shuttle theory" suggests that CRABP is involved in the transport of retinoic acid from the cytosol to nuclear receptors causing enhancement of retinoid action (Mehta et al, 1982; Takase et al, 1986; Wang & Gudas, 1984). The second theory is the "sequestration theory" which suggests that CRABP functions to limit the interaction of retinoic acid with its nuclear receptors (Maden et al, 1988; Boylan & Gudas, 1991).

Two families of nuclear retinoid receptors have been identified - retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Petkovich et al, 1987; Giguere et al, 1988; Mangelsdorf et al, 1990). RARs and RXRs both have three receptor types - α, β, and γ - and are thought to transcriptionally activate target genes by binding to specific DNA sequences termed retinoic acid response elements (RAREs), which are generally located upstream from the sites of transcriptional initiation (Glass et al, 1991; Leid et al, 1992). RARs bind with high affinity to both all-trans retinoic acid and a stereoisomer of it, 9-cis retinoic acid, while RXRs only bind to the 9-cis form with high affinity (Allenby et al, 1993).

Recent data has indicated that RXRs form heterodimers with RARs and other receptors in the steroid/thyroid superfamily, and that RXRs coregulate the activity of RARs on their response elements (Leid et al, 1992). It is possible that the formation of RAR-RXR heterodimers may be responsible for the diversity in the transcriptional response to retinoic acid. Heterodimers may generate diversity at two levels in the retinoid signalling system - at the level of the RAREs or at the level of the trans-activational process (Leid et al, 1992). Such diversity may explain, in part, the mechanisms of retinoic acid action within the cell.

4:6. SUMMARY OF RESULTS.

In the study reported here, no attempt was made to determine if CRABPs were present in B16F1M2 cells or to assay any changes induced by the presence of retinoic acid. Similarly, it was outwith the scope of the study to investigate the expression of the nuclear retinoic acid receptors.

One of the first steps in the formation of metastatic deposits is the adhesion of cells from the primary tumour mass to the extracellular matrix. In the instance of tumours such as melanoma, the extracellular matrix in question is the basement membrane. The ability of retinoids to modify this adhesion may lead to a further understanding of this step in the metastatic cascade.

When the effect of retinoic acid-pretreatment on B16F1M2 melanoma cells was examined with respect to adhesion to individual components of the extracellular matrix, it was found that adhesion to type IV collagen and laminin/nidogen was inhibited, but the ability of the cells to adhere to fibronectin remained essentially unaffected. The inhibition was dependent on both time and retinoic acid concentration. Adhesion to type I collagen was also examined and was found to be relatively unaffected by retinoic acid pretreatment.

Most treatments utilizing the inhibitory effects of retinoids on the metastatic process require a regular supply of the compound. Thus the effect of treating the cells for two days with retinoic acid, then removing it from the system was investigated as a function of adhesion to either type IV collagen or laminin/nidogen. It was found that removal of the retinoid resulted in an increase in cell adhesion to both substrates to levels approaching those of the control. If the cells were grown for two days then had retinoic acid added, adhesion to the substrates was reduced.

Examination of the effect of retinoic acid pretreatment on murine melanoma cell adhesion to individual components of the extracellular matrix provided a limited amount of information. In vivo the individual components of the extracellular matrix interact with each other to form the intact structure, thus the next step in the investigation was to consider the effect of retinoic acid pretreatment on

the adhesion of B16F1M2 cells to an intact extracellular matrix. It was not possible to synthesize a basement membrane in vitro, thus the extracellular matrix used was that deposited by endothelial cells. Pretreatment of the murine melanoma cells with retinoic acid resulted in an inhibition of cell adhesion. The composition of the deposited matrix was not determined, however, if the main components are type IV collagen and laminin/nidogen, then the results generated from the studies involving the intact extracellular matrix concur with those obtained with both type IV collagen and laminin/nidogen. When dishes were coated with both type IV collagen and laminin/nidogen, cell adhesion was again inhibited by the pretreatment of B16F1M2 cells with retinoic acid. This again supports the findings using the intact matrix.

Treatment of B16F1M2 cells with retinoic acid at a concentration of 10^{-6}M results in a decrease in the growth rate of the cells and a reduction in cell numbers. Thus it is possible that the results obtained are due to a cell density effect rather than to retinoic acid pretreatment of the cells. Cell seeding densities were adjusted such that, when harvested, the number of cells in the retinoic acid-treated cultures was the same as for those from control cultures. To double check the density effect, a series of experiments were carried out to determine the effect of density on cell adhesion. Cells seeded at the normal seeding density but cultured for 2 days, rather than 4 days, were found to adhere much more slowly and at a reduced rate to fibronectin, type IV collagen and laminin/nidogen.

Cells were also seeded at a much lower density than normal and cultured for 4 days. Their adhesion to the three substrates fibronectin, type IV collagen and laminin/nidogen followed a pattern very similar to that obtained for control cultures. This may be a function of the B16F1M2 growth pattern in addition to a density effect.

Tumour cells not only interact with the basement membrane, they also make contact with other cells - both homotypic and heterotypic in origin. Such cell-cell interactions are important in the formation of cell aggregates and may afford the metastatic cells some protection against the host defence mechanisms. Retinoic acid pretreatment of B16F1M2 cells was found to inhibit their adhesion to

monolayers of B16F1M2 cells (homotypic cell-cell adhesion) and endothelial cells (heterotypic cell-cell adhesion). If the B16F1M2 monolayers were also pretreated with 10^{-6}M retinoic acid, there was no significant change in the pattern of inhibition.

The formation of homotypic cell-cell aggregates was also investigated. Pretreatment of B16F1M2 cells with retinoic acid decreased the size of the aggregates and the cell clumps were more easily disrupted by mechanical means. This would suggest that retinoic acid has an effect on cell-cell adhesion processes with, perhaps, some modulation of cell adhesion molecules. The effect of retinoic acid on heterotypic adhesion is also of interest as tumour cells are thought to interact with and modulate host cells. This may involve an initial adhesion event which, in turn, may stimulate or inhibit the production of various compounds by the host cells.

The positioning of the glycosaminoglycan side chains of proteoglycans on the surface of the cell make them possible candidates for a role in the cell adhesion processes. In fact, the hyaluronate receptor, CD44, is a proteoglycan with chondroitin sulphate chains. It is possible that modulation of cell adhesion by retinoic acid may, in part, occur by altering the expression of the cell surface glycosaminoglycans. Examination of cell surface glycosaminoglycans of retinoic acid-pretreated B16F1M2 cells did reveal changes in expression. A change in the degree of sulphation of the molecules was detected as was a change in the chondroitin sulphate:heparan sulphate ratios. However, it is unlikely that this correlates to the results obtained for cell adhesion. An attempt was made to determine whether or not the isolated glycosaminoglycans would bind to affinity columns prepared with either fibronectin or laminin/nidogen, but this proved unsuccessful and no further correlation was attempted.

Once the tumour cells adhere to the basement membrane, they must traverse it. This involves the dissolution of the matrix by the production of degradative enzymes. The ability of B16F1M2 melanoma cells to produce the degradative enzymes collagenases types I & IV, plasminogen activators and glycosidase was determined. The enzymes were selected for the following reasons. The collagens are the structural components of the extracellular matrix and as such

breakdown of these would undermine the integrity of the structure. Glycosidases act on the glycosaminoglycans which are thought to function, in part, as reservoirs for growth factors and small peptides in the matrix. Any alteration in their structure may bring about the release of these factors which would, perhaps, be of use to the metastasising cell. Plasminogen activators are serine-proteinases which can act on various substrates. Their role may be to cause partial degradation of the matrix allowing easier access for the more specific enzymes.

To determine if B16F1M2 cells were capable of degrading the matrix, intact extracellular matrix was radiolabelled with ^{35}S (glycosaminoglycans) or [^{3}H]-proline (collagens). Breakdown products labelled with ^{35}S were released by the cells, but this was inhibited by pretreatment of the cells with retinoic acid in a concentration-dependent manner. However, although ^{3}H-labelled compounds were released, the degradation of the matrix was essentially unaffected by retinoic acid pretreatment. This is in contrast to other findings and as such requires further investigation.

When specific substrates were used to determine the production of individual degradative enzymes, similar results were obtained. Intracellular glycosidase activity was inhibited by retinoic acid and this was concentration-dependent. Conversely, extracellular glycosidase activity was slightly increased in the presence of retinoic acid up to a concentration of $10^{-7}M$, but was reduced to approximately base levels at $10^{-6}M$. An attempt was made to examine type I and type IV collagenase production, but this proved unsuccessful. No activity could be detected for either enzyme.

Plasminogen activator activity was found to increase if the cells were pretreated with retinoic acid. This was the case for both intracellular and extracellular levels. Why this should occur is unclear.

The effect of tumour cells on the host cells may be of importance in the metastatic process. For example, B16F1M2 melanoma cells were found not to produce hyaluronate, one of the requirements for cell migration. These cells may stimulate the surrounding host cells to produce tracts of hyaluronate to allow

migration. Fibroblasts, when suspended in a type I collagen gel solution and in the presence of serum, will ellicit gel contraction by reorganising the collagen to form fibrils, and this can be used as a model dermal equivalent. The effect of B16F1M2 serum-free conditioned-medium on the process of contraction was observed. Serum-free tumour cell-conditioned medium brought about gel contraction in a similar manner to control gels containing serum. This contraction was inhibited if the medium was conditioned in the presence of 10^{-6}M retinoic acid. This would suggest that retinoic acid can alter fibroblast function in such a way as to prohibit their ability to reorganize the collagen. The ability of tumour-conditioned medium prepared in the presence or absence of 10^{-6}M retinoic acid to modulate fibroblast glycosaminoglycan expression was also examined.

B16F1M2 cell-conditioned medium was found to greatly increase the amount of hyaluronate produced by the fibroblasts. This was slightly decreased by the presence of 10^{-6}M retinoic acid, but this level was still above that from control cultures. This would suggest that B16F1M2 cells are indeed capable of stimulating host fibroblasts to produce hyaluronate and that retinoic acid has little or no effect on this stimulation. The sulphated glycosaminoglycans expressed by the fibroblasts were decreased in the presence of tumour cell-conditioned medium, but the decrease was slightly reduced if the medium was conditioned in the presence of retinoic acid. It is unclear if this is related to the observed inhibition of collagen gel contraction. However, it would suggest that B16F1M2 cells produce factor(s) which have a similar effect to those found in serum. How, exactly, retinoic acid acts on the production and function of the factor(s) is unclear.

4:7. POSSIBLE FUTURE WORK.

1. The work presented here was carried out on one melanoma cell line and used only one retinoid. Thus further investigation is required using other melanoma cell lines and other forms of retinoid. This would allow comparisons to be made on both the effectiveness of retinoids as possible compounds for use in the inhibition of metastisis and on their usefulness within different cell lines.

2. The production of collagenases by B16F1M2 melanoma cells requires further investigation. The results presented in this thesis are in contrast to other reports found in the literature and the reason(s) for the differences is of importance in the metastatic cascade.

3. The observed increase in plasminogen activator activity after retinoic acid-pretreatment is of interest. Further investigation may reveal whether the increase is due to an increase in enzyme production or to a decrease in enzyme inhibition. This may also incorporate the role of plasminogen activator inhibitors in the process.

4. The mechanisms of cell adhesion to the extracellular matrix provides another possible project. The expression of the various cell adhesion molecules and how their expression is affected by retinoic acid is an area of importance. Any alteration in the expression of these molecules may be associated with the metastatic process of the melanoma cells, and possibly give an indication as to the mode of action of retinoic acid within this particular system.

5. Also in relation to adhesion, the role of cell surface proteoglycans requires further investigation. Affinity work was attempted during the course of the project, but proved to be unsuccessful. Further isolation of the surface proteoglycans using different methods may allow identification of potential adhesion substrates recognised by these molecules.

6. No attempt was made to investigate the expression of either the nuclear retinoid receptors or the cellular retinoic acid binding proteins by B16F1M2 melanoma cells. The effect of retinoic acid pretreatment on the expression of these receptors and binding proteins may provide useful information in the elucidation of the mechanisms involved in the metastatic cascade.

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