THE CELLULAR RESPONSE TO GLUCOCORTICOIDS IN HUMAN ASTROCYTOMA

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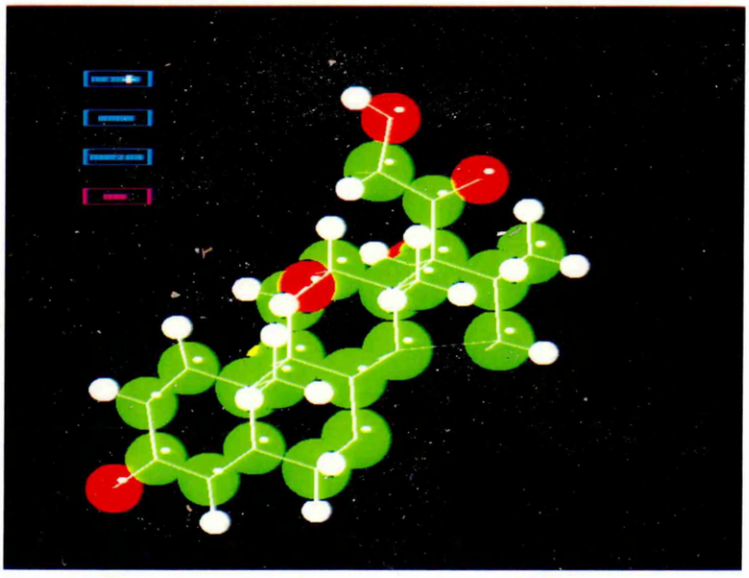
THE STRUCTURE OF DEXAMETHASONE



Key

- carbon
- hydrogen
- oxygen
- fluorene

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SUMMARY

Glucocorticoids have been widely used in reducing the oedema associated with brain tumours and post-operative pain. In vitro, they have been shown to inhibit growth of several different cell types, for example lung tumours and gliomas. Glucocorticoids have also been found to encourage immature malignant glioma cells to differentiate. This was measured by a reduction in immature properties, such as plasminogen activator activity, and the appearance of differentiated characteristics, such as glutamine synthetase activity.

Malignant cells have been proposed to arise from a breakdown in cell communication between the cell and the environment. As the cell's surface is the first point of contact between the cell and the environment, it has been postulated that an alteration in the composition of the plasma membrane may lead to a breakdown in cell communication. Carbohydrates are of particular interest as they are mainly situated on the outside of the membrane, and in spite of their relatively small proportion by weight they can cover the entire surface of the cell. Alterations in both glycoproteins and proteoglycans have been reported to differ between normal and malignant cells. Thus this alteration in surface carbohydrates may be important in explaining the malignant cells' abnormal behaviour.

In the present investigation dexamethasone and methylprednisolone were shown to reduce the saturation densities and labelling indices in a number of early passage and continuous cell lines. It was shown that the glucocorticoids exert this effect through a cytostatic rather than a cytotoxic process. This was shown by a clonogenic assay where no decrease in the number of colonies was observed in the cells which had been pretreated with either dexamethasone or methylprednisolone. The glucocorticoids were observed to have a biphasic response: at low cell concentrations they enhanced growth while at high cell densities they inhibited growth. In the absence of serum both steroids at

2.5×10^{-9}M increased the saturation density to the level reached in serum.

The exact mechanisms by which the glucocorticoids exert these effects have not yet been fully elucidated. A breakdown in cellular communication has been suggested by the abnormal behaviour of malignant cells. Normal cells continue proliferating until cell-cell continuity is established, whereas malignant cells are seen to continue proliferating and possess the ability to grow on top of one another. This implies that interactions at the cell surface are important in normal growth control. In vitro the cell's environment has been shown to influence cell shape, proliferation and differentiation, which suggests that the cytoskeleton may play an important role in these processes. An intricate matrix of microfilaments has been shown to exist between the interior of the cell, the cell membrane and the extracellular matrix. Proteoglycans and glycoproteins have been shown to be associated with this matrix in normal cells. Thus, an alteration in the proteoglycan and glycoprotein composition may lead to a breakdown in cell communication. Proteoglycan and glycoprotein composition has been reported to differ between malignant and normal cells.

It was asked in the present investigation whether the glucocorticoids exerted their cytostatic effect through altering the molecular composition of the cell surface. Examination of the iodinated glycoproteins of the cell by gel electrophoresis did not reveal any consistent change, however gel exclusion chromatography of protease digests of the cell surface showed that the presence of glucocorticoids increased the incorporation of ^{3}H glucosamine. Ion exchange chromatography showed that the presence of glucocorticoids increased the proportion of material associated with the cell relative to the released fraction and decreased the proportion of hyaluronic acid compared to the sulphated glycosaminoglycans.

It was concluded that the glucocorticoids were modifying the surface of malignant cells to resemble that of their normal counterparts in terms of proteoglycans. This alteration in

proteoglycan composition might account for the alteration in the cell's physiological activity.

CHAPTER 1

GENERAL INTRODUCTION

The aim of this report is to gain a deeper understanding of how glucocorticoids exert their cytostatic effect at the cellular level in malignant glioma cell lines. Glucocorticoids have been widely used for many years to reduce intracerebral pressure associated with conditions such as brain tumours (Selker et al, 1979). In addition to symptomatic improvements, it has recently been shown that these agents may directly reduce the size of the tumour itself. In vitro, glucocorticoids have been shown to inhibit growth of several different cell types, for example lung tumours (M^{C}Lean et al, 1986) and gliomas (Freshney et al, 1980a). However, the exact cellular mechanisms through which these effects are exerted are still elusive.

The composition of the extracellular matrix and the plasma membrane have been found to have profound effects on cell behaviour. It was initally asked whether the malignant cells' behaviour was due to an alteration in the composition of these structures. If so, do the glucocorticoids exert their cytostatic effect by altering the composition of the plasma membrane and the extracellular matrix to resemble that of normal cells?

An attempt was made in this report to investigate these questions by examining the effect of glucocorticoids on the composition of glycoproteins and proteoglycans associated with the plasma membrane and the extracellular matrix. Before these questions can be addressed, the mechanisms involved in normal cell growth need to be understood.

Characteristics of growth control

1. Normal cellular behaviour

1.1. Growth Control In Normal Tissues

The organs of the body all grow to a certain size and then cease to grow. Some, however, have the capacity to regenerate in response to injury. Such a response has allowed normal growth regulatory mechanisms to be probed.

Removal of cells from a confluent monolayer *in vitro*, as happens when a wound is produced, results in the cells at the periphery of the site undergoing a burst of proliferation and migrating into the "denuded area" until cell-cell continuity is re-established (Martz and Steinberg, 1971). The tissue continues proliferating until the cells attain their original thickness and density, which may involve a temporary overshoot in cell number. An increase in DNA synthesis in this system is detectable within a couple of hours after injury, while mitotic activity reaches a peak within a couple of days. These experiments suggest that cell-cell interactions are extremely important in controlling normal cell growth.

1.2. What are the factors involved in such a process?

The size of mature tissues is maintained by the number of cells undergoing mitosis exactly equalling those lost through either cell death or cell migration. The majority of cells - with the exception of haematopoietic cells in the bone marrow, basal cells in the epidermis of the skin and the lining of the GI tract - are not cycling. However, these non-cycling cells can be encouraged to start dividing, when an organ such as the liver is damaged (Goss, 1978b).

Surgical removal of part of the liver triggers a wave of mitotic activity in the remaining piece until the organ attains its original size. However, the rate of mitosis differs among the different cell types found in the liver. The peak mitotic rate is

reached by parenchymal cells at around 24 hours, Kupffer cells a day later, and endothelial lining cells 3-5 days after the parenchymal cells. Such observations pose two questions which are relevant to theories of growth control. What factor(s) stimulates the liver cells to divide and controls the extent to which regeneration occurs, and what are the signals which inhibit further proliferation once the correct organ size has been attained? The above system provided some of the first evidence for the existence of growth factors in the circulation.

Bucher (1963) was able to demonstrate, using an elegant experiment, that one of the many factors responsible for the induction of mitosis is present in the blood, by using a pair of rats which had their circulations surgically connected. If two-thirds of one of the livers was excised, then mitosis increased in both the damaged and unaffected livers. Fisher et al in 1971 carried out experiments using a series-arrangement of transplanted and host liver remnant tissues, in which they diverted portal blood through a partial liver transplant in a partially hepatectomised rat. The transplant receiving the portal blood responded in the same way as the liver remnant in the non-transplanted host. However, the response in the host liver remnant, which was in series with the transplant, was considerably reduced. Therefore, it was concluded that the transplant used up most of the mitogenic factors present in the portal blood, leaving little available to stimulate the host's liver. This led to the view that the promotion of mitogenic activity in the liver was not the result of decreased concentrations in a negative feedback system such as is found in the skin (Goss, 1978a).

Further evidence of a positive, rather than a negative control system, came from <u>in vitro</u> experiments carried out by Paul et al (1972) using foetal rat liver cells. He found that these cells responded to serum from partially hepatectomised rats by increasing the uptake of [^{3}H] thymidine and [^{3}H] leucine, whereas serum from normal rats caused no such response. It seems unlikely that a negative control system, such as a chalone, would result in this increased uptake of nucleic acid and protein precursors. In

experiments carried out by Czeizel et al (1962) the effects of radiation and bone marrow injections on rat liver regeneration was studied. Injection of unirradiated syngeneic bone marrow into irradiated animals restored liver regenerated growth to normal, while an excess of bone marrow given by injection produced excessive regeneration, ie. the bone marrow appeared to over-ride the normal homoeostatic controls. This implies that the hormonal factors responsible for liver regeneration are not produced by the liver itself, but probably by some "central" machinery which controls tissue proliferation and organ size within the body.

1.3. Characteristics of growth in vitro

Tissue culture provides one of the few models where the various components described in the previous section can be investigated individually, as it allows the environmental conditions to be closely controlled. This method has the potential for comparing growth characteristics in normal and malignant cells of similar lineage, and at similar growth rates.

Growth of most mammalian cells in vitro requires supplementation of a chemically defined medium with serum. The complex nature of the serum has made the elucidation of the various components, which promote and inhibit cell proliferation, a difficult and time consuming occupation. Under conditions of serum restriction, the cells become arrested in the GO/G1 phase, (see Figure 1), of their growth cycle and DNA synthesis is reduced, (Lindgren and Westermark, 1977). However, these cells can be encouraged to continue proliferating in the presence of fresh serum, which results in higher final saturation densities. For many cell types, saturation densities have been found to be directly proportional to the concentration of serum in the culture medium. This implies that cellular proliferation is regulated by the availability of mitogenic factors present in the growth medium. Growth factors such as EGF* (1µg/ml) can stimulate cells to proliferate in the absence of serum.

EGF is a potent polypeptide mitogen for epidermal cells in vivo and for a variety of cultured cells in vitro, whose mitogenic

* epidermal growth factor

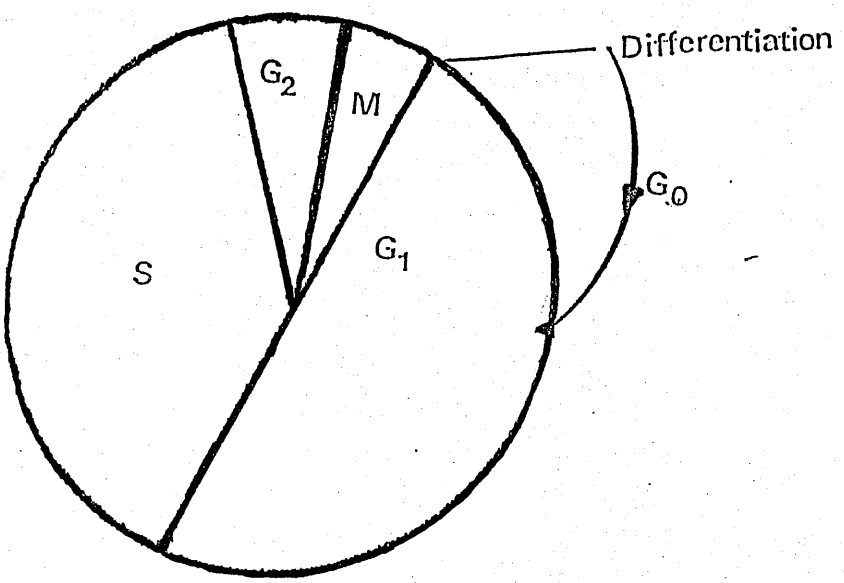


Figure 1

A diagrammatic representation of the mammalian cell cycle taken from Plumb and Wright, 1985. Mitosis (M) is normally completed within 1 hour and is the final phase of the cell cycle. Upon completing mitosis the cells can either remain in the proliferative compartment (G1) or can leave the cell cycle and start to differentiate. The length of the G1 phase is variable, 3 hours in rat small intestine and 95 hours in mouse epidermis. Some cells enter a resting phase (Go) from which they can return when circumstances are appropriate. The synthetic phase (S) is normally about 8-12 hours for most mammalian tissues, it is this phase where DNA doubles.

effect is mediated through a specific receptor (Jakobovits, 1988). The initial identification of EGF was by its ability to induce premature eyelid opening in newborn mice, along with observations that it was involved in the development of embryonic lung in rabbits (Catterton et al, 1979).

A cell will interact with adjacent cells and with the structural components of the extracellular matrix, via the external surface of its plasma membrane (Nicolson, 1976). This membrane is the barrier between the cell and the extracellular environment. It contains the cell's antigenic and chemical determinants along with receptors for signal molecules whose information must be transduced through the lipid bilayer. The exact mechanisms through which these cellular interactions take place have not yet been fully elucidated. However, it has been established that cell development requires the surface membrane being able to receive and transmit regulatory signals from its environment (Bissel et al, 1982). This suggests that any alteration in the composition of the plasma membrane may result in a breakdown in the normal cell's development and result in the appearance of large quantities of immature cells.

1.4. Influence of the extracellular matrix on cell shape, proliferation and differentiation.

In vivo, it is the extracellular matrix (ECM) which is important for maintaining order throughout the tissue. The composition of this matrix varies between different regions of the body (Hay, 1984). The differences in the biochemical composition of the extracellular matrix from various sources has been correlated to its various functions. For example, in cartilage the extracellular matrix combines the tensile strength of type II collagen fibres with compressive resilience, provided by the water holding properties of hyaluronic acid and proteoglycans with long side chains, whereas in bone, the tensile strength contributed by type I collagen is combined with rigidity through deposition of calcium phosphate crystals in the extracellular matrix. Connective tissue cells, such as chondrocytes and dermal

fibroblasts are completely surrounded by their extracellular matrix. Some cells are separated from adjacent connective tissue by a specialised sheet of extracellular matrix, known as the basement membrane or basal lamina. A basement membrane may completely surround individual cells, as in the muscle, or make contact with only one cell surface, as in the epidermis. Some extracellular matrix constituents are widely distributed: type I collagen is found in skin, tendon, cornea, ligaments, bone and internal organs. Other constituents have a more restricted distribution: type X collagen is specific to hypertrophic cartilage and laminin is only found in basement membranes.

The substrate on which the cells are grown can profoundly influence cell morphology and behaviour as shown by the following example. Corneal epithelial cells plated onto a plastic substrate adopt a flat, monolayer appearance whereas on collagen gels they become stratified with cells in the basal layer adopting a cuboidal shape (Gospodarowicz et al, 1978). The latter cellular appearance is similar to that found in vivo, emphasising the importance of the substrate in relation to the cell's shape.

Such alterations in cell shape can profoundly influence both cell proliferation and differentiation. In order to control the degree of cell spreading, cells were plated onto substrata coated with different concentrations of poly 2-hydroxy-ethyl methacrylate (polyHEMA) (Folkman and Moscona, 1978). As the polyHEMA concentration is increased, cell spreading and proliferation are progressively inhibited. This reduction in cell proliferation is accompanied by a decrease in DNA synthesis. Precise control of cell shape in the absence of intracellular contacts is achieved by plating individual cells onto adhesive islands of various sizes (O'Neill et al, 1986). On the largest island, cell spreading is maximal but becomes progressively restricted as the island size is reduced, this is accompanied by a reduction in the amount of DNA being synthesised by the cell. The latter concept is reinforced by the increase in DNA synthesis upon mechanically stretching epithelial cells (Brunette, 1984). By plotting the proportion of cells synthesising DNA against the degree of spreading, a classic

dose response curve was obtained (O'Neill et al, 1986), suggesting that a direct relationship between DNA synthesis and the degree of spreading exists.

Cell polarity can also be influenced by an alteration in cell shape. Upon plating fibroblasts and epithelial cells onto a Type I collagen gel, the fibroblasts were seen to become highly elongated and enter the gel, whereas the epithelial cells remained on top of the gel and became polarised both morphologically and functionally (Hay, 1984; Schor, 1980).

Cell shape, in addition to cell polarity and proliferation, can influence cell differentiation. This is illustrated in the following examples. Friend cell-fibroblast hybrids when grown in suspension are spherical in shape and could be induced to express haemoglobin, whereas adherent clones which possess a flatter morphology could not express haemoglobin (Allan and Harrison, 1980). Benya and Shaffer (1982) demonstrated that chondrocytes in tissue culture can produce collagen type II, a specific differentiated characteristic, when grown in a suspension culture but not when grown as a monolayer.

Changes in cell shape can in turn lead to an alteration in the cell's biological activity as illustrated by the following experiment carried out by Gospodarowicz et al (1978). Corneal epithelial cells grown on plastic are sensitive to fibroblast growth factor (FGF), whereas, on collagen-coated dishes they respond to EGF rather than FGF. The latter response is similar to that found in vivo. It is therefore quite clear from these experiments that the substrate is important in vitro for maintaining cell shape and hence its biological activity.

1.5. Growth control at the molecular level

The fact that the cell's environment can alter its shape, which in turn can influence cell proliferation and differentiation, suggests that the cytoskeleton may play an important role in these processes. Singer (1979) was among the first investigators to illustrate a continuity between the interior of the cell, the cell membrane and the extracellular

matrix. Immunofluorescence microscopy using antisera against actin, tubulin and other cytoskeletal proteins has revealed a complex organisation of cytoskeletal fibres inside the cell, many of which seem to be associated with the plasma membrane (Bissell et al, 1982).

The microfilaments extend the contact point through the cytoplasm to the nucleus. There is also a mesh of fine filaments termed micotrabeculae (Wolosewick and Porter, 1976) from the nucleus to the plasma membrane with distinct connections to each. While many filaments are associated with the nucleus, the best example is seen with intermediate filaments (Lazarides, 1980). In fibroblasts treated with colcemid, vimentin filaments, visualised by immunofluorescence, collapse to form a perinuclear cap, which suggests a connection with both the nucleus and the microtubules. Modulation of actin assembly results in changes in cell shape and hence in the synthesis, assembly and organisation of other cytoskeletal proteins (Ben-Ze'ev, 1985).

The relationship between cell spreading and the cytoskeleton is illustrated elegantly in the following experiment. When suspended cells are plated in the presence of high concentrations of lectin which cross links cell surface carbohydrates and immobilises the cells, or alkaloids which disrupt the microtubular or microfilamental cytoskeletal systems, cell spreading is effectively inhibited but the cells are still able to attach to the substrate. Under these conditions the rate of protein synthesis recovers in the control monolayer level in about 6-8 hours and this is not markedly affected by the limited degree of cell spreading. This suggests that protein synthesis recovery is triggered by establishing only a few contact points between the cell and the substrate and that extensive cell spreading is not a prerequisite.

A close association has been shown between fibronectin and either collagen fibres on the exterior of the cell or intracellular proteoglycans such as heparan sulphate (Woods et al, 1984) on the interior. This results in an interaction between the extracellular fibrous network and the cytoskeleton through

mediation of fibronectin and its cell membrane receptor. This in turn influences cell-cell and cell-matrix interactions. However, the reverse situation also exists where an alteration in the cell-cell or cell-matrix interactions may influence changes in the cytoskeleton.

Thus, the above results suggest the existence of an intricate feedback system where cell shape and the extracellular matrix can play an important role in controlling cell proliferation and differentiation (Figure 2). It also illustrates a possible pathway, at the cellular level, through which information can be sent either between the cell and the extracellular matrix, or, from cell to cell within a tissue. Such a feedback system will be mentioned again in relation to proteoglycan and glycoprotein composition (Chapters 3 and 4).

1.6. How do alterations in the cytoskeleton lead to alterations in gene expression?

Although a change in cell shape may be a consequence rather than a cause of changes in differentiated gene expression (Horton and Hassel, 1986), evidence does exist which suggests that an alteration in the cytoskeleton does lead to alterations in gene expression. Polyribosomes and elements of the Golgi and rough endoplasmic reticulum are specifically associated with the cytoskeleton, therefore alterations in the cytoskeletal structure could influence mRNA translation and protein transport. Lenk et al (1977) showed that polysomes were associated with the cytoskeletal framework extracted in detergent. Fulton et al (1980) and Cervera et al (1981) have provided valuable evidence for a significant functional role for this structural association. They have demonstrated that the translation of mRNA coding for tubulin and polio virus takes place on polysomes attached to the cytoskeleton. Furthermore, the state of polymerisation of the cytoskeletal components influences the rate of translation. mRNA appears to be bound to the cytoskeleton while being translated but afterwards is released from the framework to become soluble. An increase in free tubulin or actin through microtubule or

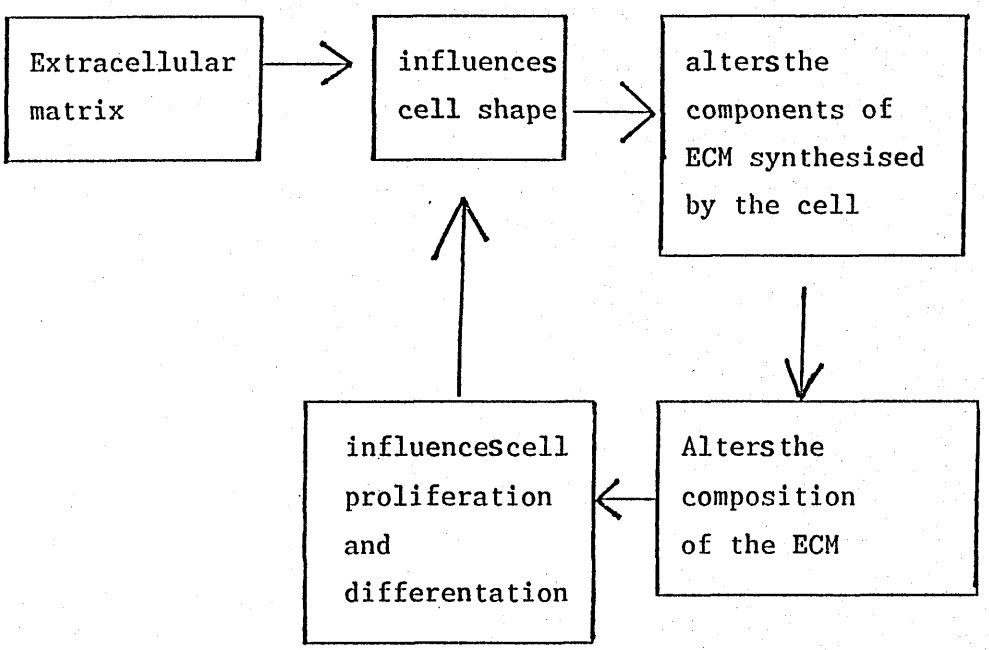


Figure 2

A schematic diagram illustrating the inter-relationship between the extracellular matrix, cell shape, cell proliferation and differentation.

microfilament disassembly can also trigger changes in gene expression.

The actual relationship between cell shape and attachment to the nature of the expressed gene products is illustrated in experiments carried out by Penman et al 1982 (refs. within Bissel et al, 1982). Upon passaging the anchorage dependent 3T6 cells in suspension culture, mRNA synthesis and turnover were found to decrease rapidly. Thus, the net result is the maintenance of a constant level of mRNA. Despite this, protein synthesis gradually decreased indicating withdrawal of mRNA molecules to an "non-translatable" pool. These cells were then grown in a monolayer culture where protein synthesis was found to rapidly increase, even when new mRNA synthesis was inhibited with Actinomycin D. Inhibitors of cell spreading were used in an attempt to elucidate the contributing effects of either attachment and/or spreading in the terms of translational and transcriptional processes. The results indicate that attachment of cells to their substratum was sufficient to reactivate protein synthesis, but spreading was necessary for reactivation of mRNA synthesis. These results thus indicate two distinct ways by which contact with substratum (or shape) can influence gene expression: cell attachment appeared to be sufficient for translational control while, spreading was necessary for transcriptional control.

Therefore, the cytoskeletal framework, in addition to its role in maintaining cell morphology might have a major regulatory role in gene expression by virtue of its close association with major macromolecular processes.

2. Malignant cell behaviour

With the exception of cells infected by oncogenic viruses, neoplastic cells produce no molecules which are not found in either normal adult or embryonic cells. For a malignant cell to survive and prosper, it must acquire products and functions which enable it to grow and divide rapidly, invade surrounding host tissues and in many cases migrate to various sites of the body via the blood and lymphatic systems. None of these processes are, in

themselves, biologically abnormal. Rapid cell growth is observed in the process of tissue regeneration in response to partial removal of or injury to the liver (1.2). "Invasion" and migration of many types of cells occurs during embryogenesis and in adults. Leukocytes are one example of cells which invade tissues in order to carry out their physiological role (Goss, 1978c). Cell migration is a prominent feature in wound healing (1.1). It may therefore be suggested that in malignant tissues, the mechanisms occurring are not abnormal as they are found either in foetal or adult tissues, but what is abnormal is that they are being exhibited at an abnormal time and seem to be uncontrolled and progressive.

Tumours are thought to arise from a breakdown in one of the mechanisms correlating cell renewal with cell loss. A good example of such a process is seen in psoriasis and excema (Goss, 1978a). Instead of cell division becoming inhibited when the epidermis has attained the correct size, the cells continue proliferating and appear as epidermal scales. The life span of normal human epidermal cells is approximately four weeks and only one cell divides at any one time in the basal monolayer. In psoriasis, the average cell divides, differentiates and is sloughed off in less than a week. The germative layer in this disease instead of being a monolayer is three cells deep and more than one cell, at any one time, is undergoing mitosis. It has been proposed that such a disease arises from a breakdown in the relationship between cell loss at the epidermal surface and cell renewal at the basal layer.

Hence, the above results suggest that malignant cells may arise from a breakdown in the controlling mechanisms between cell loss and cell renewal.

2.1. Characteristics of malignant cells

Malignant cells, in general, do not possess material which is not found at some stage in the normal cell's development, therefore, they may be abnormal in the respect that they display material which is not normally seen at that particular period in

time. Malignant cells are seen to possess characteristics of foetal cells, as illustrated by the re-expression of foetal antigens. Tumours derived from gut epithelium have been found to produce in some instances, large quantities of carcinoembryonic antigen (CEA), while tumour cells derived from liver or yolk sac tissue may produce alpha-fetoprotein (AFP) (Sugarbaker et al, 1982). Both of these substances are normally expressed in foetal tissues and are absent in the corresponding mature tissues. This relationship between malignant and foetal cells has been found to exist in terms of both functional activity and morphology, and is most prominent in the more aggressive tumours.

Malignant cells are able to escape the normal constraints imposed on them by their environment, and this is observed in vivo by the appearance of growths (Martz and Steinberg, 1971 and Lindgren and Westermark, 1977). Malignant cells can grow more efficiently at lower serum concentrations in vitro than normal cells. Tucker et al (1981) There is a tendency with time for tumour cell populations to increase their growth rate and to show further evidence of escape from local control mechanisms. Usually this appears not to reflect a shortening of the cell cycle time, but rather an increase in the "growth fraction" i.e. an increase in the number of cells within the neoplastic population actively proliferating (Westermark, 1973). This leads to a decrease in the number of cells undergoing differentiation. In some circumstances this further escape from growth regulation may be related to an altered response to circulating hormones, through either loss of specific receptors or an increase sensitivity to growth factors (Gross et al, 1983).

2.2. How do malignant cells arise?

Malignancy has been found to be reversible in a number of tumour types, as illustrated in neuroblastomas (Prasad, 1983), squamous carcinomas (Pierce and Wallace, 1971) and leukemias (Gootwine et al, 1982). Thus, it may arise from specific genes being activated at the wrong time. Malignant cells may theoretically arise from either:-

(i) A block occurring in the natural maturation pathway. This pathway may be regarded as a series of steps through which the cell gains more and more differentiated characteristics, until it becomes fully differentiated (Sartorelli, 1985). If the body regulates tissue size through a negative feedback system, a reduction in the number of fully differentiated cells present will result in an increase in the number of immature cells entering the maturation pathway. A block occurring in this pathway by whatever means, would prevent the immature cells from progressing through to their fully differentiated state. As the body is unable to detect these partially differentiated cells this would result in more and more cells entering the maturation pathway, which results in an increase in the number of partially differentiated cells present. As this block may occur at any point along the maturation pathway, this may explain the large heterogeneity found amongst tumour cells of similar lineage (Yates and Stephens, 1987)

OR

(ii) The oncogenic stimulus may act on fully differentiated normal cells in such a way as to change their properties to that of malignant cells. As these cells continue to proliferate, they might gradually lose some of their differentiated characteristics. This concept is illustrated by an elegant experiment carried out by Rabes et al (1970), where rats were treated with diethylnitrosamine, a carcinogen which induces liver tumours, and the rate of mitosis in the livers were investigated. Initially, when the mitotic index in the parenchymal cells remained at the control level, the response to hepatectomy was normal. However, as the mitotic index in the parenchyme cells of the carcinogen-treated animals began to increase due to neoplasia, the response to partial hepatectomy was correspondingly reduced. Thus, as malignant cells proliferate there may be a corresponding loss of normal growth control.

2.3. What changes at the molecular level would bring about these abnormal properties?

Two areas have been proposed where such changes can occur:-
(1) Alteration in the composition of the plasma membrane (Nicolson, 1976). As the plasma membrane is the first point of contact between the cell and its environment, it is important in passing information into and out of the cell.
OR
(2) Alteration in the composition of the extracellular matrix. Experimental evidence has suggested three areas where such a breakdown may occur :-
(i) Reduction in the quantity of fibronectin present. The concentration of fibronectin in malignant cells is less than that in normal cells (Hynes, 1981).
(ii) Increase in the quantity of high molecular weight fucose-glycopeptides (Van Beek et al, 1978) and degree of sialation (Warren et al, 1972). Differences are found in the linkage region between the polypeptide and carbohydrate and also in the number of side chains attached to the mannose cores (see Chapter 3). Considerable evidence exists suggesting a possible correlation between the degree of glycopeptide sialation and malignancy.

Glycoproteins comprise 80% of all cell carbohydrates and are mainly situated on the outside of the cell's membrane. In spite of their relatively small proportion by weight, the surface carbohydrates can cover the entire surface of the cell and thus form the first point of interaction with other cells and the extracellular matrix. Due to their large structural variability, glycopeptides can theoretically carry large quantities of information (Kornfelt and Kornfelt, 1980). Van Beek et al, 1978 found that glycoproteins from malignant glia cells were more highly sialated than their normal counterparts. Such changes in the degree of sialation in the plasma membrane may influence cell-cell and cell-matrix interactions through alterations in surface charge, this may lead to a breakdown in the continuity between the interior of the cell and the plasma membrane.

(iii) Proteoglycan production; Proteoglycans differ from the glycoproteins described above in the following way. Proteoglycans are much larger and usually consist of 90-95% carbohydrates by weight, in the form of many long unbranched glycosaminoglycan chains whereas glycoproteins contain a lower proportion of carbohydrate, (1-60% by weight) in the form of numerous, relatively short (>15 sugar residues), branched oligosaccharide chains which often contain terminal sialic acid residues. They are highly charged molecules due to the presence of carboxyl, sulphate and hydroxyl groups on the glycosaminoglycan molecules (Smets and Van Beek, 1984). High concentrations of hyaluronic acid have been associated with a variety of malignant tissues (Satoh et al, 1973).

3. BRAIN TUMOURS

3.1. Why study brain tumours?

Gliomas and primitive neuro-ectodermal tumours account for the majority of childhood neural tumours, occur in young adults rather than the elderly and are almost invariable fatal within two years of diagnosis. One of the major problems with this disease is that although the lesion may be microscopic, the neurological effects produced by both the tumour and conventional therapies can be significant (Kornblith et al, 1982). Therefore, an alternative treatment with fewer side effects would be advantageous. It is hoped in this report that by studying the differences between malignant and normal cells, a new site for chemotherapy may emerge.

3.2. Current treatment of brain tumours

A variety of methods are commonly used in treating brain tumours (Kornblith et al, 1982).

(i) Surgery to remove as much of the tumour as possible.
(ii) Radiotherapy to destroy the remaining tumour fragments.
(iii) Chemotherapy to prevent further replication of the remaining neoplastic cells.

Unfortunately, the effectiveness of each of these treatments is not often complete. Surgery may be virtually impossible without destroying vital areas of the brain, due to the invasive capacity of the tumour. The dose of radiation required to destroy all the glioma cells will often cause severe neurological disability through destroying normal cell function. Chemotherapeutic drugs are generally cytotoxic to both normal and malignant cells (Sartorelli, 1985). An ideal therapeutic method would consist of drugs which are able to differentiate between normal and malignant cells, without affecting normal cell function. Cytostatic drugs, such as glucocorticoids, are thought to be advantageous in this respect as they reduce the growth rate of malignant cells without affecting that of normal cells as the latter cells already exhibit density dependency.

Glucocorticoids have been widely used in relieving the symptoms of brain tumours, but the exact cellular mechanism(s) through which this is achieved are still elusive. Glucocorticoids have been shown to exert a cytostatic rather than a cytotoxic effect on the growth of malignant glial cells in vitro (Guner et al, 1977) and to induce the immature cells to differentiate (Almazan et al, 1986). However, recent in vivo evidence with lung cells suggests that these drugs may directly reduce the size of the tumour in vivo (M^{C}Lean et al, 1986). Glucocorticoids are thought to exert their effects at the nuclear level by combining with cytoplasmic receptors as depicted by Figure 3 (Alexis,1987). Braunschweiger et al (1978) suggested that this reduction in cell proliferation with methylprednisolone was due to a block occurring in the G1 phase of the cell's growth cycle. The cells already in the growth cycle are committed to complete the entire cycle before they can be affected. The latter effect is reversible, upon removing the glucocorticoid the cells are seen to synchronously progress through the growth cycle.

3.3. Concept under investigation

The concept under investigation in the present study is to find whether the cytostatic effect of glucocorticoids is exerted, at the cellular level, through altering the carbohydrate composition of malignant cells. Malignant cells have not been found to possess material which is not normally found at some stage in the normal cell's life cycle. This suggests that production of these materials themselves are not abnormal but it is the mechanisms regulating their production which are at fault. Since carbohydrates occupy a strategic position on the cell's surface, and their composition differs between normal and malignant cells, it was decided to investigate the carbohydrate composition of glioma cells in the presence and absence of glucocorticoids.

Malignant cells have been reported to possess larger molecular weight fucose-glycoproteins, which are more highly sialated than their normal counterparts. This relationship has

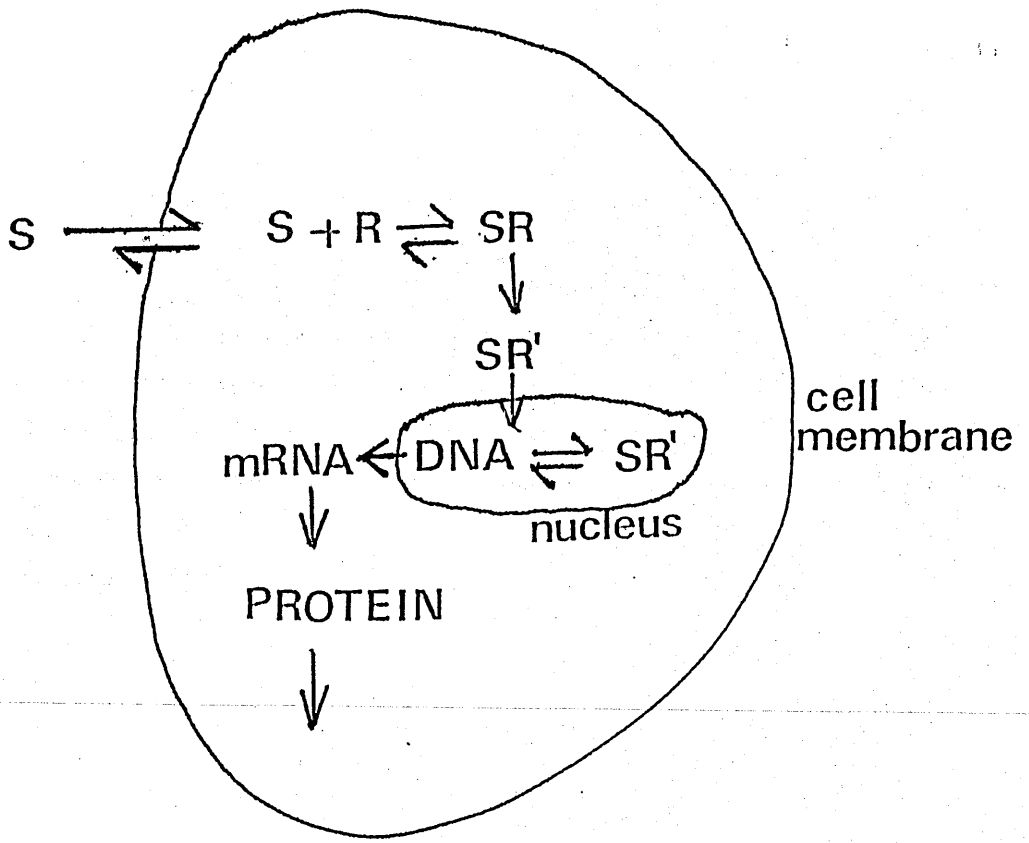


Figure 3

Mechanisms of steroid/receptor interaction

The steroid interacts with cytoplasmic receptors and the resultant conformational changes result in the complex having an increased affinity for the high-affinity acceptor sites on the chromatin, and translocation occurs. The presence of the complex in the nucleus activates the transciption of mRNA. Taken from Alexis, 1984.

been correlated by Poste and Nicolson (1980) to metastasis. Glimelius et al (1979) found that gliomas differed from glia in terms of their proteoglycan composition; malignant cells generally possess a higher proportion of hyaluronic acid compared to sulphated proteoglycans. Work carried out in this laboratory (Freshney et al, 1980a) showed that increased sialation of glycoproteins in gliomas could be reduced with dexamethasone. Previously, this difference between normal and malignant cells could be diminished by treating the malignant cells with neuraminidase (Warren et al, 1972; Van Beek et al, 1978). Therefore, it was asked whether the glucocorticoids were exerting their effect through altering the glycoprotein and proteoglycan composition of the malignant cells.

In this laboratory, McLean et al (1986) have previously shown that glucocorticoids exert their effect through inducing malignant cells to become more differentiated, both morphologically and functionally. Dexamethasone was observed to encourage glioma cells to become more differentiated in function, as illustrated by the increase in differentiated characteristics such as, γ-amino butyric acid (GABA) uptake and reduced plasminogen activator activity. Thus, it may be asked whether the glucocorticoids were exerting their cytostatic effect by inducing the malignant cells to become more differentiated through altering the carbohydrate composition at the cellular level.

This question was looked at in this report in the following way:-

(i) The second chapter looks at the cytostatic effect of dexamethasone and methylprednisolone on saturation densities and labelling indices. The cell's differentiated appearance was monitored in relation to its plasminogen activator activity. A decrease in plasminogen activator activity was found to accompany the re-expression of density inhibition in malignant glioma cultures.

(ii) Chapters 3 and 4 deal with the glucocorticoid's effect at the cellular level. Chapter 3 deals with the effect of glucocorticoids on glycoprotein composition. Glucocorticoids were found to reduce the high molecular weight glycoproteins

characteristic of malignant cells. Chapter 4 deals with the effect of glucocorticoid on proteoglycan composition. Glimelius et al (1979) have investigated the proteoglycan composition between malignant and normal glia cells, and found that malignant cells generally contained higher quantities of hyaluronic acid compared to their normal counterparts. A similar result was obtained by Satoh et al (1973) using hamster embryo fibroblasts transformed with either Herpes Simplex Type-2 or Simian Virus-40. (iii) Chapter 5 discusses how the cytostatic effect of glucocorticoids may be related to an alteration in either glycoprotein or proteoglycan composition in either the plasma membrane or the extracellular matrix. If the altered carbohydrate composition at the cellular level can account for the abnormal physiological properties of the malignant cells, then this type of investigation may reveal a new target site for chemotherapy.

CHAPTER 2

INTRODUCTION

INVESTIGATING THE CYTOSTATIC EFFECT OF GLUCOCORTICOIDS

In this chapter the cytostatic effect of glucocorticoids will be investigated in relation to saturation densities and labelling indices.

1. Growth characteristics of normal glia and malignant glioma cultures in vitro

Normal glial cells derived from adult brain have slow initial growth rates in culture and a finite *in vitro* life span of about 40-60 generations (Yates and Stephens, 1987). These cells are stable in culture and do not undergo spontaneous transformations into continuous or morphologically altered cell lines. Foetal glial cells adapt much more rapidly than normal glial to growth *in vitro* although their life span is short, about 6-8 generations in the majority of cases. Normal glia cells show density dependent growth inhibition in monolayer cultures where the majority of the cells were retained in the G0/G1 phase (see Figure 1) of the cell's growth cycle even in the presence of fresh serum. The mitotic rate of these cells is low (1-5%) (Martz and Steinberg, 1971; Lindgren and Westermark, 1977). This plateau phase can be maintained for months with a high percentage of viable cells provided the medium is renewed weekly.

Malignant cells can be grown in tissue culture with relative ease and a small percentage (20-40%) of the early passage lines derived from biopsy material may become permanent cultures, that is, appear capable of indefinite proliferation (Yates and Stephens, 1987). Gliomas are comprised of a heterogeneous cell population ranging from highly differentiated to undifferentiated cells. They attain higher saturation densities than normal glia, and only a small percentage of these malignant cells at confluence are retained in the G0/G1 phase of the growth cycle, i.e.

inhibition is not complete (Westermark, 1973 and Lindgren and Westermark, 1977). This accounts for both the higher saturation densities and labelling indices. However, a pseudo-plateau phase may be reached by these malignant cells in vitro where the rate of cell division exactly equals the rate at which the cells are being sloughed off into the medium. These malignant cells were found to be more susceptible than their normal counterparts to chromosome breakage, non-disjunction and ploidy changes (Nowell, 1986).

In general, malignant cells are generally defined by the pattern of molecular gene activation and "silencing". It is therefore necessary to consider the differences between these cells and their normal counterparts in terms of the deletion "switching off" of specific genes and the inappropriate expression of others (Nowell, 1986; Sartorelli, 1985). The observed effect is the loss of some differentiated characteristics, such as glutamine synthetase activity, and the appearance of less mature ones such as plasminogen activator activity (Frame et al, 1984). However, different tumours vary considerably in relation to the period of time and sequence in which these various aspects of tumour progression become apparent. In some instances, properties of far advanced malignancy may be established before the neoplasm reaches macroscopic size, while well differentiated slow growing tumours may persist for many years before undergoing a transition to a more aggressive form.

2. Proliferative control of normal glia and malignant gliomas.

The regulation of proliferation in both human glia and glioma cells has been studied in vitro (Freshney et al, 1980a and b). Westermark et al (1973) have shown that astrocyte-like cells, derived from an adult human brain, are subject to stringent control mechanisms in vitro. At confluence, only 1% of normal glia were found to synthesise DNA at any one time, while the addition of fresh serum resulted in only a transitory increase in DNA synthesis (Lindgren and Westermark, 1977). In contrast, 10-20% of either glioma or virus transformed glia-like cells, were still synthesising DNA upon reaching confluence. This reflects

the higher saturation densities reached by these malignant cells.

Plating normal glia onto a confluent monolayer of either glia, foetal human intestinal epithelium or fibroblasts, showed that proliferation was only inhibited by the glial substrata (MacDonald et al, 1985). Thus, proliferation was only decreased by plating these normal cells onto a confluent monolayer of a similar lineage. This suggests that there must be some type of communication between cells of similar type. Malignant glioma cells under similar conditions continued proliferating, thereby suggesting that they were able to escape, to a certain extent, from the constraints imposed on them by their environment. As previously mentioned in the general introduction, this increase in proliferation did not appear to reflect a shortening of the cell cycle time, but rather an increase in the "growth fraction" (Ford and Maizel, 1986). In some circumstances, this type of reaction may be due to an altered response of the cell to circulating hormones through loss of specific receptors, for example Gross et al, (1983) have shown a correlation between the down regulation of EGF receptors and the increase in plasminogen activator activity. The proliferative index, as shown by the labelling index, is a useful diagnostic tool in assessing the aggressiveness of brain tumours. Hoshino (1981) found that patients with brain tumours which had a labelling index of greater than 5% generally had less than a year to live, while those with labelling indices of less than 1% had a chance of more prolonged survival. This suggests that a drug which reduces the proliferative activity of malignant tissues may be a useful therapeutic tool in enhancing the life span of these patients.

3. The relationship between cell proliferation and differentiation

Experimental data from transformed cell lines shows that a loss of differentiated characteristics is accompanied by an increase in cell proliferation (Sartorelli, 1985). Thus, it may be proposed that by encouraging the malignant cells to become more differentiated this may decrease the abnormal behaviour of these cells. This concept has been previously studied in this

laboratory by looking at the maturation of glioma cell lines in relation to astroglial differentiation markers such as high affinity amino acid transport and glutamine synthetase activity (Frame et al, 1984; M^{c}Lean et al. 1986).

Kato et al (1981)

Glia maturation factor (GMF), isolated from cow's brain, was found to stimulate cell proliferation and maturation of normal glia but prevented overgrowth once the cells had reached confluence (Lim et al, 1981). GMF has been chemically characterised as consisting of a group of acidic proteins existing in multiple molecular sizes, the smallest of which is 23,000 daltons. These results suggest that GMF has the ability to restore contact inhibition, possibly through encouraging the cells to become more differentiated.

4. Plasminogen Activator

Plasminogen activators (PAs) are a specific group of serine proteolytic enzymes that specifically convert plasminogen to the active proteinase plasmin (Saksela, 1985; Dano et al, 1985). Plasmin has a broad substrate specificity and can break down a variety of tissue proteins. The best characterised substrate for plasmin in vivo is fibrin. There are two major types of PA, they differ from each other with respect to tissue distribution, molecular and immunological properties: a tissue type PA (t-PA) and a urokinase-like PA (u-PA). The u-PA is a 54 kDa enzyme composed of two disulphide-linked 30 and 24 kDa subunits (Dano et al, 1985). The native t-PA in contrast is a single 70 kDa polypeptide chain (Rijken and Collen, 1982; Edlund et al, 1983). Both the u-PA and t-PA are synthesised as proenzymes (Saksela, 1985; Dano et al 1985). Exactly how the pro-PAs are converted to active forms is not clear. The majority of cell lines cultured in vitro secrete the u-PA.

U-PA is thought to be responsible for the activation of plasminogen present in the extracellular tissues. In addition to u-PA being fibrinolytic, plasmin can degrade the non-collagenous matrix components such as laminin and fibronectin (Liotta et al, 1981). This will be mentioned again in Chapter 3. Both foetal

cells and malignant cells possess a high u-PA activity (Roblin and Young, 1980). The increased levels of u-PA has been associated with their ability to degrade the extracellular matrix in vitro (Liotta et al, 1981).

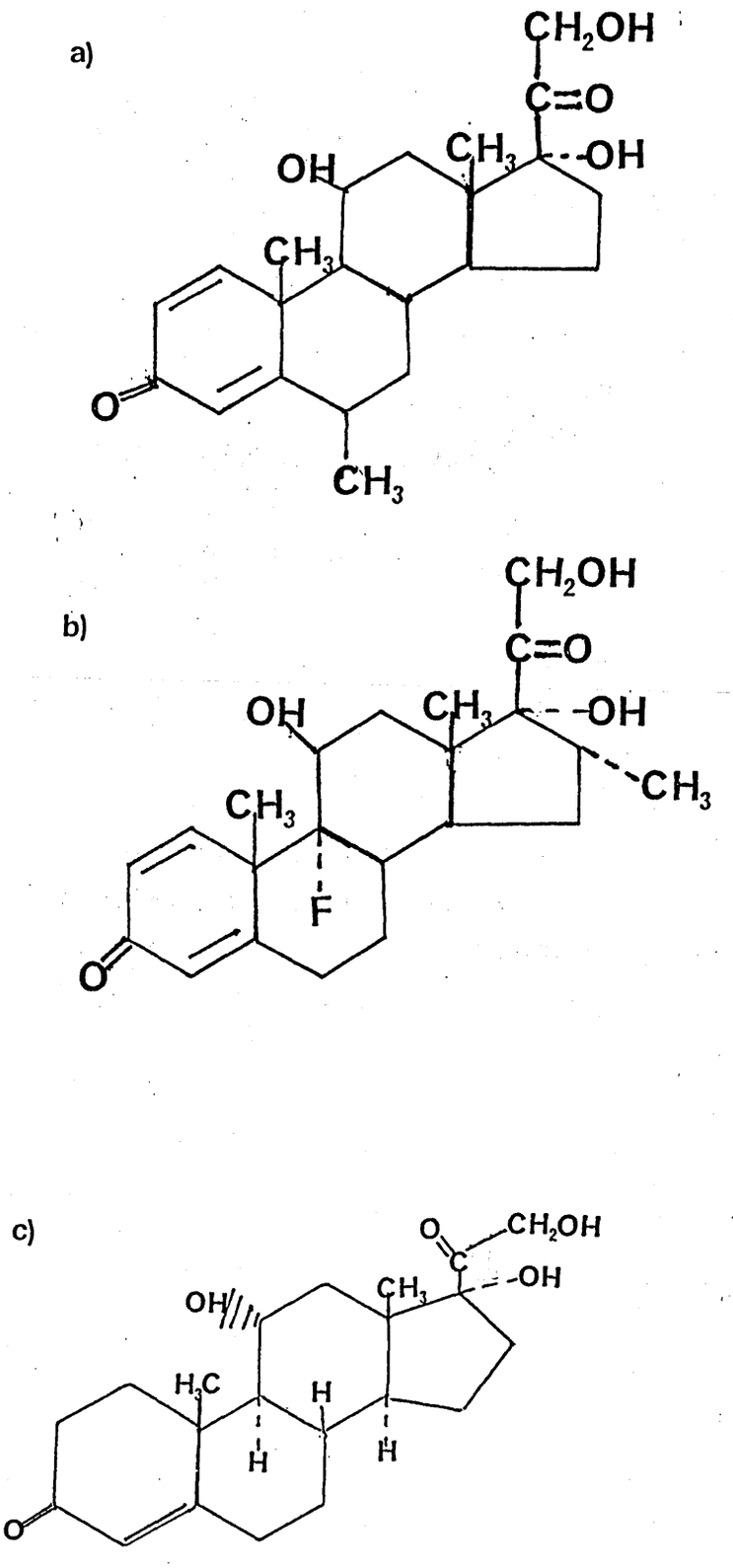
Dexamethasone has been shown by McLean et al, (1986) to inhibit u-PA activity. The glucocorticoid was found by Seifert and Gelehrter, (1978) to exert its activity by inducing the production of an inhibitor. This is clearly illustrated in the following experiment. PA activity was measured in Triton-X-100 cell extracts on ^{125}I fibrin-coated plates. When extracts of dexamethasone treated cells were mixed with extracts of control cells, u-PA activity was drastically reduced. Whereas, variant cells of rat hepatoma cells were fully resistant to inhibition of u-PA activity by dexamethasone. These variants were found to have no demonstrable inhibitor activity, as u-PA production in these cells was still sensitive to inhibitors produced from wild-type cells.

Gross et al, (1983) have shown an inverse relationship between EGF activity and PA activity. They found using Human A431 epidermoid carcinoma cells that an enhanced rate of EGF receptor inactivation is closely correlated with an increase in cellular PA activity. Blocking PA synthesis or activity by cycloheximide or dexamethasone prevented down-regulation of the EGF receptor. The removal of EGF from previously down-regulated cells resulted in the recovery of total cellular EGF binding activity with a concurrent loss of cellular PA activity. Thus, the effect of dexamethasone may be inversely related to the mitogenic effect of growth factors such as EGF.

The above results suggest that glucocorticoids may be exerting their cytostatic effect through encouraging the cells to become more differentiated. This is accompanied by a reduction in the rate of proliferation, however, the reverse situation may also exist i.e. by inhibiting proliferation, differentiation becomes reduced. In this chapter, the cytostatic effect of dexamethasone, methylprednisolone and 11-α-epihydrocortisone were investigated in terms of saturation density and labelling index. The structure of

these drugs are shown in Figure 4.

Figure 4



A schematic representation of the structure of a) methylprednisolone, b) dexamethasone and c) 11-α-epihydrocortisone. Taken from Mealey et al, 1971.

MATERIALS AND METHODS

TISSUE CULTURE

1. Cell lines

The cell lines used in this investigation were:-

Cell line	Passage level	Pathology	Origin
G-UVW	continuous	Anaplastic Astrocytoma	Institute of Neurological Sciences Glasgow
G-IJKt	"	"	"
G-CCM	"	"	"
G-ELL	"	"	"
BG-14	8	"	"
BG-28	7	Ependymoma	"
BG-30	6	Parieto-occipital lobe	W.G.H. Edinburgh
BG-35	2	Astrocytoma	Inst. Neurological Sciences Glasgow
BG-84	5	Recurrent malignant glioma	"
BG-103	10	"	"
EWLU	unknown	normal human foetal lung fibroblast.	Southern General Hospital, Glasgow

The passage number of the early passage cell lines is denoted by by the number after the cell name. All materials, unless otherwise stated, came from BDH and were of ANALAR grade. The growth medium unless otherwise stated, was a 1:1 mixture of Ham's

F10 (Flow Labs. ltd) and Dulbecco's modification of Eagle's Basal Medium (Gibco), i.e. F10/DMEM.

Solutions:-

Holding medium:-

Growth medium	500ml
Penicillin	125,000 units (Glaxo)
Streptomycin	125mg (Gibco)
Gentamycin	25mg (Gibco)

PBS:- Phosphate buffered saline without Ca^{2+} and Mg^{2+} (Dulbecco 'A'), (Oxoid).

Dissection PBS:- PBS containing penicillin, streptomycin and gentamycin at the above concentrations.

Phenol red free medium:-

HBSS-Phr	95ml
Vitamins	1ml
MEM AA	2ml
NE AA	1ml
Glutamine(200mM)	1ml
Glucose	1ml
HCO3	1.1ml
NaOH to pH7.2	

2. Making primary cultures from biopsy material

Biopsies were aseptically collected into holding medium. The tissue was finely chopped, washed three times in dissection PBS and the pieces were transferred into culture flasks, about 20-30 pieces per $25cm^2$ flask, containing holding medium with 10% serum and 0.5ml of crude collagenase (2000 units/ml; to give a final concentration of 200 units/ml ;Sigma) and incubated at 37^0C. Eighteen hours later the medium was removed, the sample was spun at 150g for five minutes and the material was reseeded in fresh

medium. The cells were then grown in growth medium, supplemented with 10% foetal bovine serum (Flow Labs. Ltd), and with a gas phase of 2% CO_2 in equilibrium with 8mM $NaHCO_3$ for approximately two weeks. After this period of time the cells were fed with medium containing no antibiotics in order to prevent the appearance of resistant organisms.

3. Maintenance of cell lines (monolayers)

Cells were trypsinised using 2ml of 0.25% trypsin (PBS) (Sigma) for 60 seconds. The trypsin solution was discarded. The cells were then incubated at 37^0C for 5-15 minutes, until they became rounded in appearance. The cells were resuspended and reseeded at $2x10^4$ cells/ml in a new culture flask (Nunc), which was gassed with 2% CO_2 and fed with fresh growth medium every 2-3 days. Early passage lines were used up to the 10th passage. A cell line whose passage exceeded 50 was assumed to be continuous. The cells were used from frozen stocks within 3 months of thawing.

4. Electronic cell counting

A homogeneous single cell suspension for cell counting was obtained by trypsin isolation. A sample of the cell suspension was then diluted 1:50 in 20ml of counting fluid using a 25ml disposable counting cup. The counting sample was mixed thoroughly and read on a Coulter counter ZB.

5. Cell freezing

Frozen stocks of all cell lines were maintained in liquid nitrogen. Cell suspensions of approximately $5x10^6$-$2x10^7$ cells/ml in culture medium containing serum and 10% dimethyl sulphoxide (DMSO) were frozen with an approximate cooling rate of 1^0C/minute for approximately 18 hours, and were then transferred to liquid nitrogen.

6. Thawing

Frozen ampoules were thawed out in water at 37^0C. The sample was suspended in fresh culture medium. The medium was changed after 18 hours in order to remove the DMSO.

7 Mycoplasma staining

Cultures fixed with 25% acetic acid in methanol, were checked for mycoplasma by treating with fluorescent DNA stain, Hoechst 33258 (Hoechst) at 0.05µg/ml for 15 minutes at room temperature. Surplus stain was then removed with two washes of distilled water. The slides were stored in the dark as the stain is sensitive to light. The detection of extra nuclear DNA by fluorescence microscopy indicated the presence of mycoplasma.

8. Clonogenic assay

Subconfluent cells were grown in flasks ($75cm^2$) in the presence of either dexamethasone (Merck, Sharp and Dohme Ltd) or methylprednisolone (Upjohn Ltd) for five days. Dexamethasone was used in the form of dexamethasone phosphate (Decadron) which is rapidly hydrolysed in the culture medium to dexamethasone (Rohdewald et al, 1987), therefore Decadron will be referred to as dexamethasone throughout this report. Cells were trypsinized (0.25%), then resuspended in 5ml of medium (F10/DMEM) and centrifuged at 1500g for 5 minutes. The pellet was then re-suspended in 5ml of fresh medium and spun once more. The cell pellet was then resuspended in 10ml of medium, 200 cells/ml were plated into 50mm petri dishes and the medium was made up to 5ml. The cells were grown for 10 days, with the medium being changed every three days. The colonies were then fixed with acetic methanol and stained with Giemsa, as described in section 10. The number of colonies per dish were recorded.

9. Saturation densities

Cells were seeded at a concentration $1x10^5$ cells on 15mm Thermanox coverslips in 24-well plates (Nunc.), where they were

either:-

(i) Grown to confluence in the 24-well plates, supplemented with 10% foetal bovine serum (Flow Labs. Ltd.) and with a gas phase of 2% CO_2 in equilibrium with 8mM $NaHCO_3$. In an attempt to prevent the medium from becoming exhausted the medium was changed every 3 days at low cell densities and daily at high cell densities.

(ii) The coverslips were transferred into 50mm petri dishes containing 4ml of growth medium two days later and grown in a humidified incubator. The cells were fed every 2-3 days.

Upon reaching confluence, saturation density was investigated by trypsinising and counting the cells as described in section (4). The saturation density was defined as the maximum cell density attainable past confluence on three consecutive days under non-limiting conditions.

10. Fixing and staining cells

Cell monolayers on either coverslips or petri dishes were fixed in the following way. In order to prevent the cells from rolling up and detaching from the substrata, the cells were exposed to increasing concentrations of acetic methanol (1:3) in PBS (50:50) each for 3 minutes, until the cells were exposed, for 5 minutes, to the pure fixative. The cell monolayer was then dried using an industrial blower. The coverslips were then mounted on glass slides using D.P.X. and were left to dry overnight. The cells were then stained by immersing either the slides or petri dishes in neat Giemsa for 2 minutes, which was then diluted in 10 volumes of water. After five minutes the slides were rinsed under running tap water until the cells could be clearly seen under the light microscope.

11. Labelling index experiments

The cells were grown to confluence on 15mm Thermanox coverslips in 50mm petri dishes in the presence of F10/DMEM containing 10% serum. They were then exposed to 1% serum containing the drug or compound being tested for one week, during which the medium was changed every 3 days. The cells were then

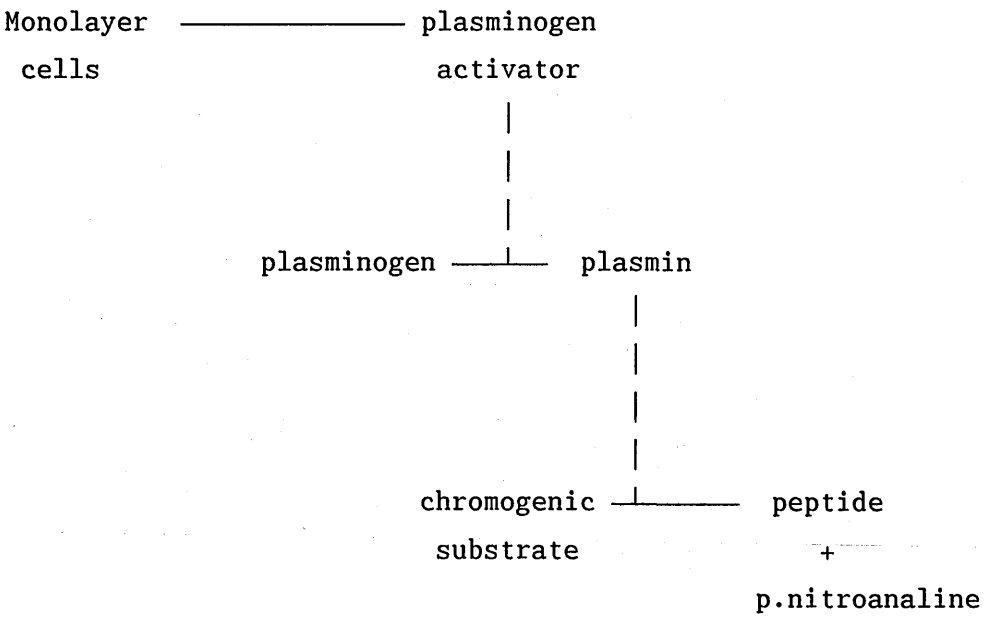
transferred to medium containing 1% serum and 5-Me-[^{3}H] thymidine 4μCi/ml (40μCi/mol, Amersham) for 24 hours. The cells were then thoroughly washed in order to get rid of the excess radioactivity. The slides were then immersed in 1) two separate solutions of 10% ice cold T.C.A.(trichloroacetic acid), each for 5 minutes, 2) distilled water for 5 minutes and 3) methanol for 5 minutes. The slides were then dried using an industrial blower.

The following procedures were carried out in the dark room using a red safe light. The slides were coated with radiographic emulsion (K12, Kodak) diluted 1:1 with distilled water. The slides were then drained vertically for 5 seconds to remove any excess emulsion and dried horizontally, and were then placed in a light-tight box containing silica gel and left at 4^{0}C for 7 days to expose. The cells were then processed, under a red safe light, by immersing them sequentially into, developer (D19, Kodak) for 5 minutes, distilled water containing 1% acetic acid for 30 seconds, fixative for 5 minutes (Ilfofix, Ilford), hypoclearing agent (Kodak) for 3 minutes and then finally washed thoroughly for 5 minutes in distilled water. The slides were then dried and stained with Giemsa according to the method in section 10.

The labelling index was determined as the percentage of nuclei labelled in relation to the total number of nuclei in that field. A cell was determined labelled if the grain count exceeded 100 grains per cell.

12. Investigating both the cell-associated and released plasminogen activator activities in glioma cell lines in the presence of glucocorticoids (Whur et al, 1980).

The assay is based on the following set of reactions:-



The p.nitroanaline produces the yellow colour the absorbance of which can be measured at 405nm and relates to the amount of plasminogen activator released by the cells.

The P.A. activity associated with both the cell monolayer and that released into the medium were both measured, using the following methods:-

12.1. Cell associated P.A. activity:-

The cells were grown to confluence in 24-well plates (Nunc) with F10:DMEM (1:1) containing 10% foetal calf serum as the growth medium in either the presence or absence of glucocorticoids. The cells were then incubated in serum free phenol red medium for 24 hours and they were then thoroughly washed with PE. 500µl of the reaction mixture, which contained 100µl of plasminogen (5Cu/ml) (Flow Labs. Ltd), 100µl of S-2251 (Flow Labs. Ltd) (5mM), 50µl poly-D-lysine (1.5mg/ml) and 250µl of Hanks balanced salt solution (HBSS) without phenol red, was added to the cell monolayer. The cells were then incubated with the latter medium for 2 hours at 37^{0}C. A 500µl aliquot was then removed and the reaction was

terminated by the addition (500µl) of 5% acetic acid. The absorbance was then read at 405nm, and the reading was corrected for both cell number and the presence of endogenous plasmin present in the sample as a result of either impurities or autocatalytic degradation. The proteolytic enzyme urokinase, which also activates plasminogen, was used as a standard in these experiments. In order to express P.A. activity in Plough units per 10^6 cells, an individual cell count was made using a duplicate plate and the result expressed as cells/well.

12.2. P.A. activity released by the cells into the medium

The cells were grown to confluence in the presence or absence of either dexamethasone or methylprednisone as described in section 8. The cells were then washed with PBS containing 1mM EDTA and then incubated in serum and phenol red free medium for 48 hours. 250µl of the latter supernatant was then incubated for 2 hours at 37°C with 500µl of the reaction mixture as described in section 12.1. above. The reaction was then terminated and the results were then expressed in Plough units per ml as previously described.

13. Extraction of glia maturation factor (Lim and O'Connell, 1982)

Fresh bovine brains were obtained from the Vet School slaughter house (Glasgow University) within 45 minutes after killing, and were transported in ice to the laboratory. The brains were cleaned of meninges and blood clots and cut up into small pieces. The brain tissue, in 150g portions, was homogenised with 3 volumes of Tris-buffer saline (0.02M Tris HCl, 0.15M NaCl, pH7.45) and homogenised for 1min in a Waring blender at 23,000rpm. The supernatant fraction after spinning the above homogenate at 23,000g for 16 hours in the ultracentrifuge was designated as the crude extract. The volume was then reduced from 350ml to 30ml using an Amicon PM10 membrane filtration unit. This material was then stored at -80°C.

In an attempt to purify the above extract, the following

protocol was followed. The supernatant was fractionated by ammonium sulphate precipitation, first at 45% then at 70% saturation. At each stage the suspension was stirred for 20 minutes and centrifuged for 30 minutes to collect the precipitate. The precipitate between 45 and 70% saturation was taken up with Tris-buffered saline at a ratio of 25ml buffer per brain (283g). The samples were then dialyzed against two changes of water for a total of 18 hours using dialysis tubing with a molecular cut-off point of 6000-8000Kda. The solution was then adjusted to contain 0.15M NaCl and 0.02M Tris at pH7.45 and applied to a G75 column, 5X93 cm in size and eluted with Tris-buffered saline at 50ml/hour. Fractions of 13.4ml were collected and tubes no. 65 to 95 were pooled. The volume was then reduced using an Amicon PM10 membrane filtration unit. Various dilutions (1:4, 1:8, 1:40, 1:80) were then added to sub-confluent cells and differentiated characteristics such as shape and fall in PA activity were noted.

RESULTS

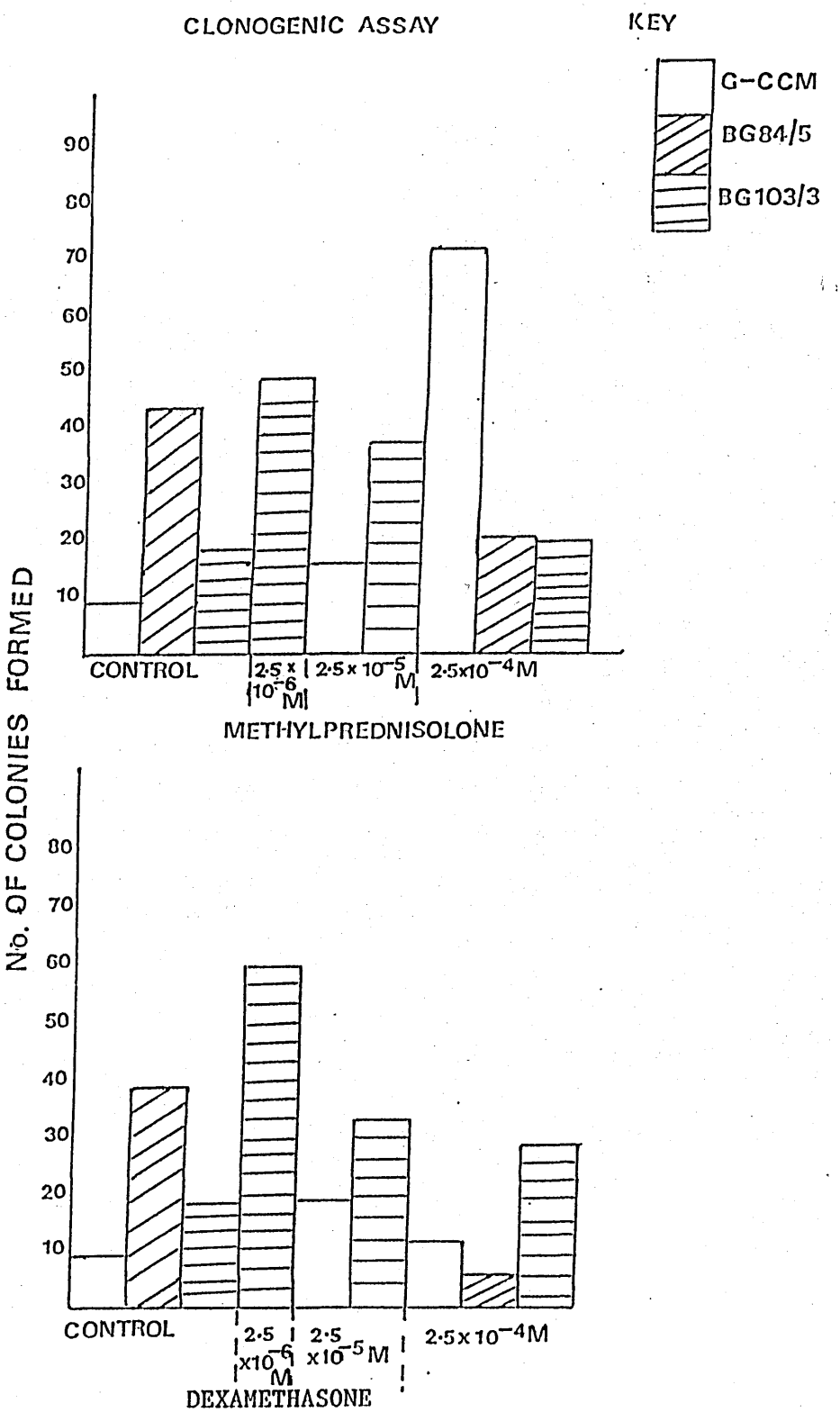
1. Investigating the cytotoxicity of dexamethasone and methylprednisolone by clonogenic assays

A clonogenic assay was used in order to investigate whether the glucocorticoids exerted a cytotoxic effect on the cell lines under investigation. These experiments were carried out as described in the methods section. Figure 5 illustrates the effect of various concentrations of both methylprednislone and dexamethasone respectively on colony formation. The majority of concentrations tested ($2.5x10^{-5}M$ and $2.5x10^{-6}M$) were not found to be cytotoxic to the cell lines under investigation, as suggested by the fact that the number of colonies formed in the presence of the steroid was never less than that of the control. However, dexamethasone and methylprednisolone at $2.5x10^{-4}M$ were found to be cytotoxic to the early passage cell line BG84/5. This concentration was much higher than would be pharmacologically attainable in vivo. The lower doses of glucocorticoids were found in general to augment survival, as seen by the increase in the number of colonies formed, and in their size as illustrated in the photographs in Figure 6.

2. Effect of glucocorticoid on saturation density

Malignant glioma cell lines characteristically reach higher saturation densities than normal glia. The photographs shown in Figure 7 are representive of how glioma cell lines "pile" up on top of one another, the extent of which varies among the different cell lines. Dexamethasone and methylprednisolone ($2.5x10^{-5}M$) were found to reduce this "piling up" effect. The following set of experiments demonstrates the cytostatic effect of these glucocorticoids in terms of saturation densities and labelling indices. Saturation densities were considered as being achieved when three similar readings were obtained on three separate days of post confluent growth.

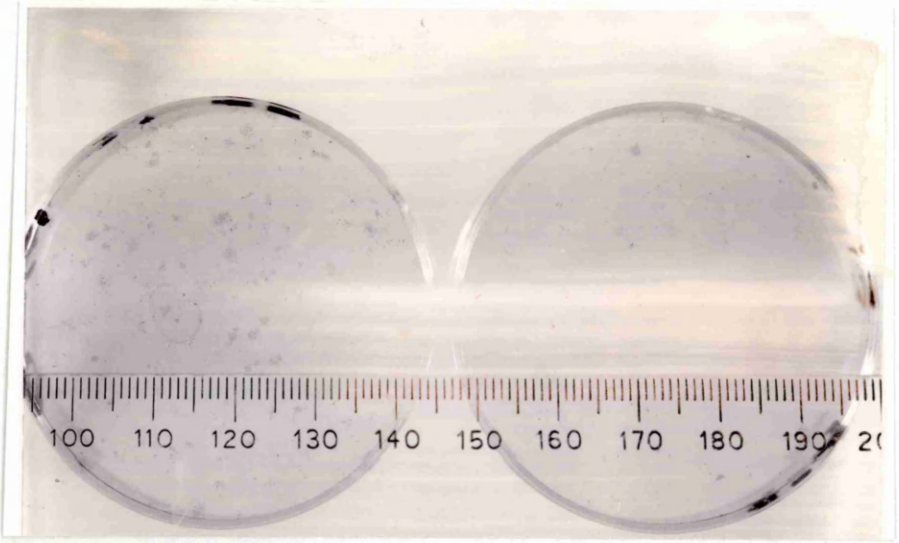
Figure 5



Effect of glucocorticoids in a clonogenic assay. Cells were grown to confluence in the presence and absence of either dexamethasone or methylprednisolone at $2.5x10^{-6}M$, $2.5x10^{-5}M$ and $2.5x10^{-4}M$. They were then trypsinized and cloned by dilution in the presence of glucocorticoids. Colonies were fixed in methanol, stained in Giemsa and counted.

Figure6

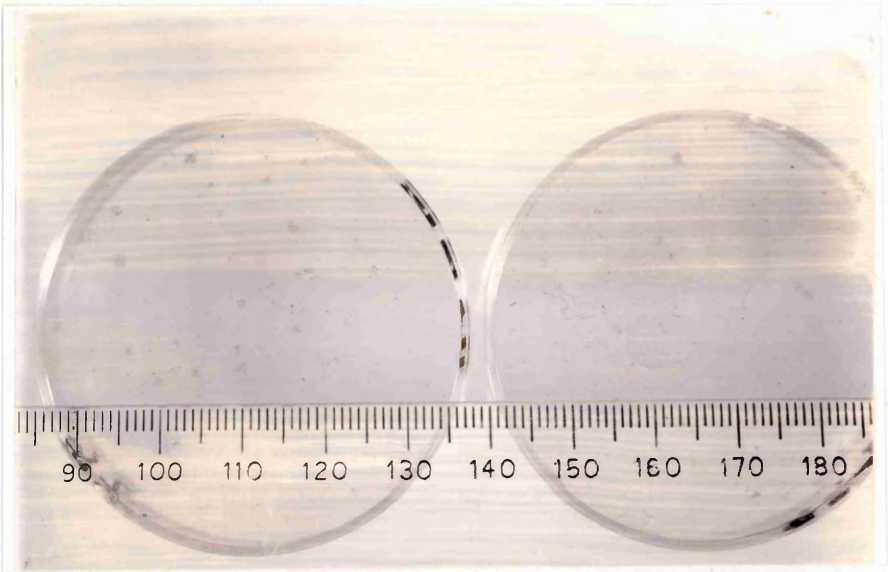
a)



IN THE PRESENCE OF DEXAMETHASONE.

IN THE ABSENCE OF DEXAMETHASONE.

b)



IN THE PRESENCE OF METHYLPREDNISOLONE.

IN THE ABSENCE OF METHYLPREDNISOLONE.

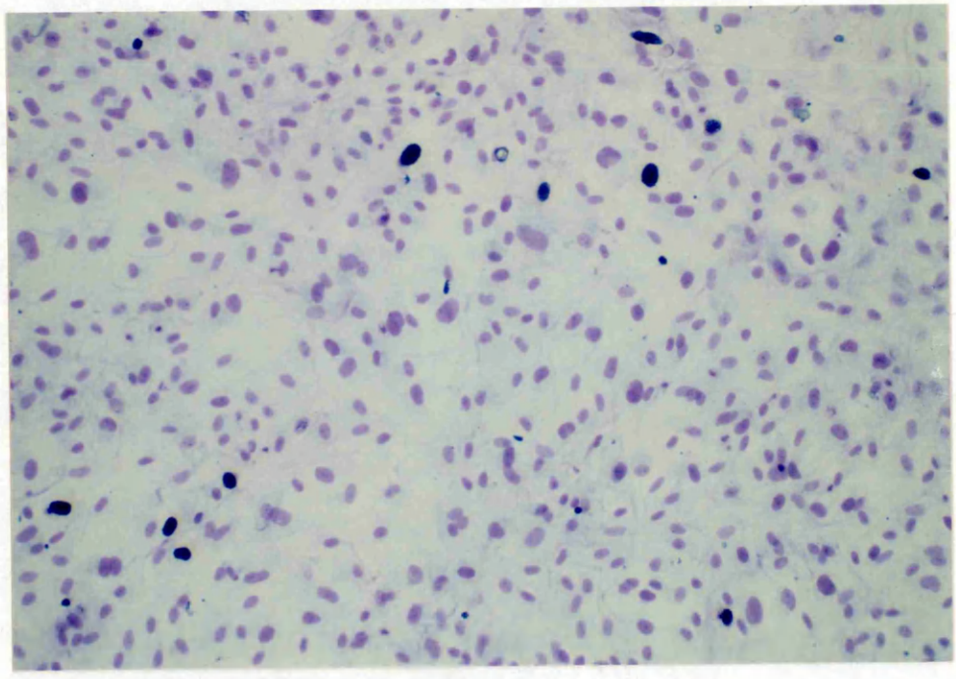
Figure 6

The photographs illustrate the effect of (a) 2.5×10^{-5}M dexamethasone and (b) 2.5×10^{-5}M methylprednisolone on a clonogenic assay using cell lines G-CCM. Culture G-CCM was cloned by dilution in the presence or absence of glucocorticoids. After 3 weeks the colonies were fixed in methanol and stained in Giemsa. The colonies produced in the presence of glucocorticoids were much larger in size and number than the controls.

Figure 7

Cell line BG84/9

a) IN THE PRESENCE OF DEXAMETHASONE.



IN THE ABSENCE OF DEXAMETHASONE.

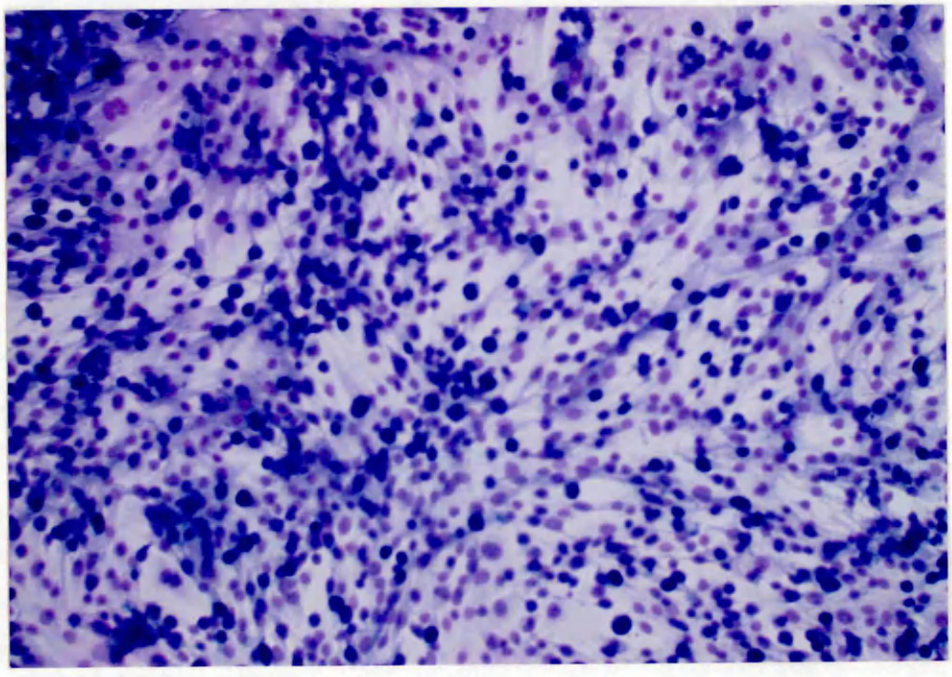


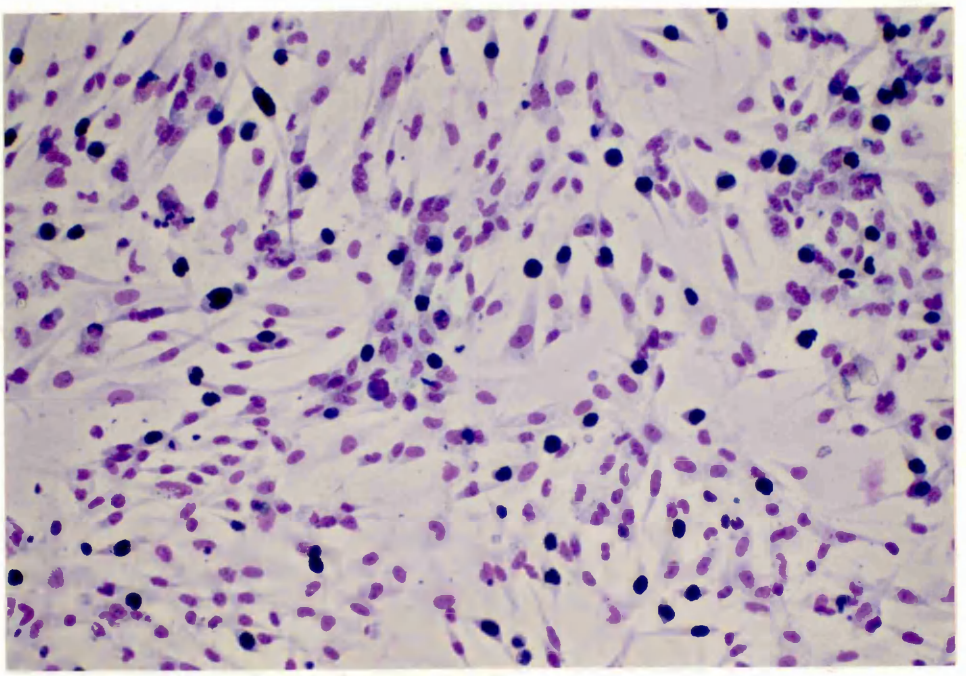
Figure 7

The photographs illustrate the effect of 2.5×10^{-5}M of dexamethasone on the saturation density of (a) BG84/9 and (b) BG105/5. Cells were seeded at 10^4 cells ml^{-1} ($5660 cm^{-2}$) on 15 mm Thermanox coverslips in 24 well plates in the presence and absence of dexamethasone at the concentrations indicated and then transferred to 50 mm petri dishes and grown for a further 5 days. The cells were then fixed with methanol. In each case the "piling-up" effect was reduced by the presence of steroid.

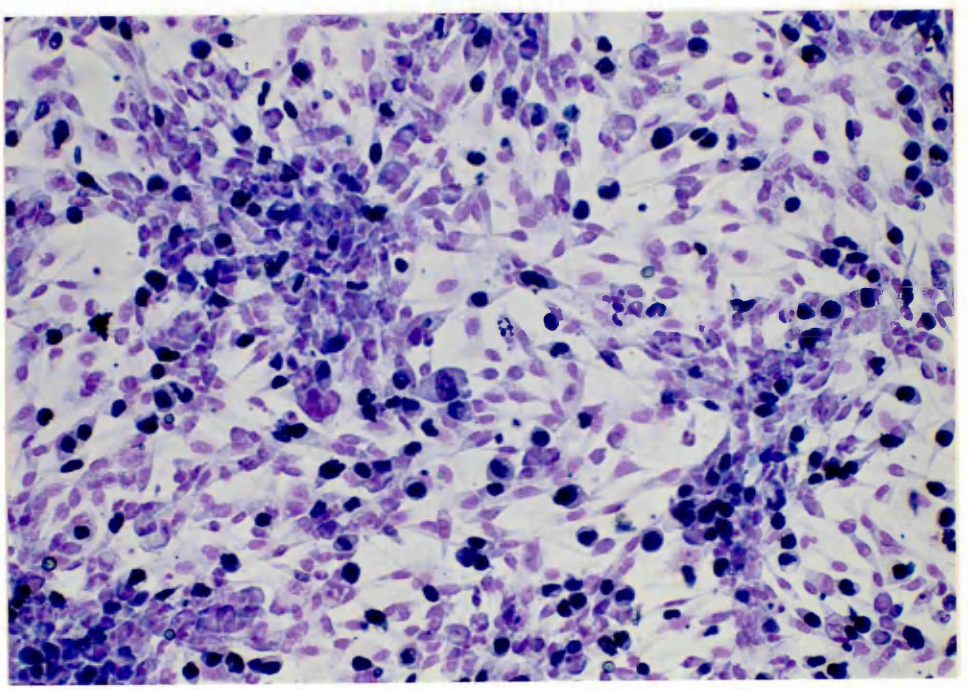
Figure 7 continued

b) Cell line BG105/5

IN THE PRESENCE OF DEXAMETHASONE.



IN THE ABSENCE OF DEXAMETHASONE.



3. Experiments carried out in 24-well plates

The effect of dexamethasone and methylprednisolone at $2.5x10^{-5}M$ on saturation densities was initially investigated using 24-well plates as described in section 9. At high cell densities the medium was found to be the rate limiting factor in terms of cell proliferation, as indicated by the colour of the medium even when it was changed daily. It was therefore decided that this method was unsatisfactory to determine saturation densities, as the results reflected both the effect due to the steroid and the cytotoxic effect produced by the low pH of the medium.

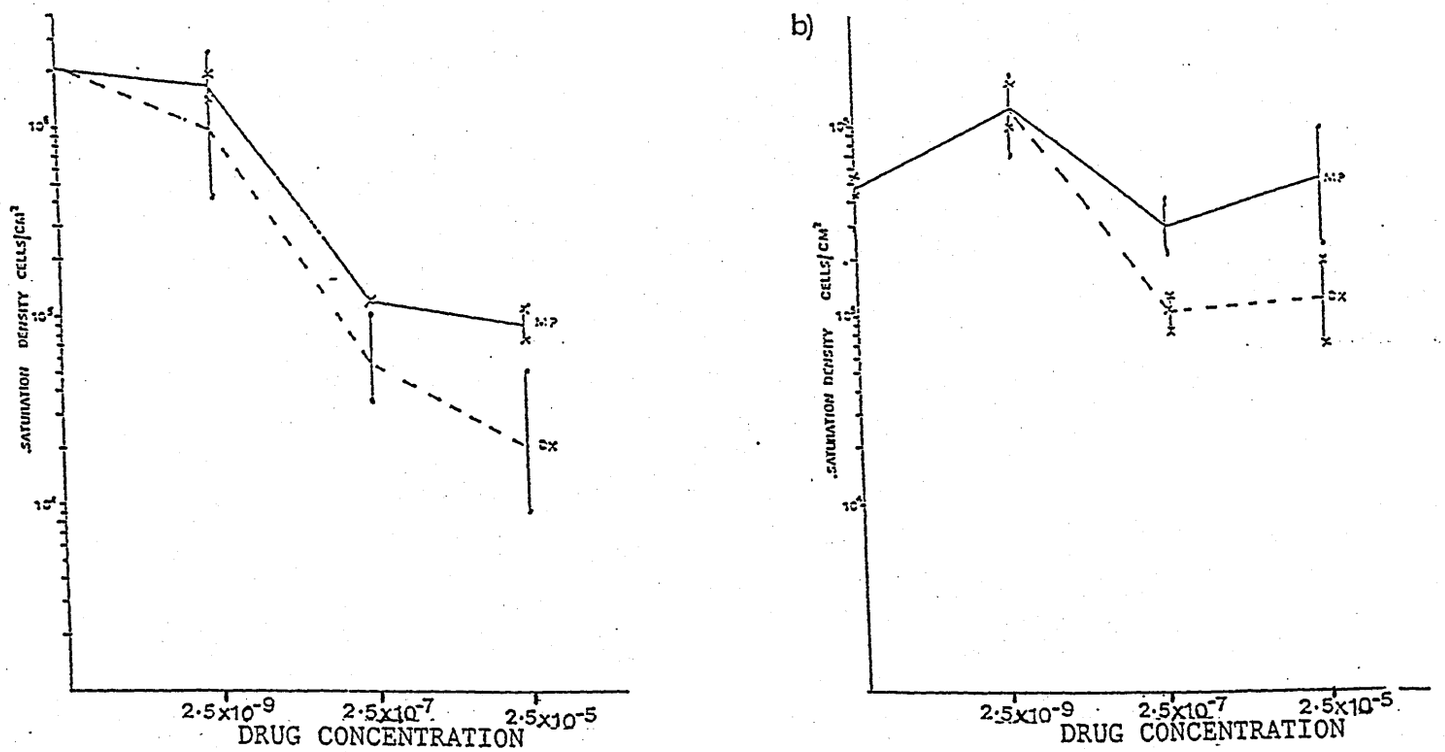
4. Density experiments using 50mm petri dishes

In order to overcome the previous difficulties, these experiments were repeated using 15 mm Thermanox coverslips in 50mm non-tissue culture petri dishes, according to the protocol in the methods section. Using this method, the medium was no longer the rate limiting factor in cell proliferation, as the quantity of medium present was always in excess of that required by the cells.

In order to test whether the presence of endogenous mitogenic inhibitors and activators in serum influenced the glucocorticoids' effect, experiments were initially carried out in both the presence and absence of serum using cell line G-IJKt (Figure 8). In the absence of serum the cells reached a lower saturation density than those in the presence of serum. Both dexamethasone and methylprednisolone were found to inhibit cell proliferation in a dose dependent way in the presence and absence of serum. However, low concentrations of glucocorticoids, eg. $2.5x10^{-9}M$, in the absence of serum were found to augment proliferation to a value, which was equivalent to that reached by the cells in the presence of serum. Thus, glucocorticoids seem to possess a biphasic response; at low concentrations in the absence of serum they augment growth while at high concentrations in the presence or absence of serum they inhibit it.

The general effect of dexamethasone and methylprednisolone on both early passage and continuous cell lines is illustrated in

Figure 8



Effect of either dexamethasone (Dx) or methylprednisolone (MP) on the saturation density of cell line G-IJKt in (a) the presence of 10% serum, and (b) the absence of serum. The cells were grown as described in Figure 7 and then trypsinized and counted. Points are means and standard errors, n=5. MP;—— Dx;- - -

Table1 Effect of glucocorticoids on saturation density in both early passage and continuous cells.

Cell line	Saturation densities cells/cm^2						
	Control	Dexamethasone (M)			Methylprednisolone (M)		
		2.5×10^{-9}	2.5×10^{-7}	2.5×10^{-5}	2.5×10^{-9}	2.5×10^{-7}	2.5×10^{-5}
G-UVW	$1.99\times10^5 \pm 0.05$	$1.78\times10^5 \pm 0.05$	$1.69\times10^5 \pm 0.07$	$1.67\times10^5 \pm 0.05$	$2.37\times10^5 \pm 0.11$	$1.77\times10^5 \pm 0.07$	$1.50\times10^5 \pm 0.05$
G-IJKt	2.50×10^6	1.40×10^6	1.00×10^5	8.80×10^4	2.50×10^6	4.60×10^5	1.50×10^5
G-CCM	$21.00\times10^4 \pm 4.90$			$13.00\times10^4 \pm 2.40$			$5.40\times10^4 \pm 0.05$
BG35/6	1.20×10^5		8.36×10^4	6.50×10^4			
BG30/4	6.10×10^5	5.50×10^4	1.83×10^5	7.05×10^4	6.05×10^4	4.60×10^4	6.31×10^4
BG28/4	7.36×10^4	5.80×10^4	3.70×10^4	5.90×10^4		6.50×10^4	4.70×10^4
BG105/5	$4.20\times10^4 \pm 0.30$			$3.60\times10^4 \pm 0.10$			$3.29\times10^4 \pm 0.14$
BG103/10	$2.80\times10^4 \pm 0.20$			$1.61\times10^4 \pm 0.07$			$1.64\times10^4 \pm 0.02$

Effect of glucocorticoids on saturation density in both early passage and continuous cell lines. Cells were seeded at 10^4 per ml (5660 cm^{-2}) on 15mm Thermanox coverslips in 24 well plates in the presence and absence of either dexamethasone. They were then transferred to 50mm petri dishes and grown till confluent. The cells were fed every three days in order that the medium did not become the rate limiting factor of growth.

Table 1. A concentration of steroid as low as 2.5×10^{-7}M was found to reduce the saturation density. In the majority of cell lines, methylprednisolone was seen to have a slightly greater effect than dexamethasone. However, larger numbers of samples are required in order that this observation can either be statistically confirmed or disproved. Both glucocorticoids were observed, in the present investigation, to reduce the saturation density, in a dose dependent manner, to approximately the same value each time within a single cell line, while the actual values differed among the various cell lines. The largest reduction in saturation density was observed in the cell lines which reached the highest saturation densities, eg 96% reduction in saturation density was observed with cell line G-IJKt.

It was postulated in the general introduction that the longer a cell grows in culture the higher the probability of that cell line transforming. In order to see whether the cell's response to dexamethasone and methylprednisolone changed under tissue culture conditions, experiments were repeated with the same cell line at two different passage levels. The results are shown in Table 2; there does not seem to be any difference in the results between the two passage levels.

Glucocorticoids have been reported to exert many of their effects through modulating the cell response to various hormones such as EGF (Baker et al, 1978). This hypothesis was investigated in terms of saturation density. Epidermal growth factor (EGF) at 1ng/ml was seen to have a mitogenic effect (Figure 9). Concentrations of dexamethasone as low as 2.5×10^{-7}M were generally found to reduce the mitogenic effect of EGF.

Thus, both dexamethasone and methylprednisolone were found to reduce, in a dose dependent manner, the saturation densities of both early passage and continuous glioma cell lines, even in the presence of a mitogenic factor. The glucocorticoids were seen to exert the largest effect on those cell lines which reached the highest saturation densities.

Table2

The reduction in saturation densities at different passage levels.

Cell line	Passage no.	% reduction in saturation density	
		DX	MP
BG103	3	-	30
BG103	10	40	41
BG105	5	14	11
BG105	10	14	11

The reduction in saturation density by dexamethasone (Dx) and methylprednisolone (MP) were investigated in early passage cell lines of different passage levels.

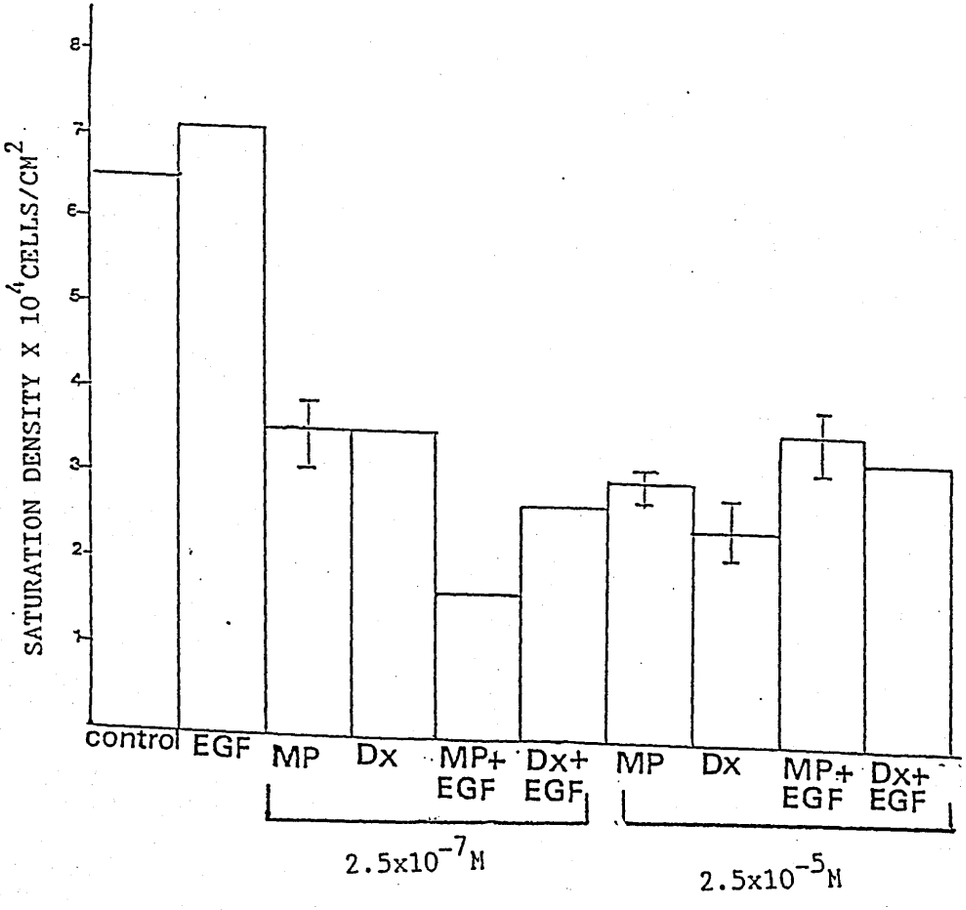


Figure 9

Effect of epidermal growth factor (EGF, 1ng/ml) on saturation density of BG30/5 in the presence or absence of either dexamethasone (Dx) or methylprednisolone (MP) at $2.5x10^{-7}$M and $2.5x10^{-5}$M.

5. The effect of glucocorticoids on labelling index

The labelling index provides a quick, efficient method for determining the proportion of cells undergoing proliferation at any one time (Maurer, 1981). The following experiments were carried out, as described in the methods section, in order to investigate whether the glucocorticoids' cytostatic effect, observed in terms of saturation densities, could also be observed in terms of the labelling index. If in the presence of glucocorticoid, the uptake of [^{3}H] thymidine by the cell was not altered, then the results obtained using the labelling index should reflect those obtained in the previous section using saturation densities. It was found in the labelling index experiments that the concentration of serum could be reduced at confluence to 1% without any obvious deterioration of the culture.

As the labelling index of sub-confluent and confluent cultures are drastically different, the cells were grown to confluence in the presence of 10% serum in order to reduce any differences which may arise from differences in cell numbers. They were then transferred to 1% serum containing the drug under investigation for 5 days, an appropriate control was carried out in the absence of drug, then the labelling index experiment was carried out. Unfortunately, the labelling indices had to be carried out within a week of reaching confluence, as some of the monolayers started to roll up and detach from the coverslips.

The labelling index of various early passage and continuous cell lines were investigated in the presence of 2.5×10^{-5}M, 2.5×10^{-7}M and 2.5×10^{-9}M of either dexamethasone or methylprednisolone. The results are shown in Table 3. In general, both dexamethasone and methylprednisolone, at concentrations as low as 2.5×10^{-7}M, were seen to reduce the labelling index in both the continuous and early passage cell lines. Thus similar results were produced in terms of saturation densities and labelling indices in the presence of glucocorticoids as seen from Table 4, however the actual magnitude of the reduction may differ. For example, dexamethasone was found to have a more profound effect in terms of saturation density and labelling index on cell line G-IJKt than

Table 3

The effect of glucocorticoids on labelling index.

Cell line	control	% labelling index in serum					
		Dexamethasone 2.5X			Methylprednisolone 2.5X		
		10^{-9}M	10^{-7}M	10^{-5}M	10^{-9}M	10^{-7}M	10^{-5}M
BG84/7	17.5	13	14	14	9.5	12	8.0
	±1.0	±0.7	±1.5	±0.2	±1.5	±1.0	±0.2
G-IJKt	21.2	3.0	1.0	2.5	2.6	6.5	6.0
	±3.6	±0.6	±0.4	±0.5	±0.4	±0.4	±6.7
G-CCM	12.95	-	9.0	10.3	10.8	-	8.4
	±0.9	-		±0.5		-	
BG30/4	13.4	5.19	6.09	5.25	8.3	-	3.79
	±0.3	±0.2	±2.0	±0.5	±0.2	-	±0.4
BG105/5	6.8	6.2	3.0	2.4	-	3.2	3.05
	±1.0	±1.0	±0.1	0.7	-	±0.5	±0.15

Illustrates the reduction in the labelling index in both early passage and continuous cell lines by either dexamethasone or methylprednisolone. The cells were grown to confluence and were then exposed to glucocorticoids for 5 days. 4µCiml^{-1} ^{3}H-thymidine was added for 18 hours and autoradiographs were prepared as described in the methods section.

Table 4

Cytostatic effect of glucocorticoids in a variety of early passage and continuous cell lines.

Cell line	%Decrease in labelling index					
	Dexamethasone			Methylprednisolone		
	2.5x 10-9M	2.5x 10-7M	2.5x 10-5M	2.5x 10-9M	2.5x 10-7M	2.5x 10-5M
G-IJKt	85	95	88	88	69	72
G-CCM	-	30	20	17	-	35
BG30/5	61	54	61	38	-	72
BG105/5	9	55	64	-	52	55

Cell line	% Decrease in saturation densities			
	Dexamethasone		Methylprednisolone	
	2.5x 10-7M	2.5x 10-5M	2.5x 10-9M	2.5x 10-7M
G-IJKt	96	96	81	94
G-CCM	-	38	-	74
BG30/5	70	88	92	89
BG105/5	-	14	-	22

Comparison of the percentage decrease in labelling index and saturation densities of dexamethasone and methylprednisolone in a variety of early passage and continuous cell lines. The results are a summary of the results in Tables 1 and 3.

methylprednisolone. The glucocorticoids were clearly seen to exert their cytostatic effect in a dose dependent manner in terms of saturation densities, but this effect was not as prominent in the labelling index experiments. Further experimentation is required to clarify this point.

In an attempt to see whether the cytostatic effect is associated with glucocorticoid activity, the labelling index experiments were repeated with 11-α-epihydrocortisone, an analogue of hydrocortisone. The glucocorticoid activity is associated with the 11-β orientation (Mealey et al, 1971), thus molecules with the 11-α orientation are devoid of glucocorticoid activity. This analogue of hydrocortisone was found to possess no cytostatic effect but the results (Table 5) suggested that it may have had a mitogenic effect as the labelling indices were increased in the presence of glucocorticoids.

Experiments were carried out to investigate whether the mitogenic effect of EGF (1ng/ml, 10ng/ml), seen in section 4 could be visualised in terms of the labelling index, and whether the presence of glucocorticoid could abolish this effect. For the exact method see section 11 in the methods section. The results are shown in Figure 10. The mitogenic effect of EGF at 1ng/ml and 10ng/ml was prominent and this was significantly reduced by concentrations of either dexamethasone or methylprednisolone as low as 2.5×10^{-7}M.

Thus, the glucocorticoid's cytostatic effect was similar in terms of saturation densities and labelling indices. This suggests that the labelling index in the present set of experiments is representive of cellular proliferation. Taken together the results suggest that the glucocorticoids are only reducing the proportion of cells proliferating at any one time and are not altering the uptake of [^{3}H] thymidine into the cells. In both sets of experiments methylprednisolone was generally observed to exert a greater effect than dexamethasone.

Table 5

A comparison of the labelling index with 11-α-epihydrocortisone compared to dexamethasone and methylprednisolone.

Cell line	Control	% labelling index in 1% serum						
		11-α-epihydrocortisone			Dexamethasone		Methylprednisolone	
		2.5x	1.25x	2.5x	2.5x	2.5x	2.5x	2.5x
		10-5M	10-6M	10-6M	10-5M	10-7M	10-5M	10-7M
G-CCM	12.95	14.8	17.4	18.7	10.3	9.0	8.4	-
	±0.9	±.42	±0.9	±1.1	±0.5	-	-	-

The above data illustrates the effect of 11-α-epihydrocortisone to that of dexamethasone and methylprednisolone on the labelling index in cell line G-CCM. The cells were grown to confluence, they were then exposed to the respective drugs for 5 days, the labelling index experiment was then carried out.

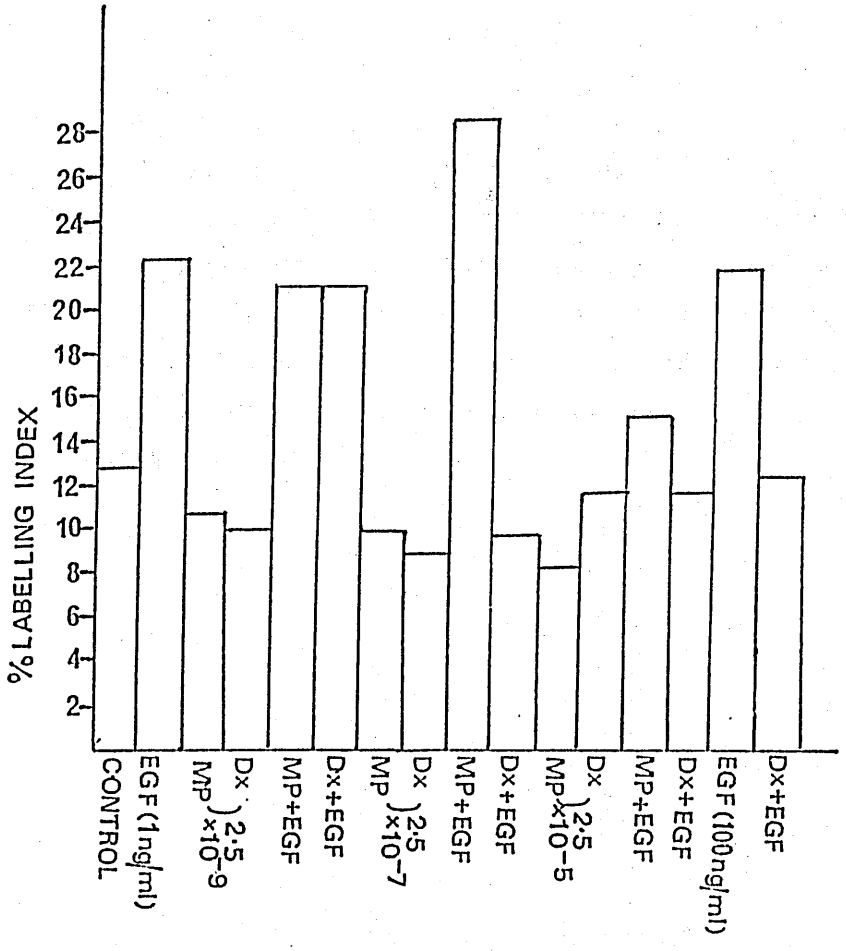


Figure **10**

Illustrates the effect of epidermal growth factor (EGF), dexamethasone (Dx) and methylprednisolone (MP) at various concentrations on the labelling index of cell line G-CCM. The various concentrations are read from the left to the right. Thus, unless stated, everything to the right of a specified concentration was carried out using that concentration.

6. Determining the number of glucocorticoid cytoplasmic receptors present in the various glioma cell lines

The question was asked as to whether the reduction in saturation densities and labelling indices were proportional to the number of glucocorticoid receptors present in the various cell lines. This concept was investigated with the help of Shelia Colven, from the Biochemistry Department at Glasgow University, using one continuous and two early passage cell lines. All the cell lines tested were shown to contain steroid receptors (Table 6). Cell line G-CCM was observed to possess ten times the number of receptors found in the other cell lines, but did not respond to the same extent in terms of the reduction in saturation density and labelling index, suggesting that no correlation exists between receptor number and response in the present experiments.

7. Plasminogen activator activity

Previous work carried out in this laboratory by Frame et al, (1984) using anaplastic astrocytomas and rat glioma cells, reported an inverse relationship between plasminogen activator activity and the cell's differentiated state. Plasminogen activator can thus be regarded as a monitor of the differentiated state of the cell. It was used for this purpose in the present study. Plasminogen activator activity was measured by the method described by Whur et al, (1980) and is described in the section 12 in the method section.

2.5×10^{-5}M of either dexamethasone or methylprednisolone was found to abolish plasminogen activator activity (Table 7), even in the presence of either epidermal growth factor or platelet derived growth factor. Thus, the glucocorticoids must be inducing the undifferentiated malignant cells to differentiate, as Frame et al (1984) associated loss of PA activity with cell differentiation. Glial maturation factor (GMF) has been previously shown by McLean et al (1986) to induce glioma cells to differentiate. However, in this study the crude brain extract (CBE) was found to elevate rather than reduce the plasminogen activator activity. The significance of this is not quite clear.

Table6

A comparison of glucocorticoid receptor number and the cytostatic effect of the glucocorticoids.

Cell line	Rec. conc. fmol/ml	DNA µg/ml	Rec. conc. fmol/mg DNA	% Reduction in saturation density		% Reduction in labelling index	
				Dx.	MP	Dx.	MP
BG103/3	59	355	166	42	41	-	-
G-CCM	682	415	1643	38	74	30	35
BG84/5	37	190	195	-	-	20	54
BG105/4	40	100	400	14	22	64	55

The number of receptors was worked out by Shelia Colvan (Biochemistry Dept., Glasgow). These results were then compared with the magnitude of the reduction in saturation density and labelling index by both dexamethasone and methylprednisolone.

Table 7

Effect of glucocorticoids and growth factors on plasminogen activator activity.

CELL LINE	CONTROL	EGF	1/8 dil. CBE	1/8 dil. CBE + EGF	MP	Dx	PDGF	MP+PDGF	Dx+PDGF	MP+EGF	Dx+EGF
BG28/4	0·15	0·26	0·51	—	0·00	0·00	—	—	—	0·00	0·00
BG14/5	0·77	0·30	4·95	8·90	—	—	—	—	—	—	—
G-CCM	4·90	6·40	—	—	0·00	0·00	3·30	0·00	0·00	0·00	0·00
G-CCM	8·10	10·10	—	—	—	—	4·70	0·00	0·00	0·00	0·00
G-CCM	12·7	12·30	—	—	0·00	0·00	23·1	0·00	0·00	0·00	0·00

Ilustrates the effect of epidermal growth factor (EGF), crude brain extract (CBE), dexamethasone (DX), methylprednisolone (MP) and platelet derived growth factor (PDGF) on plasminogen activator activity. The results are expressed in terms of P.U./10^6 cells.

DISCUSSION

The glucocorticoids were seen to exert a cytostatic rather than a cytotoxic effect on the various glioma cell lines under investigation, which confirms the results reported by Guner et al (1977). Glucocorticoids were found to reduce the saturation density in a dose dependent manner, but this effect was most prominent in terms of saturation densities. This posed the question as to whether the technique investigating saturation densities was more sensitive than that used in measuring the labelling indices. Further experimentation is required in order to answer this question. In the absence of serum, the lower concentrations of glucocorticoid (2.5×10^{-9}M) were found to augment cell proliferation, while higher drug concentrations were found to inhibit it. This suggests that glucocorticoids exert a biphasic effect, at low concentrations in the absence of serum they augment cell growth while at higher concentrations they inhibit growth. There was a corresponding loss in plasminogen activator activity, which suggests that the glucocorticoids had induced the immature malignant cells to differentiate.

In an attempt to observe if the cytostatic effect is associated with the glucocorticoid activity, the labelling index experiment was carried out using 11-α-epihydrocortisone. This analogue was not found to have any cytostatic effect on cell line G-CCM. On the contrary, the preliminary experiments suggested that the latter compound may be mitogenic. Thus, the present results suggest that the cytostatic property of the steroids resides in the same part of the molecule as glucocorticoid activity (Mealey et al, 1971).

The clonogenic assay illustrates that the glucocorticoids were not exerting their reduction in saturation density through a cytotoxic effect, as the number of colonies formed in the presence of glucocorticoids, for the majority of cell lines, were always greater than that of the controls. Malignant glioma cells were seen to attain higher saturation densities than normal glia, by growing on top of one another, as illustrated in the photographs

in Figure 7. Normal monolayer cells remain roughly at the same cell density upon reaching confluence, providing that they are fed regularly, whereas malignant cells continue proliferating, though at a slower rate than that of sub-confluent cultures (Westermark et al, 1973).

Dexamethasone and methylprednisolone were both observed to reduce the saturation density in a dose dependent manner. In the absence of serum, a low steroid concentration ($2.5x10^{-9}M$) was seen to augment rather than inhibit growth. This biphasic response has been previously reported in lung carcinoma and glioma cultures (M^{c}Lean et al, 1986). Throughout this investigation the glucocorticoids were found to reduce each cell line by approximately the same value each time, however the exact values differed from one cell line to another. It could be asked whether the latter values are representative of the densities achieved by their normal counterparts. This concept is very hard to investigate unless malignant and normal cells of a similar lineage are available. In the presence of either dexamethasone or methylprednisolone, BG30/4 plateaued consistently at about $4-6x10^{4}$ cells/cm^{2}, while G-IJKt plateaued around $9-13x10^{5}$ cells/cm^{2}. The most pronounced reduction in saturation density was observed in those lines which reached the highest saturation densities, for example G-IJKt. These results may be explained by the following hypothesis. If glucocorticoids exerted their effect through the re-expression of density inhibition, the largest effect would be expected to occur in those cell lines which were able to overcome this factor to the greatest extent. The results obtained in the present investigation favours such a concept as the cells which respond the least to density inhibition would be expected to attain the highest saturation densities, and it was on these cell lines which glucocorticoids had their greatest effect.

The labelling index is a quick and efficient method by which the cell's proliferative activity can be measured. The principle of this technique involves the dividing cells taking up exogenous [^{3}H]thymidine (Maurer, 1981; Fukuyama, 1985). The cells were only exposed to the radioactivity for approximately 18 hours, this time

is less than the cells doubling time, which is approximately 30 hours, in order to minimise the quantity of radioactivity taken up by quiescent cells where it is used to repair DNA. Otherwise the results would suggest that a much higher rate of cell proliferation was occurring. The labelling index can be influenced by the ratio of exogenous to endogenous thymidine present. Endogenous thymidine is incorporated from a pool of nucleotides which are largely fed by the thymidylate synthetase reaction, while exogenous thymidine is incorporated exclusively through the salvage pathway (Figure 11). Fluctuations in the intracellular precursor pools can markedly stimulate or reduce the rate of incorporation of exogenous thymidine, without corresponding changes in the actual rate of DNA synthesis. It was hoped to eliminate this factor by measuring the labelling index and not the total [^{3}H] thymidine incorporated. Thus, the percentage of labelled nuclei should reflect the general proliferative activity of the cells under investigation.

In the present experiments, dexamethasone and methylprednisolone were seen to reduce the labelling index in both the early passage and continuous cell lines. This cytostatic effect of glucocorticoids was previously reported by Freshney et al, (1980a). This may account for the re-expression of saturation density, but without normal cells of the same type this is a hard concept to investigate. For future work it would be desirable to repeat the above experiments using a larger cell population in order that the results could be statistically analysised.

As glucocorticoids are thought to exert their major effect through cytoplasmic receptors (Alexis, 1987) it was decided to investigate whether the degree by which the glucocorticoids reduced the saturation densities, corresponded to the number of cytoplasmic receptors present in the various cell lines. In the present experiments, the results suggest that there may be no correlation between the number of receptors present and the reduction in saturation density. This conclusion was reached from the following argument:-

Cell line G-CCM was found to possess ten times the number of

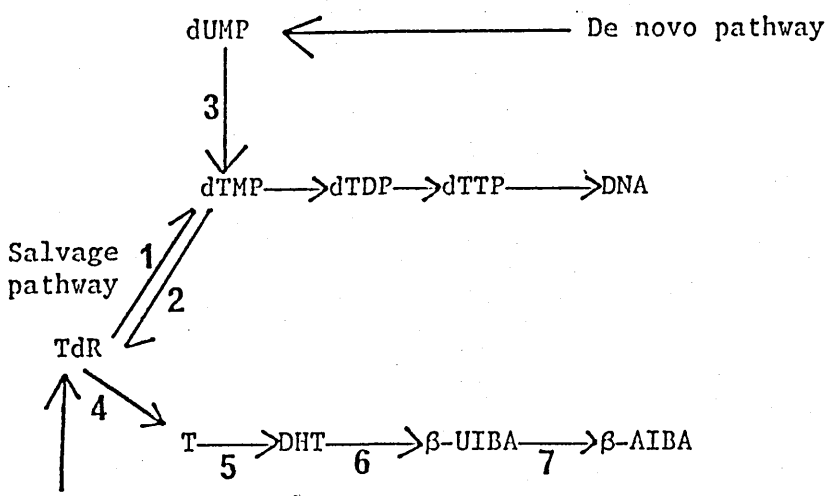


Figure 11

Metabolic pathway of thymidine (TdR). Substrates: dUMP, deoxyuridinemonophosphate, dTMP, dTDP, dTTP, deoxythymidinemono, di , triphosphate; T, thymidine; DHT, dihydrothymidine; β-UIBA, β-ureidoisobutyric acid, β-AIBA, β-aminobutyric acid.

Enzymes:1, thymidinekinase: 2, thymidine phosphorylase; 3, thymidine synthetase; 4, thymidine hydrolase; 5, thymidine dehydrogenase; 6,DHT hydrolase;7, decarbamylase. Taken from Maurer, 1981.

receptors than the other cell lines tested (Table 6), however it did not produce a corresponding decrease in saturation density. If anything it produced a similar reduction to that of the early passage cell lines. Thus, the present results show no correlation between receptor number and saturation densities. If a correlation did exist between receptor number and saturation densities, a larger reduction in saturation density would have been expected in cell line G-CCM.

It must be stressed that the above conclusion may not necessarily be true if the number of receptors actually present in each cell line was in excess of that required to produce the maximum reduction in saturation density. This can be explained as follows, if 100% occupancy is required to produce the maximum reduction in saturation density, then by plotting drug concentration against saturation density a dose-response curve would be obtained. Whereas, if only a small proportion of the receptors in all the cell lines were required to be occupied in order to produce the maximum reduction in saturation density, the dose-response curve would be masked if the concentration of dexamethasone added was in excess of that required to produce the response. This hypothesis could be tested by repeating the above experiments in the presence of various concentrations of an irreversible antagonist for the dexamethasone receptors, by making the excess receptors present unavailable to the glucocorticoid then any relationship between receptor number and reduction in saturation density would be obvious.

It could also be postulated that the glucocorticoids may exert their cytostatic effect through inducing the immature glioma cells to become more differentiated. Such a mechanism has been discussed by Sartorelli (1985). This would result if the following hypothesis is correct. Malignant cells resemble foetal cells in both morphology and biological properties (see general introduction). The transition from foetal to mature cells is gradual, thus if a block occurs in this pathway this would result in the production of immature cells (Sartorelli, 1985). If the "body" regulates cell number by only monitoring the number of

fully differentiated cells present, the latter situation would result in the "body" being unable to detect the partially differentiated cells present, thus it would encourage the passage of more and more cells into the maturation pathway. Thus, dexamethasone and methylprednisolone could be exerting there cytostatic effect through encouraging the cells to pass this "block" and progress through the cycle. The glucocorticoids were seen in the present investigation to reduce the PA activity of the malignant cells which emphasises that the glucocorticoids are encouraging the malignant cells to express more differentiated characteristics. This agrees with work carried out in this laboratory by McLean et al, (1986). They found that glial maturation factor also encouraged the immature malignant cells to differentiate. However, in the present study we did not find this to be the case. It is not clear why this inconsistency exists.

CHAPTER 3

INTRODUCTION

SURFACE CARBOHYDRATES OF TUMOUR CELLS

In this chapter the effect of glucocorticoids will be investigated in relation to the glycoprotein composition. Malignancy has been correlated with an increase in the proportion of high molecular weight fucosylated peptides, in particular with an over-sialation (Smets et al, 1984; Berthier-Vergnes et al, 1985). Freshney et al (1980b) demonstrated that this difference could be diminished by dexamethasone, which suggests that the glucocorticoid is altering the abnormal glycoprotein composition of malignant cells to resemble closer that of glia-like cells derived from normal brain. Therefore, it may be asked whether the glucocorticoids are exerting their cytostatic effect observed in Chapter 1 through altering the glycoprotein composition of malignant cells.

1. General structure of glycoproteins

Glycoproteins vary greatly in size, type and degree of glycosylation (Smets, 1984). This diversity in their structure may reflect their large range of biological functions. The carbohydrate residues are situated asymmetrically on the outer surface of the plasma membrane. The monosaccharides predominantly occurring in membrane glycoconjugates consist essentially of :-

(1) neutral sugars : mannose, galactose, fucose

(2) amino sugars : glucosamine, galactosamine

(3) acetylated derivatives : N-acetyl glucosamine
N-acetyl galactosamine

(4) sialic acids : collection of nine-carbon sugars with different substitutions on the amino and hydroxyl groups.

Membrane glycoproteins can be subdivided into three groups according to the type of linkage present between the sugar and protein:-

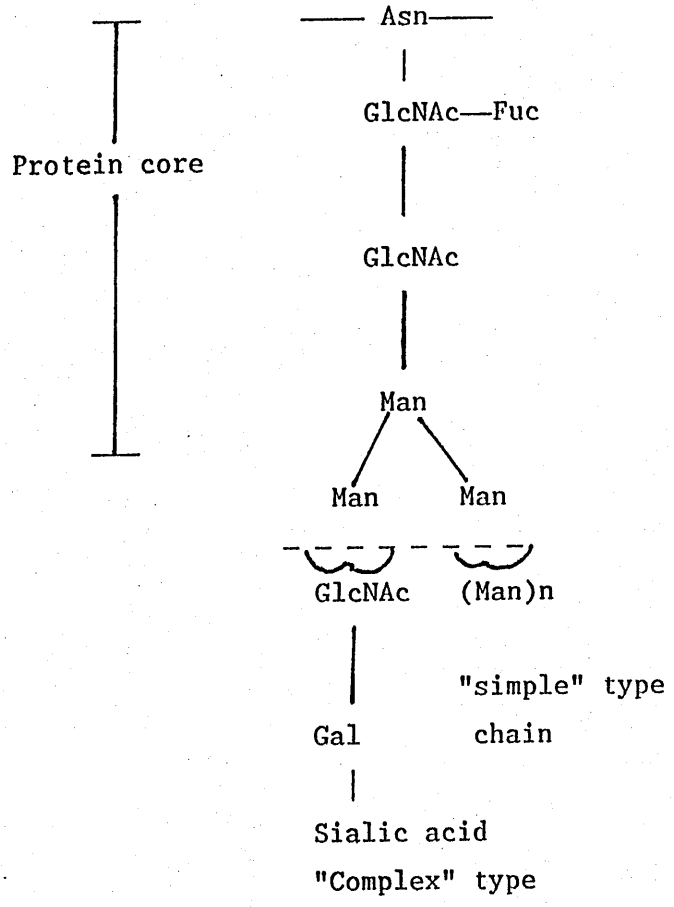
(i) 'N-linked sequences', in which an N-acetylglucosamine residue is linked to the side chain amino group of asparagine (Figure 12a). This group can be further subdivided into the so-called "complex-type" and "simple-type" sugars. Both groups contain a common core region. The "complex-type" contain several branches, with different monosaccharides associated with the protein core, whilst the "simple type" contain simple mannose residues. These glycopeptides are assembled on a lipid carrier, dolichol phosphate, as a branched dolichol oligomannose. This compound contains two N-acetyl glucosamine residues in the linkage region, with dolichol and three glucose units in the periphery of one of the branches. The high-mannose oligosaccharides are then transferred to the apoprotein in the rough endoplasmic reticulum. Before protein synthesis is complete, the glycan is transferred to the peptide where the carbohydrate chain can either remain unchanged, or may lose its outer mannose branches and gain new branches containing either N-acetyl glucosamine, galactose or sialic acid residues, through a complex interplay of glycosidases and glycosyltransferases (Hunt, 1979) forming the complex N-linked oligosaccharide moieties. Phosphorylation can then take place on some of the mannose residues present in these molecules. This type of linkage is commonly found in membrane and serum glycoproteins.

(ii) 'O-linked' sequences, in which N-acetyl galactosamine is linked to the side chain hydroxyl group of serine (Figure 12b). This type of linkage is found in secretory mucus.

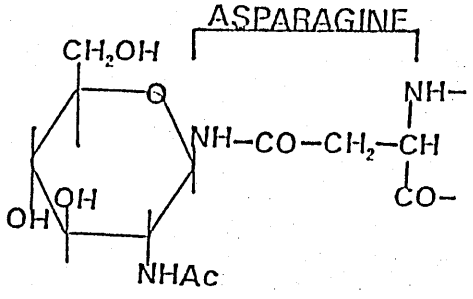
(iii) 'O-linked' sequences, in which a xylose residue is bound to serine, (Figure 12c) are found in proteoglycans (Chapter 4). Lipid intermediates are not involved in the biosynthesis of these structures. The sugars are added directly to the protein by sequential transfer from sugar nucleotide precursors (Hook, 1984).

Glycoproteins possess the potential of carrying large

Figure12


Asn
GlcNAc—Fuc
Protein core
GlcNAc
Man
Man
Man
GlcNAc
(Man)n
"simple" type
chain
Gal
Sialic acid
"Complex" type

b)


ASPARAGINE
CH2OH
O
NH–
NH–CO–CH2–CH
CO–
OH
OH
NHAc

c)

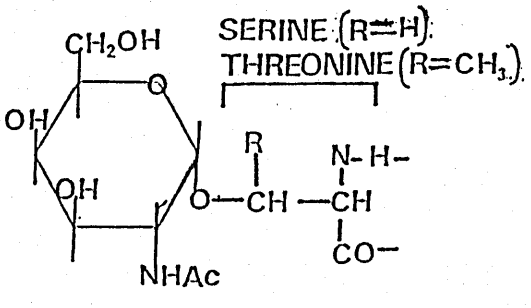

SERINE (R=H)
THREONINE (R=CH3)
CH2OH
O
OH
OH
R
N-H-
O—CH —CH
CO–
NHAc

Figure 12

(a) A schematic representation of N-glycosidically-linked oligosaccharide complex in secreted glycoproteins. The core protein either contains mannose residues (i.e "simple-type") or a "complex -type" which contains several branches containing different monosaccharides, which may carry terminal sialic or fucose units. A single glycoprotein may have both types of chain elongation and up to four chains can branch off from the mannose units of the core. Taken from Smets, 1984.

Abbreviations are:-
GlcNac., N-acetyl glucosamine; Gal., galactose; Man., Mannose; Fuc., fucose; Asn., asparagine

(b)"N-linked sequences"

Glycopeptide bond involving an N-glycosidic linkage between asparagine and N-acetylglucosamine. Taken from Smets, 1984.

(c) "O-linked sequences"

Glycopeptide bond involving an O-glycosidic linkage between serine or threonine and N-acetyl galactosamine. Taken from Smets, 1984.

quantities of information due to their high degree of heterogeneity which arises mainly from:-

(i) the degree of branching

(ii) the fact that the monosaccharides can be joined to each other through any of the several hydroxyl groups present

(iii) that the C-1 linkage can have either an α- or β- configuration.

2. Laminin and fibronectin

Laminin and fibronectin are two major glycoproteins which are thought to play an important role in cell adhesion (M^{c}Carthy et al, 1985). Fibronectin is about 220kDa and forms a class of multifunctional glycoproteins (Hynes et al (1978)). It is widely distributed in tissues throughout the body in an insoluble tissue form and a soluble plasma form. Most of its functions are assigned to domains of the polypeptide chains of the molecule (Yamada, 1981).

Fibronectin promotes spreading of a variety of cell types: fibroblasts, chondrocytes and adipocytes (Spiegelman and Ginty, 1983; West et al, 1979). Addition of fibronectin to pre-adipocytes prevents the cells from rounding up during differentiation and the cell subsequently fails to express lipogenic enzymes. Laminin is about 900kDa, comprised of three disulphide-linked polypeptide chains termed A (405kDa), B_1 (225kDa) and B_2 (205kDa) (Von der Mark and Kuhl, 1985) and is localised in basement membrane structures (M^{c}Carthy et al, 1985). Laminin appears very early in development being detected in the morula stage of the mouse embryo. Laminin like fibronectin has multiple binding activity to other matrix components including basement membrane collagen and proteoglycans as well as the surface of many cells. Laminin promotes attachment of cells that synthesise laminin in vivo, but the shape adopted depends on the origin of the cell: Sertoli cells on laminin have a columnar morphology, while Schwann cells are elongated (Kleinman et al, 1984). Both glycoproteins have been found to promote adhesion of cells to collagen.

It could be postulated that if glucocorticoids influence the

binding affinity of malignant cells then they may exert their effect through modulating the quantity of either fibronectin or laminin being synthesised by the cell. This hypothesis was investigated in the present investigation using antibodies which recognise laminin and fibronectin respectively.

3. Glycoprotein distribution in vivo

Membrane glycoproteins occur commonly in vivo as dimers or oligomers in which the subunits may be linked either by disulphide bonds or by non-covalent interactions. The transferrin receptor is comprised of four very similar glycoprotein subunits bound by disulphide bonds, while, the HLA-DR antigens, (HLA-histocompatibility antigens), consist of two dissimilar non-covalently linked glycoproteins. Transmembrane glycoproteins are usually orientated with the amino terminus on the outside of the cell and the carboxyl terminus on the inside (Figure 13). However, the reverse orientation has also been found, for example band 3 glycoproteins of erthyrocytes, may have the reverse orientation, that is the carboxyl on the outside and the amino terminus on the inside (Smets et al, 1984; Lennarz W.J, 1980).

4. Glycopeptides in relation to differentiation and proliferation

Cellular proliferation has been found to alter both the quantity and type of glycopeptides produced by the cell. Proliferating hamster cells contain a greater quantity of higher molecular weight glycopeptides compared to quiescent cultures (Buck et al, 1971). In addition to this alteration in the glycopeptide size, the actual quantity of glycopeptides being produced is also affected. Confluent non-growing cells produced a larger proportion of the "complex-type" rather than the "simple-type" glycopeptides which are normally produced during active proliferation (Sasak et al, 1985). Differentiation can also decrease the quantity of the higher molecular weight glycopeptides present (Freshney et al, 1980b). A good example of this is seen with human myeloid leukaemia HL60 cells (Smets and Van Beek, 1984).

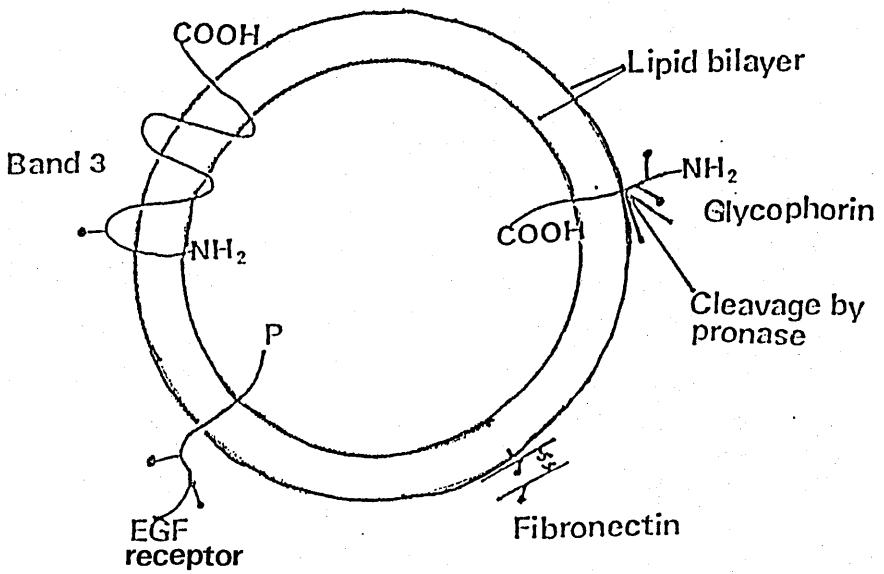


Figure 13

Transmembrane glycoproteins are usually orientated with the amino (NH_2) terminus on the outside of the cell and the carboxyl terminus on the inside. Glycoproteins normally span the bilayer once only but Band 3 glycoprotein of erythrocyte membranes traverses the bilayer several times with the carboxyl and amino ends of the polypeptide in reverse orientation to most other membrane glycoproteins interacts with a specific membrane receptor. Sugar residues are shown as ♦. Pronase cleaves glycopeptides on the external surface of the plasma membrane at the point illustrated in the diagram. Adapted from Lennarz, 1980.

5. Surface carbohydrates in malignant cells

Cancer tissues have been found to be comprised of higher molecular weight glycoproteins than their corresponding normal tissues (Van Beek et al, 1978; Smets et al, 1984). Such alterations have been reported to have no effect on the adhesive properties of these molecules. This difference in terms of glycoprotein composition can be reduced by pretreating the malignant cells with neuraminidase. This suggests that the above difference between malignant and normal cells was due to an increase in sialic acid concentration, which may arise from an increase in sialyltranferase activity. Warren et al (1972) were the first to report an increase in sialytransferase activity, suggesting a correlation between sialytransferase activity and proliferation in both transformed and normal proliferating cells. However, Grimes et al (1971) found decreased levels of sialyltransferase activity in 3T3 cells transformed by the SV40 virus. This correlates with the absence of the more highly sialated membrane glycoproteins in these cells.

Along with the size of the glycoproteins changing, Berthier-Vergnes (1985) found that the distribution of these glycoproteins differed between cell lines of differing tumourigenicity: 87% of the total sialic acid residues produced by highly metastatic lines were associated with the cell's surface, compared to only 53-55% in low tumourigenic lines. This suggests that highly tumourigenic lines may not necessarily possess a greater quantity of sialic acid residues than corresponding "normal" cells, but it is their cellular distribution which differs. Sialic acid residues were found to be cell-associated in the highly tumourigenic cells, whereas, in the lower tumourigenic cells they were generally released into the extracellular matrix. This increase in the degree of sialation has been associated with the cell's inability to respond to its external environment. Fusion of genetically transformed mouse L-cells, where the genetic deficiency is in the fucosylation of surface glycopeptides and normal mouse cells (Smets et al, 1984), results in only the

hybrids possessing the normal cell's surface carbohydrate composition being able to communicate normally. Poste and Nicolson (1980) correlated the above increase in sialic acid residues with metastasis. Thus, malignancy may be correlated to an increase in the proportion of cell-associated sialic acid residues, but not necessarily with an increase in total glycoprotein production. Thus, by altering the glycopeptide composition of the external plasma membrane, the cell's biological properties may be dramatically altered.

The loss of surface fibronectin has generally been correlated with acquisition of tumourigenic potential in chemically and virally transformed cells, although some exceptions have been reported (Hynes, 1981). It may be asked whether this reduction in fibronectin is due to a decrease in the rate of synthesis of fibronectin or an increase in the rate at which this glycoprotein is excreted from the cell. Biosynthesis of fibronectin by transformed cells is lower than that by normal cells, but in general this reduction in synthesis is not sufficient to account for the reduction in surface levels of this glycoprotein. Increased turnover rates and the decreased ability of transformed cells to bind fibronectin may also contribute to the reduced surface levels of fibronectin in malignant cells.

6. How can these alterations affect cell behaviour?

As discussed in the general introduction, the cytoskeleton may be important in terms of cell proliferation and differentiation. It was asked whether these alterations could account for the malignant cell's abnormal behaviour. Transformed cells generally have less ordered arrays of microfilaments. In particular, they usually lack, or have reduced levels of, microfilament bundles or "stress fibres" characteristic of many *in vitro* cells (Allred and Porterk, 1975). When fibronectin is added, arrays of microfilaments re-appear, which suggests that these cells are unable to synthesise sufficient quantities of fibronectin. This type of experiment suggests some form of transmembrane effect of the extracellular fibronectin on the

intracellular microfilaments. Such an effect may be postulated to produce no noticeable effect until the tumour is of significant size. This arises because the tumour is initially surrounded by normal cells, so a reduction in the production of fibronectin by the few tumour cells is insignificant. However, fibronectin production becomes important when the tumour mass is of significant size. In contrast, a reduction in the ability of these malignant cells to bind fibronectin could be important at the early stages of tumour formation.

Along with the quantity of fibronectin changing, Wagner et al (1981) found that the actual structure of fibronectin altered. Transformed cells, compared to their normal counterparts, contained a larger number of branches per core protein along with a higher degree of sialation, however they showed that this alteration in structure had no effect on the glycoproteins' adhesive properties.

7. Alterations in the composition of carbohydrate can affect gene expression.

As cancer is caused, at the molecular level, by changes in DNA, the alterations in carbohydrate composition mentioned in the previous section may be related to a direct effect on gene transcription and cell behaviour. Transformation by oncogenic viruses usually appears as a single step process, probably by the en bloc transfer of the relevant transforming genes (Smets et al, 1984). Several studies with temperature-sensitive mutants of transforming viruses have revealed that the expression of the viral genome is required for the emergence of tumour associated carbohydrate changes (Warren et al, 1972). The entire viral gene is not always required for transformation. De Leij et al (1982) studied carbohydrate changes following transfection of rat kidney cells with restriction enzyme fragments of Adenovirus 12 DNA. Transfection of a small left hand fragment (0-7.2%) resulted in immortalised and morphologically transformed non-tumourigenic cells, whereas, the entire E1 region (0-11.5%) was required for the manifestation of the tumourigenic phenotype and for expression

of the characteristic changes in glycopeptides.

Several oncogenes have been identified which are implicated in malignancy. Altered glycosylation has been demonstrated in NIH 3T3 cells transformed by transfection with DNA from human neuroblastoma and bladder carcinoma cell lines. The oncogenes responsible have been identified as N-ras and C-Hras-I respectively (Santer et al, 1984).

8. The effect of glucocorticoids on glycopeptide composition

Freshney et al (1980b) have previously shown that pretreating glioma cell lines with dexamethasone resulted in these cells acquiring a glycopeptide composition similar to that of their corresponding normal cells. This effect was similar to that found by Van Beek et al (1978) using neuraminidase, which suggests that the glucocorticoid produces its effect by reducing the quantity of sialic acid on surface glycoproteins. McLean et al (1986) suggested that the glucocorticoids may be exerting their effect through inducing the immature malignant cells to become more differentiated, which may be expressed by an alteration in the composition of the glycoconjugates. Alternatively, the glucocorticoids could directly reduce the sialytransferase activity.

9. Aim of Chapter:-

The aim of this Chapter was to investigate the effect of dexamethasone and methylprednisolone on glycopeptide composition. This was investigated in two different ways. Initially the composition of glycopeptides was investigated by Gillian Hunt (Royal Victoria Hospital, Newcastle) using gel electrophoresis. As the composition of the extracellular glycopeptides may be important in determining the biological behaviour of the cell, the composition of these glycopeptides was specifically looked at using gel exclusion chromatography.

Materials and Methods

Dexamethasone and methylprednisolone were used at a concentration of 2.5×10^{-5}M throughout this chapter, as this concentration was observed in Chapter 2 to produce the maximum decrease in saturation density. At this concentration any effect the glucocorticoids have on the glycopeptide composition should be clearly seen. The glucocorticoids were seen in Chapter 2 to have a similar effect on both the continuous and early passage cell lines, the continuous cell lines G-CCM and G-IJKt were arbitrarily chosen for the following investigation. As the following methods do not differentiate between glycoproteins and proteoglycans the mixture will be referred to as glycoconjugates throughout this chapter.

1. Radioiodination of monolayer cultures

This method was carried out by Gillian Hunt (Royal Victoria Hospital, Newcastle). The cell layer was pre-incubated in phosphate-buffered saline (PBS), pH 7.2 for 15 minutes at 37^{0}C, followed by a final PBS rinse to remove adherent serum proteins. 3ml of 5mM glucose in PBS was then added to each flask, followed by 1mCi of carrier free Na ^{125}I, 2.5µg of glucose oxidase and IV lacto-peroxidase. These solutions were mixed gently and were left for 10 minutes at room temperature. The reaction was terminated with 5ml of phosphate-buffered iodine (PBI), containing 0.137M NaI and 2 mM phenyl-methyl-sulphonyl-fluoride (PMSF) a protease inhibitor. The monolayer was then rinsed 3 times in PBI-PMSF. The cells were scraped off in a small quantity of PBI-PMSF and centrifuged to obtain a cell pellet. The pellet was then solublised by boiling in 0.3ml of 0.01M sodium phosphate buffer, pH7, containing 1% (w/v)SDS, 1% (w/v) mercaptoethanol and 2mM PMSF for 10 minutes. Sucrose was added to give a 30% w/v final solution. The extract was stored at -20^{0}C.

30µl aliquots of extracts of the above solution were run on 6% w/v cylindrical polyacrylamide gels (6mmx140mm). The running

buffer contained 200mM sodium phosphate buffer pH 7.2, 0.2% (w/v) SDS and 0.05% (w/v) bromophenol blue as a tracking dye. 1mm slices of the gel were then cut and counted for radioactivity. The counts per slice were expressed as a percentage of the total radioactivity recovered per gel. The molecular weights of the labelled proteins were determined by comparison with molecular weight markers on the gel.

2. Gel exclusion chromatography

Cells were grown in 23.8cm^2 (5.5cm) petri dishes to 70% confluence. They were then incubated in either dexamethasone or methylprednisolone (2.5×10^{-5}M) in the presence or absence of 1ng/ml EGF for 5 days. The cells were then treated in the following way:-

(i) Cells were exposed to 5 µCi/ml [^{3}H] glucosamine (NEN; 29Ci/mmol) for 16 hours.

(ii) Medium was removed and stored at -80^{0}C. The cells were then incubated with 1ml of 1mg/ml of pronase (Sigma) on day 1, followed by 0.2ml of 10mg/ml pronase solution on days 2, 3 and 4. The pronase extract was then centrifuged for 5 minutes to remove the precipitated material. This extract was stored at -80^{0}C.

(iii) In order to separate the unincorporated [^{3}H] glucosamine from that incorporated into glycoproteins, the above solutions were passed through a C_{18} cartridge (Whatman Co.). The cartridge was activated by passing 2ml of methanol (HPLC grade) through it, then rinsed with 5ml of distilled water. The sample was passed through the cartridge followed by some distilled water and the red colour of the medium was seen to be retained at the top of the cartridge. The sample was then washed out with 3ml of a solution containing 0.1% TCA and 50% acetonitrol (HPLC grade), and 0.5ml of this exudate was then counted in 10ml of Ecoscint in the scintillation counter (Packard). The remaining solution was then lyophilised and stored at -80^{0}C.

(iv) The glycoproteins were separated using a TSK-G3000SW column at 4^{0}C. A 100µl sample of each of the above solutions was then run through the column and 0.5ml fractions were collected. These

samples were then added to Ecoscint, thoroughly mixed and counted on the scintillation counter. The results were standardised by multiplying up by the dilution factor and expressed as counts per 10^6 cells.

Note: In all the above results 90% of all the radioactivity put on the column was recovered. The missing 10% was probably dispersed throughout the column exudate, appearing as background. Therefore, the area under the curves should represent the quantity of radioactivity taken up by the cells.

3. Detecting the quantity of either laminin or fibronectin in the presence or absence of glucocorticoids

In order to detect whether dexamethasone or methylprednisolone affected the quantity of either fibronectin or laminin present, the following protocol was followed.

The protocol is based on the principle that a 1^o antibody is added which detects the appropriate glycoprotein. The staining is amplified by using a 2^o antibody which binds to the 1^o antibody and peroxidase anti-peroxidase (PAP) molecules then bind to the 2^o antibody. The PAP molecules are then visualised via a substrate-chromagen reaction with diaminobenzidine (DAB; Sigma) (Figure 14). All the antibodies were obtained from Dakopatts.

Cells were seeded at approximately $2X10^4$ cells in either Lab Tek slides (0.4ml) or in 50mm petri dishes (4ml). These cells were then grown in the presence or absence of either $2.5X10^{-5}M$ dexamethasone or methylprednisolone. The cell monolayer was then fixed using cold methanol as described in section 9. The antibodies used in this section were obtained from Dakopatts:-

1) For fibronectin, the 1^o antibody was mouse anti-fibronectin, the 2^o antibody was anti-mouse attached to peroxidase.

2) For laminin the 1^o antibody was rabbit anti-laminin, the 2^o anti-rabbit linked to peroxidase.

The fixed monolayer was incubated at 37^oC with the 1^o antibody for 20 minutes, washed thoroughly with PBS, incubated for 20 minutes with the 2^o antibody, washed thoroughly with the PBS then incubated with peroxidase anti-peroxidase for 20 minutes then

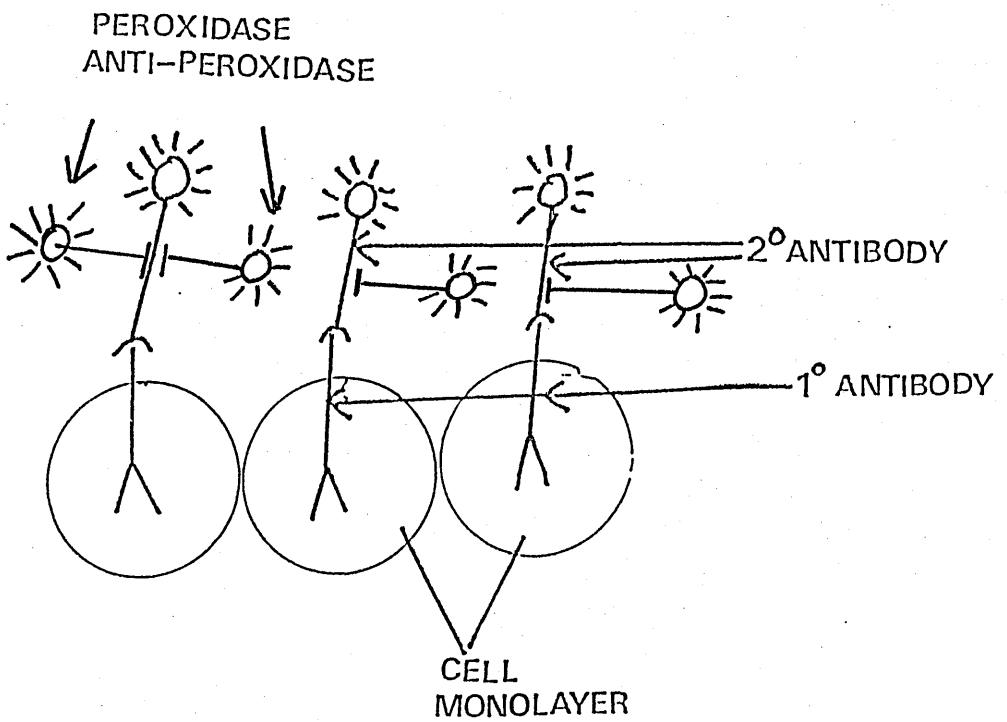


Figure 14

A schematic diagram of the technique used to detect the presence of both laminin and fibronectin. The peroxidase enzyme is visualised via a substrate-chromagen reaction.

finally washed thoroughly with PBS. The cells were then exposed to 0.5mg/ml of DAB in PBS until the brown stain could be seen, they were then washed thoroughly with PBS before being mounted. The quantitation of laminin and fibronectin was arbitrary.

RESULTS

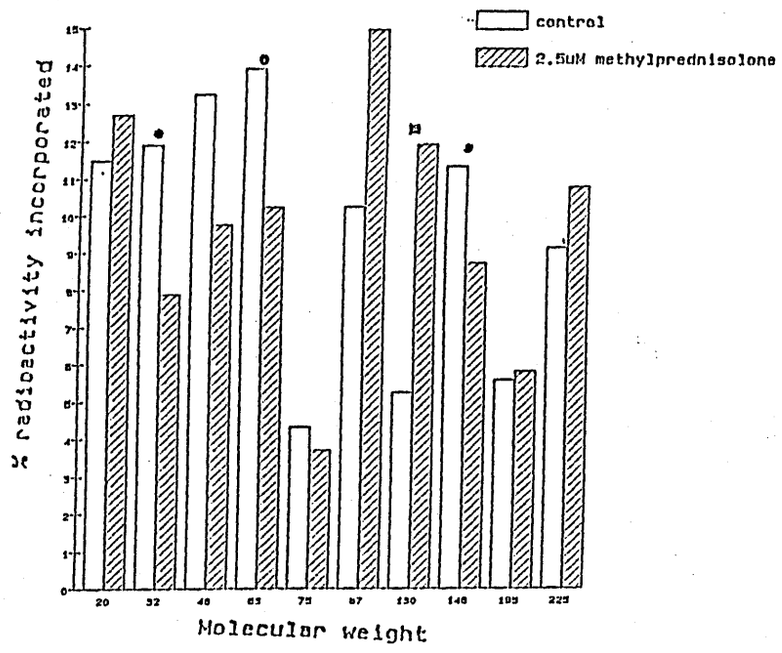
1. Investigating the effect of glucocorticoids on the molecular weight distribution of glycoconjugates

In collaboration with Gillian Hunt (Royal Victoria Hospital, Newcastle), cell surface carbohydrates were examined in the presence of methylprednisolone using polyacryalmide gel electrophoresis. Confluent cultures of G-CCM and G-IJKt, were grown in the presence or absence of 2.5×10^{-5}M methylprednisolone, iodinated by the lactoperoxidase technique and separated using gel filtration chromatography as described in the methods section.

The results obtained are shown in Figure 15. In cell line G-CCM, glucococorticoid reduced the quantity of glycoconjugates of molecular weight 225kDa (possibly fibronectin) and 20kDa, while increasing the 63kDa component. There was a slight decrease in the 87KDa species, but this was not statistically significant. In cell line G-IJKt the drug reduced the quantity of 148kDa, 63kDa, 48kDa and 32kDa glycoconjugates while increasing the proportion of the 130kDa and 87kDa species. Thus, there is no evidence for a consistent change in the membrane glycoproteins in these two cell lines.

2. Separating glycoconjugates by molecular weight gel exclusion chromatography.

In the previous section, intact glycoconjugates were examined. This section deals with the glycoconjugates on the surface of the cell which are accessible to pronase digestion (Figure 13). Prelabelled glycoconjugates were separated using a T.S.K. column, as described in the methods section, which is is a quick and efficient method for separating proteins according to their molecular weight (Kato et al, 1980). Two fractions were investigated, the medium which contained the material secreted from the cell and the pronase digest which contained the cell associated material.



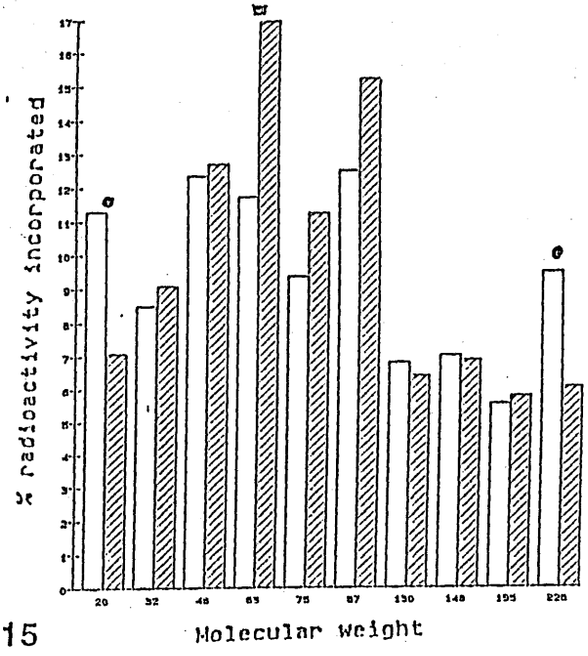


Figure 15

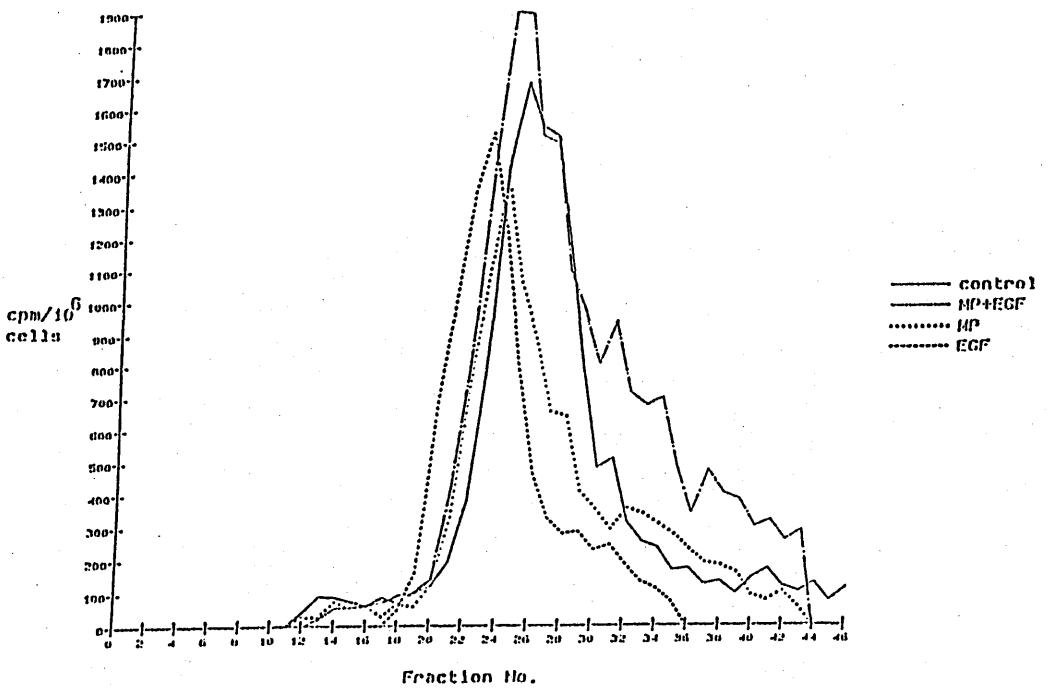
Data obtained from radioiodination of surface glycoproteins by Gillian Hunt (Newcastle). Illustrating the effect of 2.5×10^{-5}M of methylprednisolone (MP) on cell line a) G-IJKt and b) G-CCM. Each cell line was grown to confluence in a $75cm^2$ flask, exposed to methylprednisolone for 5 days post confluence and the iodinated as described in the methods section. In (a) n=5 for the control group and 4 for the glucocorticoid group, while in (b) for the control group and 5 for the test group. The means were statistically compared by a two sample t-test where ◐ $p<0.05$ and ■ $p<0.01$.

2.1 Cell line G-CCM:

In cell line G-CCM dexamethasone was found to increase the quantity of glycoconjugates present in both the medium and pronase fractions (Figures 16 and 17). Methylprednisolone produced a similar effect on the pronase fraction, but had the opposite effect on the medium extract. Dexamethasone increased the range of glycopeptides eluting in both fractions, as depicted by the broadening of the elution peak, and the increased area under the curve as shown in Table 8. Methylprednisolone was seen to produce a similar effect on the pronase fraction, however it reduced the quantity of glycoconjugates eluting in the medium extract as seen from the narrower width of the elution peak. EGF was seen in the medium fraction of both cell lines to effectively shift the peak to the left, which indicates the presence of higher molecular weight glycoconjugates. This shift by EGF was diminished in cell line G-CCM in the presence of methylprednisolone, but was augmented by dexamethasone.

2.2 Cell line G-IJKt

Both dexamethasone and methylprednisolone were seen to increase the quantity of material present in the medium extract, as seen by the broadening of the peak and the increase in the area under the curve (Figures 18, 19 and Table 8). However, dexamethasone was seen to possess a greater effect than methylprednisolone. Dexamethasone was seen to increase the quantity of the glycoconjugates in the pronase fraction, which was similar to its effect on the medium fraction, however methylprednisolone showed no effect. In the pronase fraction, the peak was seen to shift to the left in the presence of glucocorticoids, indicating the presence of higher molecular weight species. Whereas, in the medium extract the glucocorticoids increased the quantity of the lower molecular weight species eluting from the column, as seen from the broadening of the peak to the right. EGF was seen in this cell line to increase the quantity and size of the glycoconjugates



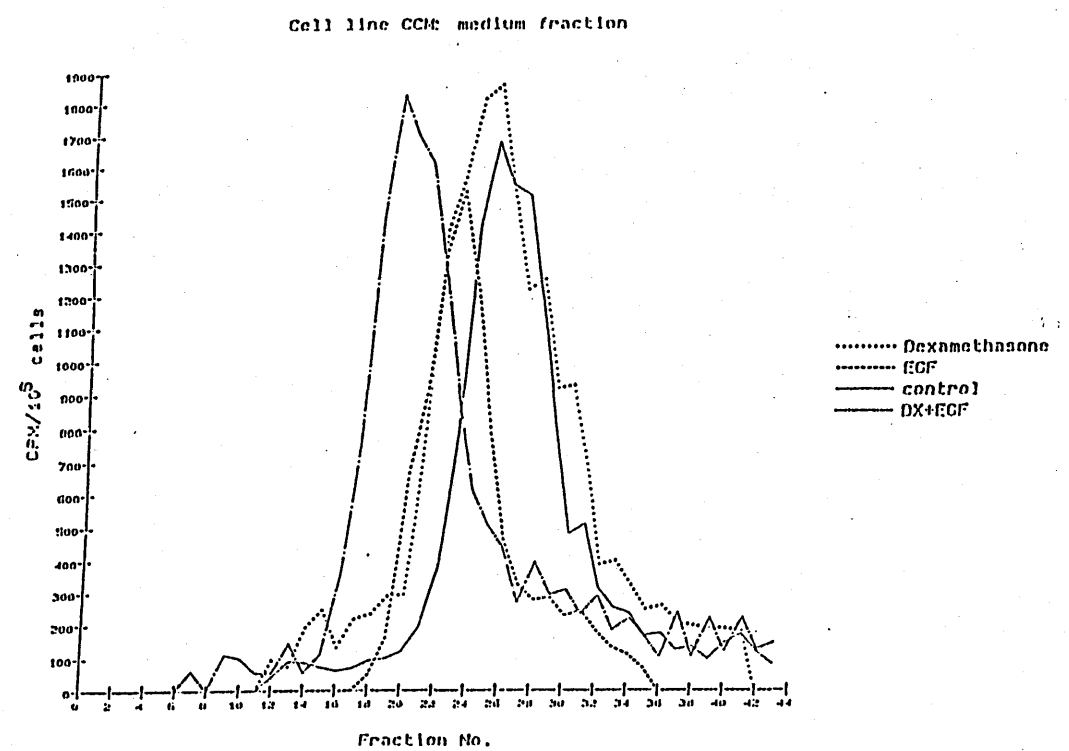
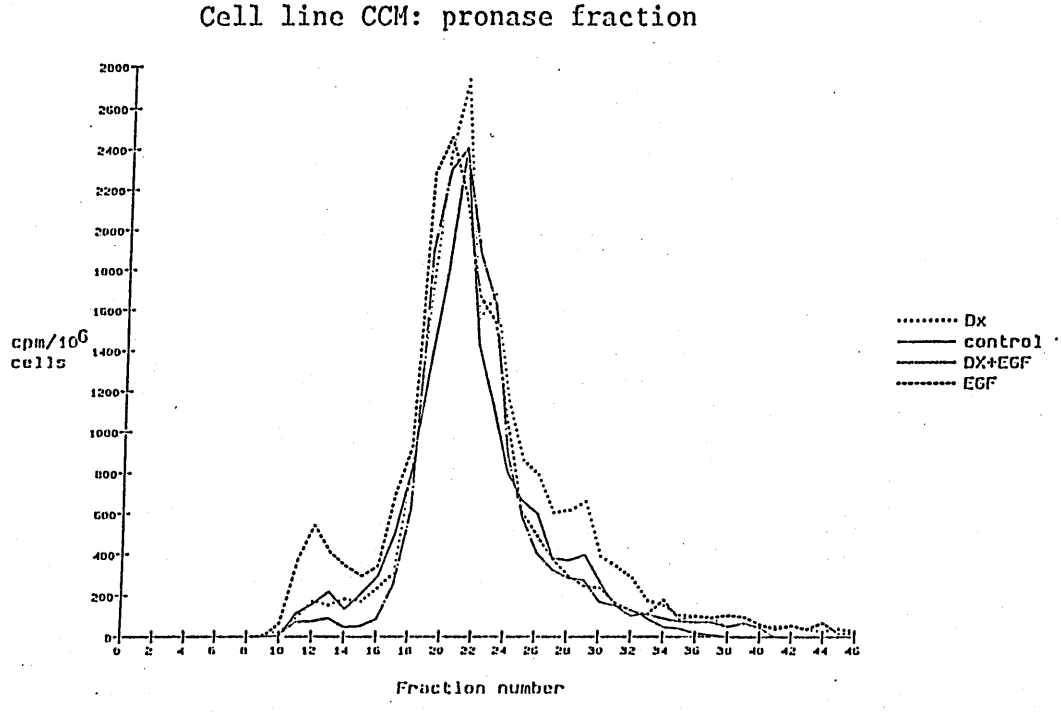


Figure 16

The effect of glucocorticoids on the glycoconjugates profile. The effect of (a) methylprednisolone (MP) and (b) dexamethasone (Dx) on the elution profile of glycoconjugates in the medium fraction of cell line G-CCM from a TSK-G3000SW gel column. The cells were grown to 70% confluence, then were exposed to glucocorticoid for 5 days, then they were exposed to 5 μCi ml^{-1} of ^{3}H-glucosamine as described in the methods section.



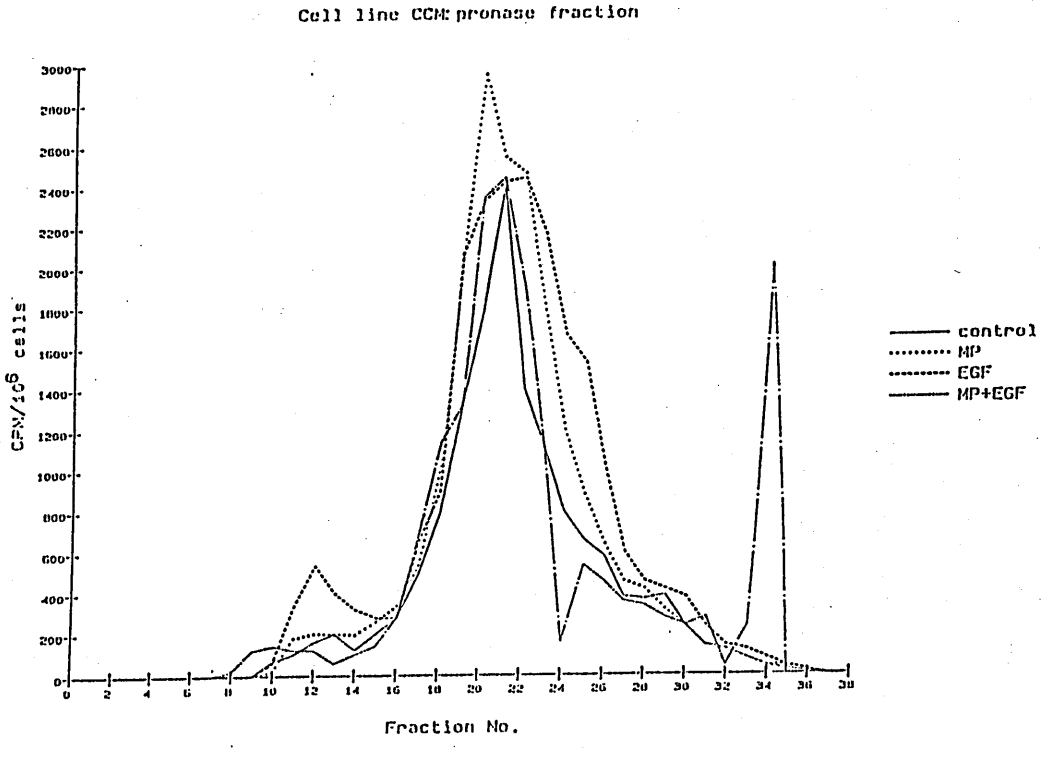


Figure 17

The effect of glucocorticoids on the glycoconjugates profile. The effect of (a) methylprednisolone (MP) and (b) dexamethasone (Dx) on the elution profile of glycoconjugates in the pronase fraction of cell line G-CCM from a TSK-G3000SW gel column. The cells were grown to 70% confluence, then were exposed to glucocorticoid for 5 days, then they were exposed to 5 μCi ml^{-1} of ^{3}H-glucosamine as described in the methods section.

Cell line G-IJKt: medium fraction

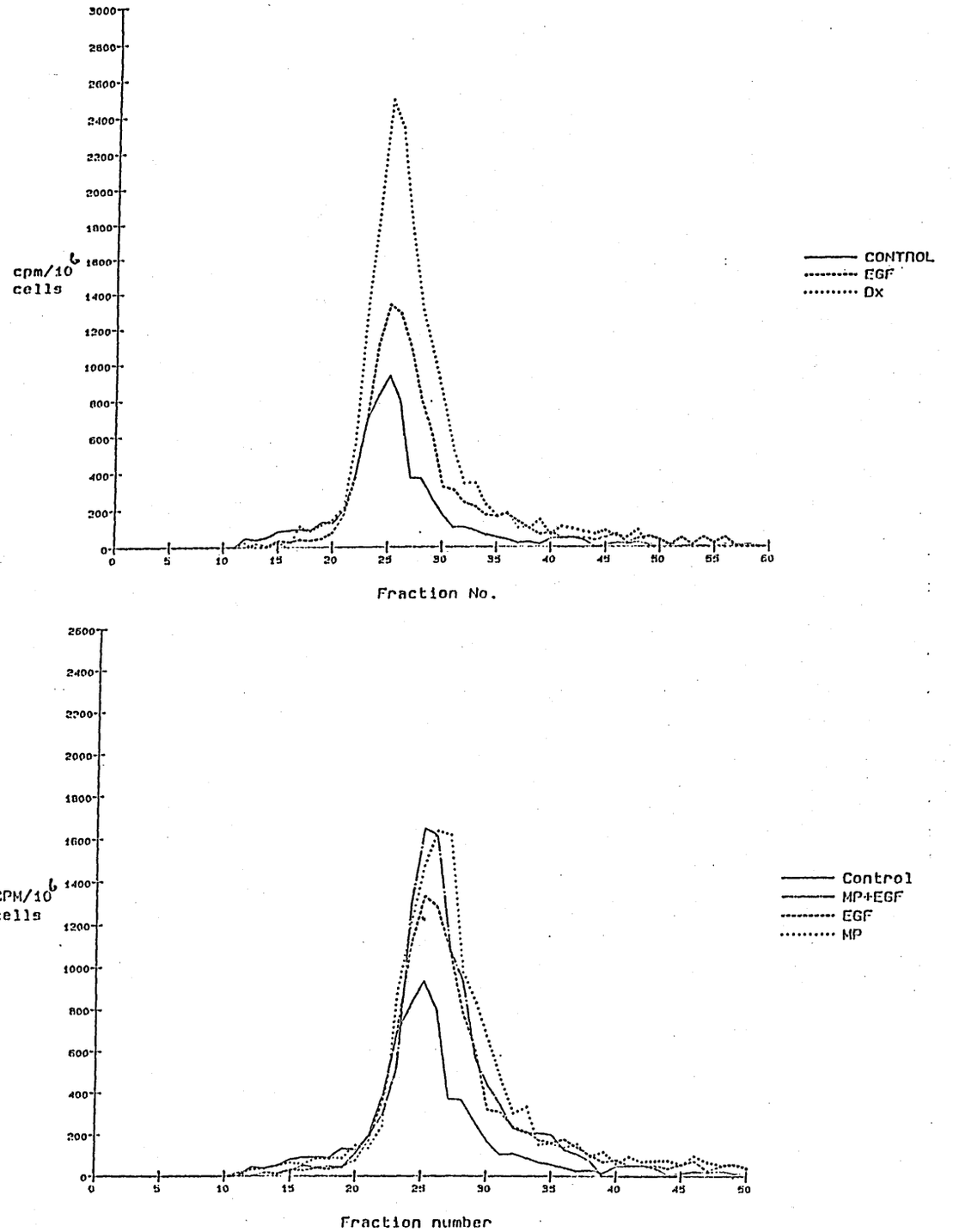


Figure18

The effect of glucocorticoids on the glycoconjugates profile. The effect of (a) methylprednisolone (MP) and (b) dexamethasone (Dx) on the elution profile of glycoconjugates in the medium fraction of cell line G-IJKt from a TSK-G3000SW gel column. The cells were grown to 70% confluence, then were exposed to glucocorticoid for 5 days, then they were exposed to 5 $\mu Ci\ ml^{-1}$ of ^{3}H-glucosamine as described in the methods section.

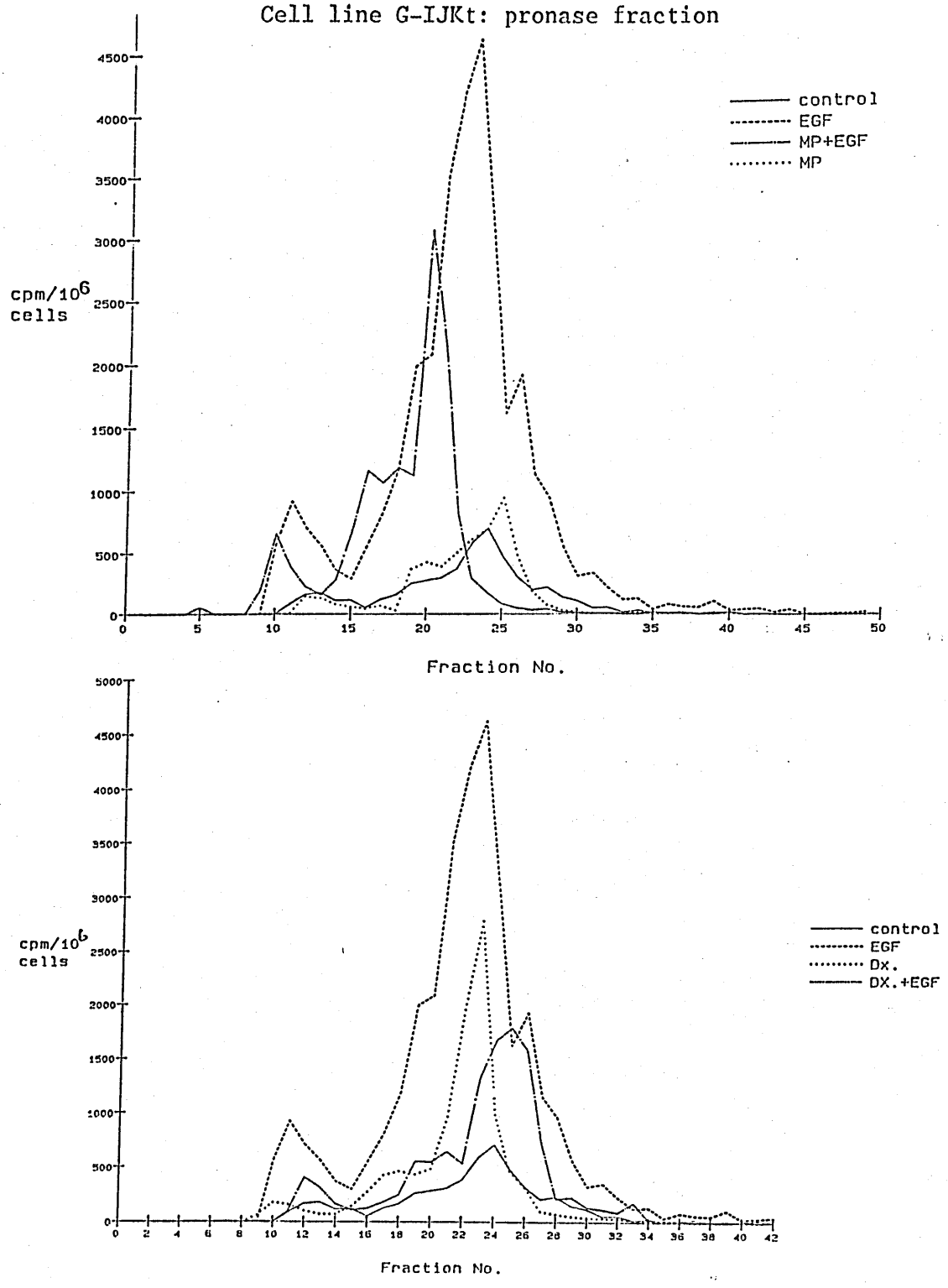


Figure19

The effect of glucocorticoids on the glycoconjugates profile. The effect of (a) methylprednisolone (MP) and (b) dexamethasone (Dx) on the elution profile of glycoconjugates in the pronase fraction of cell line G-IJKt from a TSK-G3000SW gel column. The cells were grown to 70% confluence, then were exposed to glucocorticoid for 5 days, then they were exposed to 5 µCi ml^{-1} of ^{3}H-glucosamine as described in the methods section.

Table 8

The effect of glucocorticoids on the quantity of glycoconjugates elluting from the TSK-G3000SW column.

Cell line	Fraction	Drug	Area under curve
G-CCM	medium	control	13,751
		Dx+EGF	16,395
		MP+EGF	19,089
		MP	10.707
		Dx	18,289
		EGF	8,154
G-CCM	Pronase	control	14,521
		Dx	18,964
		MP	21,453
		EGF+DX	15,175
		EGF	18,749
		MP+EGF	17,976
G-IJKt	medium	control	6,696
		Dx	17,103
		EGF	10,459
		MP+EGF	10,495
		MP	13,445
G-IJKt	pronase	control	5,174
		EGF+Dx	12,086
		EGF	32,239
		Dx	10,617
		MP	5,425
		EGF+MP	14,108

Illustrates numerically the area under the glycoconjugate peaks elluted from the TSK-G3000GW column. This was worked out by adding up all the counts within that peak. (Dx) dexamethasone, 2.5×10^{-5}M; (MP) methylprednisolone, 2.5×10^{-5}M and (EGF) epidermal growth factor, 1ng/ml.

present in both the medium and pronase fractions. In the presence of dexamethasone, the shift in terms of molecular weight by EGF was reduced.

3. Investigating the effect of glucocorticoids in relation to the quantity of either fibronectin or laminin produced by the cell

An example of fibronectin positive cells is shown in Figure 20 with cell line BG84/5. Quantification of the results presented some difficulties since they depended on assessing the intensity of the staining, which is very subjective. Thus, it proved extremely difficult to try to ascertain whether the presence of either 10µg/ml or 1µg/ml of dexamethasone or methylprednisolone altered the intensity of the staining. The staining was not found to be uniform throughout the slides: it increased towards the edges of the coverslips. The results obtained suggested that dexamethasone may have reduced the quantity of laminin present in the glioma cell lines, while in terms of fibronectin the results were inconclusive.

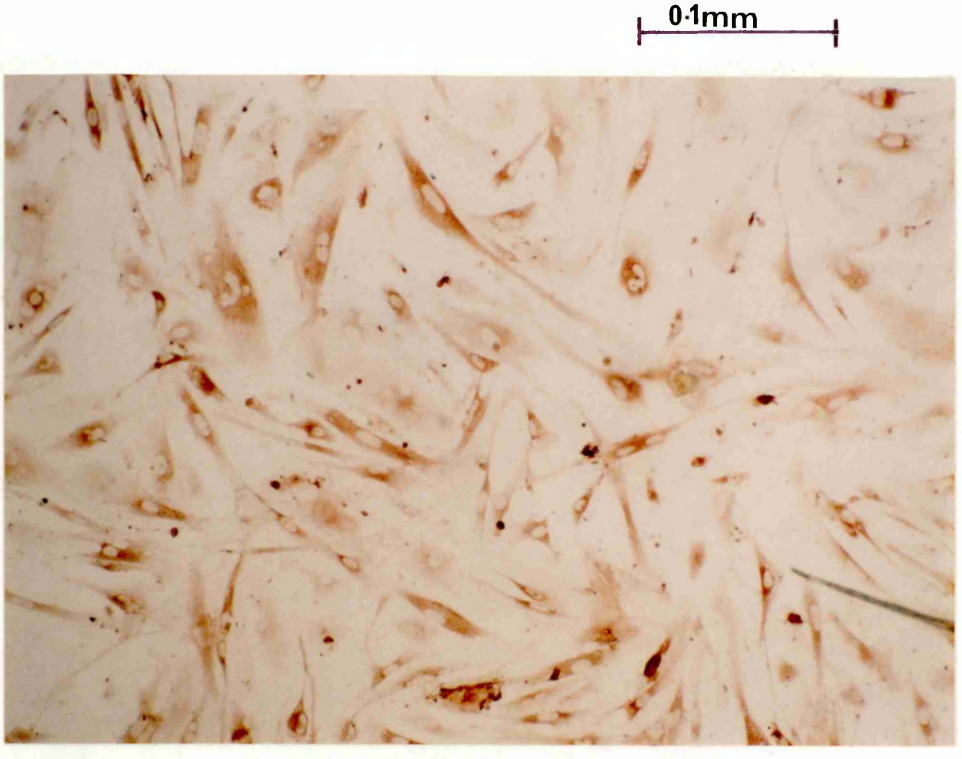


Figure 20

The photograph illustrates staining for fibronectin in cell line BG84/5.

DISCUSSION

Malignancy has been correlated with an oversialation of the glycopeptides (Van Beek et al, 1978; Warren et al, 1972). It was therefore asked whether alteration of the glycopeptide composition of malignant cells would diminish the abnormal behaviour of malignant cells. Freshney et al (1980b) showed that an oversialation in malignant cells could be reduced by treating the cells with dexamethasone. Thus, it was hoped to observe in this chapter an alteration from high molecular glycoconjugates to lower molecular weight species in the presence of either dexamethasone or methylprednisolone.

The results obtained by Gillian Hunt, looking at the composition of intact glycoconjugates, illustrated that both methylprednisolone and dexamethasone did induce alterations in the composition of glycoconjugates produced by the cell. However, both cell lines differed in terms of their actual response. Thus, these experiments would have to be repeated using a variety of different cell lines before any conclusive decisions can be made.

The composition of the surface glycoconjugates was investigated using a TSK-G3000SW gel exclusion column, as described in the methods section. Upon separating the glycopeptides in the pronase digest using a TSK-G3000SW gel exclusion column changes in both the size and quantity of the glycoproteins were detected in the presence of either dexamethasone or methylprednisolone. With respect to the medium extract, both dexamethasone and methylprednisolone were seen to produce changes. Dexamethasone was found to increase the total quantity of glycoconjugates present in the medium fraction of both cell lines, whereas methylprednisolone had the opposite effect on cell line G-CCM. However, dexamethasone was seen to have a more pronounced effect than methylprednisolone on cell line G-IJKt, which corresponds with the results obtained in the last section with respect to cell proliferation. In the pronase extracts, the glucocorticoids were seen to increase the quantity of glycoconjugates present, this effect was more prominent in cell

line G-IJKt. It is very hard to interpret the effect of epidermal growth factor in the present experiments.

Thus, dexamethasone and methylprednisolone were observed to alter the quantity of glycopeptides on the surface of the malignant cells. However, no consistency in the alterations were observed between the two cell lines tested. These experiments need to be repeated with a larger number of different cell lines before any conclusions can be drawn. The sensitivity of this technique was insufficient to detect small changes in molecular weight. It was therefore concluded that a more sensitive method was required. As glycopeptides comprise a vast range of different species, it was decided that it may be easier to investigate the above relationship by looking specifically at one particular group of molecules. It was therefore decided to investigate the composition of proteoglycans as these molecules have been shown to have profound effects on normal cell behaviour.

CHAPTER 4

INTRODUCTION

PROTEOGLYCAN AND GLYCOSAMINOGLYCAN PROFILES IN RELATION TO MALIGNANCY

The aim of this chapter is to investigate the effect of glucocorticoids on proteoglycan composition and whether such an effect could account for the cytostatic effect of the glucocorticoids observed in Chapter 2.

1. STRUCTURE OF PROTEOGLYCANS

Proteoglycans are comprised of glycosaminoglycan (GAG) chains bound to a protein core via a linkage region (Figure 21). The GAG chains consist of repeated disaccharide sequences containing either glucuronic acid or iduronic acid residues and an amino sugar, which can either be N-acetyl glucosamine or N-acetyl galactosamine (Hook, 1984; Kraemer, 1975; Lindahl and Hook, 1978). There are no formal criteria for identifying proteoglycans, however seven different glycosaminoglycans families have been identified according to their sugar residues:- hyaluronic acid, chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate, heparan sulphate, heparin and keratan sulphate (Lindahl and Hook, 1978; Hook, 1984; Poole, 1986). They are comprised of linear polymers of alternating A and B monosaccharide units (Figure 22)

1.1 Hyaluronic acid proteoglycan

Hyaluronic acid is the largest GAG molecule of all the seven groups, (molecular weight exceeds 10^6 daltons in normal tissues), and exists as a linear structure. It is the only GAG group which is not found sulphated or bound to a protein core. Under physiological conditions the carboxyl group dissociates to form hyaluronate. The literature on hyaluronic acid however does not differentiate between the acid and salt forms, therefore both will be referred to as hyaluronic acid throughout this report. Turley

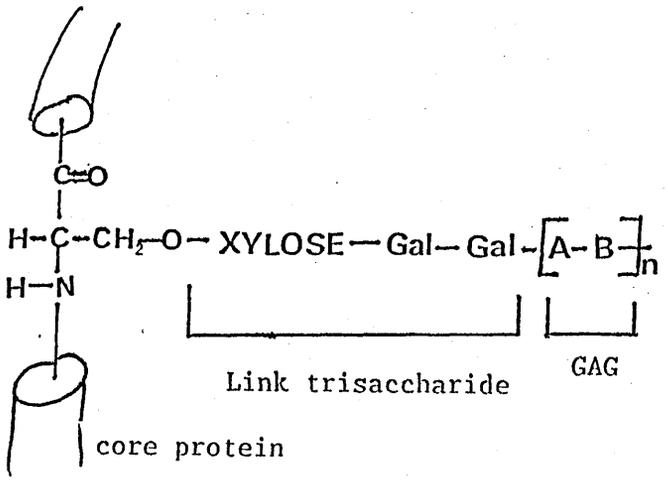


Figure 2.1

Schematic drawing of the linkage between a glycosaminoglycan chain (GAG) and the protein core. A specific linkage sequence at the end of the GAG chain is bonded to a serine of the protein core.

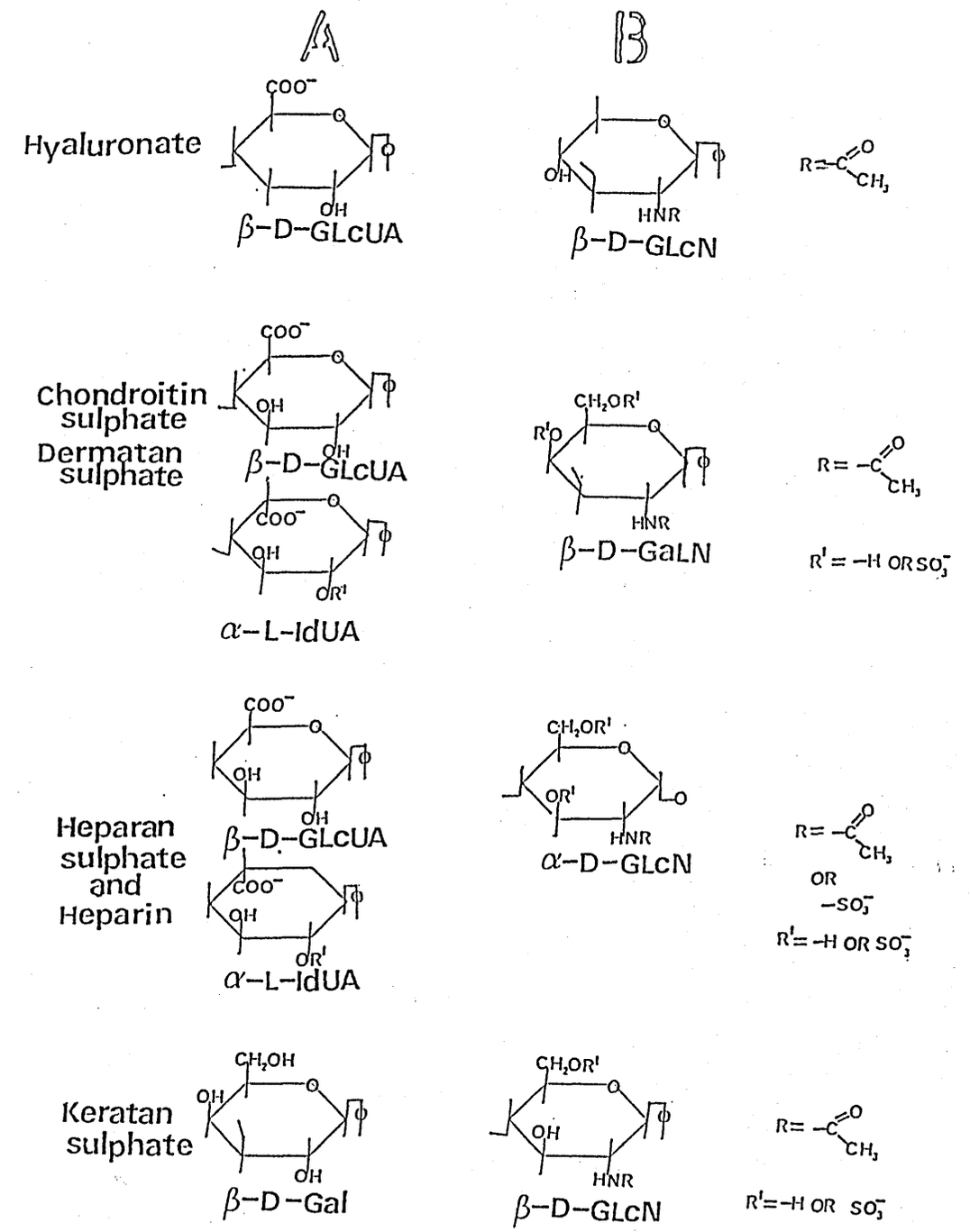


Figure 22

A schematic drawing of the molecular structure of the seven different glycosaminoglycan groups. The abbreviations are: GlcUA, glucuronic acid; IdUA, iduronic acid; GlcN, galactosamine; Gal, galactose.

and Roth (1979) proposed that hyaluronic acid may be synthesised at the cell's surface, while Prehm (1983(a), 1983(b)) using teratocarcinoma cells, suggested a stepwise addition of UDP-N-acetyl glucosamine and UDP-glucuronic acid alternatively to the reducing end of the polysaccharide at the plasma membrane. The UDP unit is released as the subsequent sugar nucleotide is added. Initiation of synthesis is thought to be independent of a protein core, resembling the mechanism for assembling bacterial polysaccharides (Hook, 1984).

1.2 Dermatan sulphate /Chondroitin sulphate proteoglycan

Dermatan sulphate is formed from chondroitin sulphate through the conversion, at the polymer level, of some or all of the glucuronic acid residues by epimerization into iduronic acid residues. The content of D-glucuronic acid residues in dermatan sulphate may range from negligible amounts to more than 90% of the total uronic acid residues. This variation in the D-glucuronic acid content introduces a considerable degree of heterogeneity: some sources of dermatan sulphate consist solely of iduronosyl-N-acetyl galactosamyl-4-sulphate disaccharide units, while the more complex form contains an extensively hybridised polysaccharide backbone where both the 4- and 6-O galactosamine residues are sulphated (Lindahl and Hook 1978). The degree of sulphation can vary considerably within this molecule and may occur at the 4 and/or 6 position of N-acetyl galactosamine or at the 2-O position of iduronic acid residues. Sulphation is carried out by sulphotransferases which utilise 3'phosphoadenylsulphate (PAPs) as the sulphate donor and catalyse the formation of ester sulphate (O-sulphate) groups at positions C_4 and C_6 of N-acetyl galactosamine residues (Hook, 1984; Kraemer, 1975; Dorfman, 1981).

1.3 Heparan sulphate and Heparin proteoglycan

Heparan sulphate has been the most widely investigated proteoglycan (Gallagher et al, 1986; Hook et al 1974; Hook, 1984). It is thought to contain the largest degree of structural variability of all the different GAG groups, which may reflect its broad range of

biological functions (see discussion section). Despite heparin and heparan sulphate containing identical residues, Gallagher and Walker (1985) have suggested that they are actually two separate families of N-sulphated GAGs. Heparan sulphate is produced by most mammalian cells. Within this molecule, N-sulphated galactosamine comprises of about 50% of the total hexosamine content, while the quantity of O-sulphated groups present varies between different tissues. Heparin, on the other hand, is produced mainly by mast cells. The majority of these residues are N-sulphated, (>80% of the glucuronic acid residues), and there is always a molar excess of O-sulphates over N-sulphates. Compared to heparan sulphate, heparin in general contains fewer N- and O-sulphated groups and reduced numbers of tri-sulphated disaccharides. They concluded that in heparan sulphate, both the iduronate and O-sulphate groups were largely confined to regions of high N-sulphated glucosamine content, while the O-sulphates were never found to be separated from the N-sulphates by more than one monosaccharide. The N-acetylated glucuronic acid groups were correlated to the regions containing low sulphated and enriched glucuronic acid residues. It is reasonable to suggest that the above structural rules may also apply to the other GAG groups. Heparan sulphate is synthesised according to a series of polymerisations shown in Figure 23. These reactions do not always go to completion, hence a mixture of the various intermediates are found in vivo (Lindahl and Hook, 1978).

1.4 Polymer formation for sulphated GAGs

Polysaccharide formation is initiated by the transfer of xylose units from UDP-xylose to serine residues in the protein core. The xylosated protein core then acts as an acceptor for successive transfer of two galactose units from their corresponding UDP sugars (Hook, 1984; Kraemer, 1975). The neutral trisaccharide xylosylgalactosylgalactose acts as a primer for growth of the polysaccharide chain. In the case of chondroitin sulphates, polymer formation involves an alternating transfer of glucuronic acid and N-acetyl galactosamine units to the non-

Polymerization of Heparan sulphate

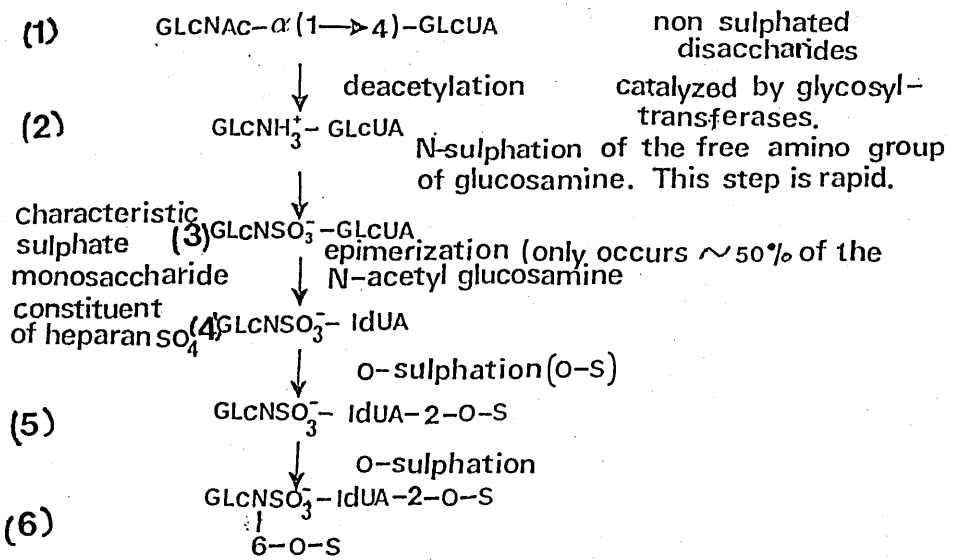


Figure 23

A schematic representation of polymerisation of heparan sulphate. Herparan sulphate is synthesised initially as a non-sulphated polymer of repeat sequences of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA). The extensive postpolymerisation modifications that occur can convert the non-sulphated disaccharides (1) into disaccharides containing 3 sulphate groups with the original GLcUA residue converted into iduronic acid (IdUA) (6). The modifications do not go to completion so (3 and 4) are found in vivo. Adapted from Lindahl et al, 1978.

reducing end of the growing polysaccharide chain, forming a chain of repeating disaccharides.

The length of the polysaccharide chains appears to be controlled by the ratio of available primers and UDP- sugar precursors present. This is clearly shown by the following example. Upon increasing the concentration of β-Xylosides, the number of primers available for chain elongation are increased and the length of the synthesised chains are reduced (Schwartz, 1979). In contrast, if protein synthesis is inhibited by cyclohexamide, the number of growing chains are reduced while the length of the polysaccharide chains are increased (Kimura et al, 1981).

1.5. Protein cores of proteoglycans

Compared to the polysaccharide GAG chains, there is relatively less information available concerning the molecular structure of the protein cores, mainly due to the denaturing conditions necessary in the purification procedures. Recent evidence suggests that protein cores are not unique entities but exist either as unsubstituted proteins or as proteoglycans. A 240kDa protein from the surface of melanoma cells, was found to exist on its own or in association with chondroitin sulphate groups (Hassel et al, 1986). Fransson (1987) found that protein cores differed in terms of their molecular weight and biological properties. Recent evidence suggests a relationship between the protein core structure and the cellular distribution of proteoglycans. Antibodies to protein core determinants have shown cross reactivity between a range of basement membrane proteoglycans, for example yolk-sac carcinoma, Engelbreth-Holmswarm (EHS) sarcoma, kidney, liver, skin and cornea basement membranes. Whereas antibodies against the rat liver membrane proteoglycans cross reacted with the cell surface but not with the basement membrane proteoglycans from either EHS sarcoma or parietal endoderm cells (Oohira et al, 1982; Fenger et al, 1984; Dziadek et al, 1985). This suggests that there may be two structurally dissimilar proteoglycan protein cores being synthesised in tissues with different distributions *in vivo*.

1.6. Proteoglycan synthesis and degradation

Proteoglycans are synthesised either through altering existing proteoglycans or by synthesising totally new molecular species. New protein cores are synthesised in the rough endoplasmic reticulum, from where they are transported into the Golgi apparatus, for the addition of new GAG chains (Hassel et al, 1986; Gallagher et al, 1986; Lindahl et al, 1986; Farquhar, 1985). The vast majority of post-translational modifications occur when the precursor protein is processed in the Golgi apparatus. These include one or more of the following:-

i) Addition of O-linked oligosaccharides onto appropriate serine and threonine residues.

ii) Addition of GAG chains onto serine and threonine residues.

iii) Conversion of high mannose N-linked oligosaccharides to complex forms.

iv) Possible processing of the protein by removal of portions of the polypeptide.

The newly synthesised proteoglycan can have a large variety of fates depending upon the cell type and function of the macromolecule. It may enter a storage granule for example, as heparin proteoglycan does in mast cells (Metcalf et al, 1980) or it may enter secretory vesicles where it may either be deposited on the cell surface as an intercalated, integral membrane component, as for heparan sulphate on rat liver plasma membranes (Kjellen et al, 1981), or, it may be secreted into the extracellular matrix as a structural component, for example heparan sulphate proteoglycan in glomerular basement membranes (Kanwar et al, 1981).

Surface proteoglycans can either be removed by being secreted into the matrix or by being endocytosed (Bienkowski and Conrad, 1984). Two different endocytotic pathways have been elucidated, one with a half life of approximately 30 minutes, while the other has a half life of approximately 4 hours (Yanagishita and Hascal, 1984). Only a small fraction of the cell's total surface proteoglycans are reported to be endocytosed, where they are

either partially degraded in the endosome and then transferred to the Golgi apparatus or are totally degraded to monosaccharides, sulphate and amino acid moieties. Enzymatic degradation (Piepkorn et al, 1988) of proteoglycans requires the presence of glycosidases to cleave the GAG side chains and proteinases to degrade the core and link proteins.

2. Distribution of proteoglycans in vivo

Proteoglycans are either found in the extracellular matrix or associated with the plasma membrane where they are either intercalated into the membrane or bound to it as illustrated in Figure 24. 85% of cell associated proteoglycans are situated on the external surface of the plasma membrane (Piepkorn et al, 1988).

2.1. Proteoglycans in the extracellular matrix

The extracellular matrix consists of a dense lattice of collagen and elastin containing proteoglycans and glycoproteins (Kramer and Vogel, 1981; Bosman et al, 1985). Heparan sulphate, heparin and a highly sulphated dextran sulphate have been implicated in playing a role in matrix stabilisation. Some *in vitro* data suggests that the binding of heparan sulphate to fibronectin is too weak to constitute a stable association *in vivo*. However, certain protease generated fragments of fibronectin were found to bind heparan sulphate more strongly than the intact molecule, implying that cryptic binding sites may exist which are only accessible after certain conformational changes in the fibronectin molecule. This conformational change may be induced by composition of the extracellular matrix.

2.2. Proteoglycan associated with the plasma membrane

(I) Proteoglycans intercalated with the plasma membrane

Recent evidence implies the existence of intercalated proteoglycans. A hydrophobic segment has been identified in the protein core associated with proteoglycans, which possesses the

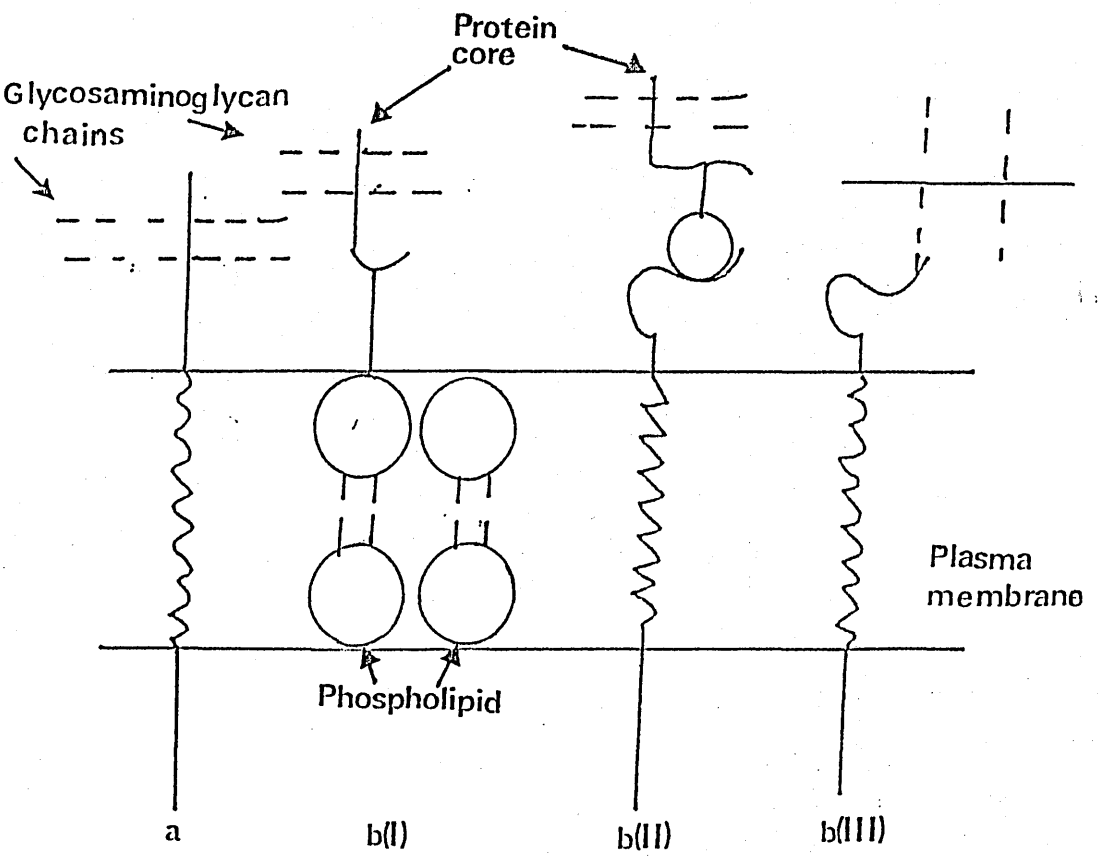


Figure 24

Taken from Fransson, 1987. Illustrates how proteoglycans are attached to the plasma membrane. They are either found:-

(a) Intercalated: The proteoglycans may be an integral plasma membrane component anchored via a hydrophobic peptide segment.

(b) Bound to the plasma membrane either via:-

(i) The core protein.

(ii) Non-covalent bonds to a receptor in the plasma membrane.

(iii) The glycosaminoglycan side chains.

ability to span the plasma membrane, while the GAG groups are situated on the outside of the membrane (Rapraeger and Bernfield, 1983; Norling et al, 1981; Kjellen et al, 1981). The existence of such a molecule has been ascertained from the following set of experiments:-

(i) Exogenous proteoglycans can be incorporated into artificial membrane vesicles illustrating the presence of a hydrophobic region.

(ii) Proteoglycans contain hydrophobic regions in the protein core, as seen from their adhesion to hydrophobic gel matrices.

(iii) Proteoglycans can be extracted from cells using 4M guanidine hydrochloride. Large aggregates can then be dissolved upon addition of detergents, thereby implying the presence of a hydrophobic region.

These protein cores are not unique to proteoglycans but are also found as free entities in the body. The above experiments provide indirect evidence for the presence of a hydrophobic region in the protein core. Direct evidence could be obtained upon identifying an intradomain region containing a lipophilic amino acid sequence. This type of proteoglycan was found to be resistant to trypsin digestion.

The intracellular proteoglycan concentration is thought to influence the physiological behaviour of the cell by acting indirectly via the nucleus. Unusually high concentrations of heparan sulphate fragments, rather than sulphated proteoglycans, were found to accumulate in the nucleus of hepatocytes at confluence. If the entry of heparan sulphate into the nucleus was inhibited, this resulted in the cell being unable to respond to contact inhibition (Ishihara et al, 1987). However recent evidence suggests that the proportion of GAGs endocytosed, compared to the net quantity produced by the cell, is very small (Fransson et al, 1987) and thus, the significance of nuclear localisation may be open to question.

(II) Proteoglycans found bound to the plasma membrane

Proteoglycans are found bound to the plasma membrane (Figure 24) through either:-

(a) Covalent links to an inositol-containing phospholipid in the plasma membrane. This type of binding was found, using endothelial cells, Chinese hamster cells and brain cells, to be reversible, and dependent on time, pH, ionic strength and temperature (Kjellen et al,1980; Oldenberg et al, 1979; Robinson and Gospodarowicz, 1984).

(b) The core protein or the glycosaminoglycan side chains.

Proteoglycan receptors have been identified on the plasma membrane by displacement studies, where endogenous GAGs are displaced by an alternative molecule, for example heparan sulphate proteoglycan can be displaced from its receptors by the addition of heparin (Kjellen et al, 1980). Heparin, heparan sulphate and iduronic-acid rich chondroitin sulphate were found to compete for the same receptor, while glucuronic-acid rich chondroitin sulphate and hyaluronic acid competed for a different receptor. These results suggest the presence of at least two distinct receptors for proteoglycans on the cells surface. However, it must be stressed that these experiments only suggest that these various compounds compete for the same receptor, but does not actually prove it. A good example where this suggestion was proved wrong, was seen when highly sulphated polysaccharides were thought to compete with low-density lipoproteins in cultured fibroblasts. However, upon analysis the LDL receptors were found to differ significantly from the GAG receptors (Goldstein and Brown, 1977). Such a result could be explained if a conformational change could occur, when a ligand binds to its receptor and results in the dissociation of a completely distinct ligand. Thus, care must be taken in analysing such data. From the proteoglycans position on the extracellular matrix and on the plasma membrane they are thought to influence cell adhesion, proliferation and migration. The ability of proteoglycans to bind to either the plasma membrane or the extracellular matrix is influenced by:-

(i) Electrostatic forces at physiological pH and ionic strength

(Underhill and Toole, 1980). With the exception of hyaluronic acid and keratan sulphate (which lack sulphate and carboxyl groups respectively), binding was found to increase with the degree of sulphation.

(ii) Molecular weight. Using hyaluronic acid moieties of different molecular weights, Underhill and Toole (1980) found that the larger molecular weight species bound more strongly to the surface of 3T3 cells than the lower molecular weight species. They explained the higher affinity for large polymers in terms of multiple-site attachment, the larger the number of receptors interacting with the polymer then the stronger the attachment (Figure 25). However, Laurent et al (1986) carrying out a similar investigation denied that the above relationship was due to multiple binding sites. They explained the results in terms of multiple exclusion: the increased binding of the higher molecular weight species was due to a larger number of sequences being recognised along the oligosaccharide by the receptor.

(iii) The degree of ionisation of the side chains, that is the partition coefficient (Underhill and Toole, 1980; Lindahl and Hook, 1978).

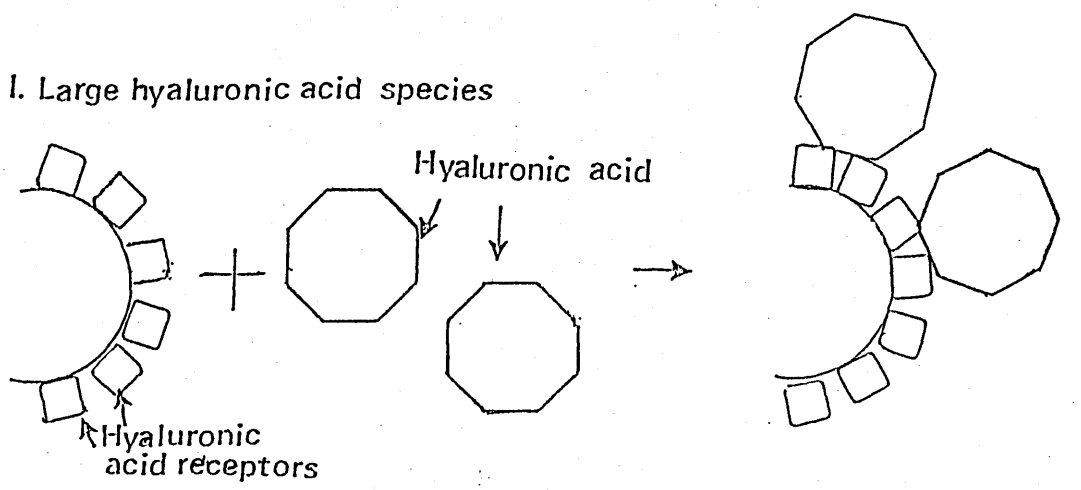
(iv) Composition of proteoglycans. L-iduronic acid residues (for example, dermatan sulphate and heparan sulphate), were found in general to bind proteins to a greater extent than residues only containing D-glucuronic acid as the only uronic acid component (for example, chondroitin sulphate).

Each of these factors were found to influence binding, however the intact proteoglycan molecule was found to interact with proteins to a greater extent than their corresponding polysaccharide chains. This suggests that the three dimensional structure may be important in terms of their biological properties.

3. Biological activity of proteoglycans

The biological properties of proteoglycans are thought to be primarily due to the glycosaminoglycan residues attached to the protein core. There are on average 50-100 disaccharide repeat

Characteristics of proteoglycan binding to receptors



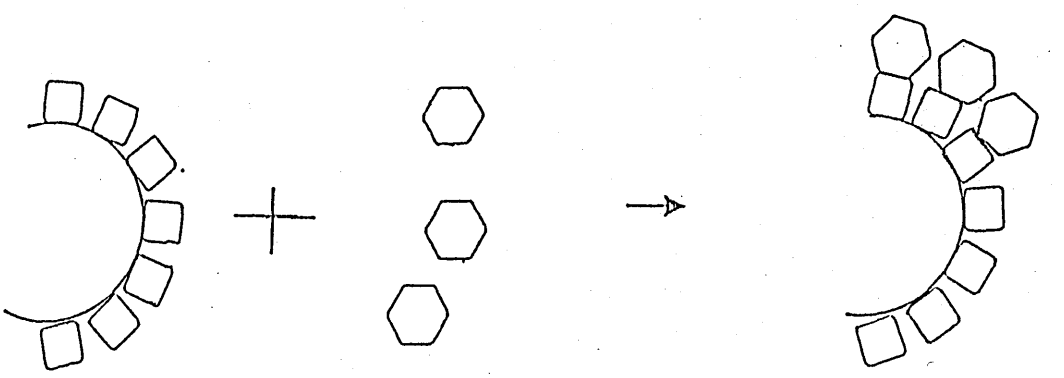


Figure 25

A schematic model of the multiple receptor occupancy model. The larger the size (and hence molecular weight) of the GAGs then the greater the number of receptors they interact with. Taken from Underhill et al, 1980.

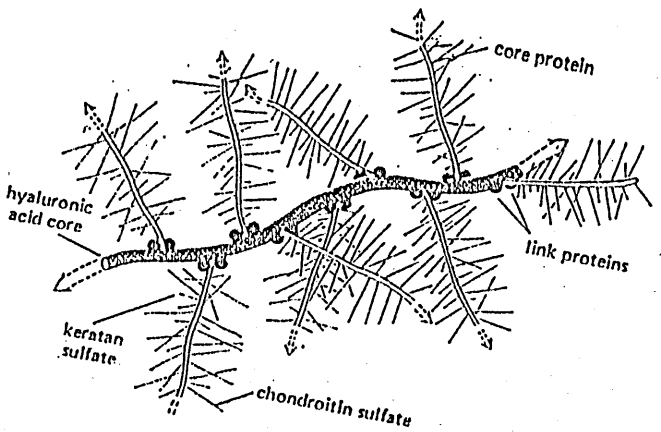


Figure 26

A schematic drawing of a large proteoglycan aggregate in cartilage of about 100 proteoglycans (taken from Stryer, 1981).

units in each GAG chain. Glycosaminoglycans, with the exception of hyaluronic acid, are not found to exist in the native form as free entities but are found bound to proteins as proteoglycans. These molecules exist in vivo as aggregates which can either be made up of one GAG group, or a combination of different groups (Figure 26). Heterogeneity is introduced into the proteoglycan molecule, as the GAG chains themselves differ in terms of molecular weight, charge and in both the quantity and spatial arrangement of the hydroxyl, sulphate and carboxyl chains (Table 9). The situation is even more complicated as GAG chains have been found to contain more than one GAG group. Sequences for both chondroitin sulphate and dermatan sulphate have been found to exist on the same GAG chain in mouse mammary epithelial proteoglycans (Rapraeger et al, 1985).

Before the profile of proteoglycans in malignant tissues can be analysed in terms of abnormal behaviour, the GAG profile of normal cells needs to be investigated in relation to the following criteria:-

(i) The degree of cellular maturation.

(ii) Differences between the logarithmic and stationary phases of the growth cycle.

(iii) The relationship between the composition of the extracellular matrix, cell shape and proteoglycan production.

(iv) The relationship between cell adhesion and the proteoglycan profile.

3.1. Proteoglycan profile in relation to cell maturation

Upon correlating proteoglycan production with malignancy, many investigators have omitted to take into consideration the stage of tissue maturation and age of the tissue, resulting in an unjustified over-generalization of results. Glant et al (1986) reported that normal cartilage showed age-related differences in terms of proteoglycan composition. Roughly and White (1980) reported that the change from foetal to the mature adult state occurred through a gradual transition rather than a sharp alteration. As cartilage tissue becomes more mature the following

Table 9

Differences in relation to charge and molecular weight of proteoglycans

Polysaccharide	Mol. wgt. Range $x10^3$	Charge/ Disaccharide unit	Site of occurance
Hyaluronic acid	4000–8000	1	synovial fluid, vitreous humor
Chondroitin–6–sulphate	5–50	1·2-2·3	Cartilage, bone
Chondroitin–4–sulphate	5–50	1·1–2·0	Cartilage, bone
Dermatan sulphate	15–40	2·0–2·2	Skin, heart valves
Heparan sulphate	50	1·1–2·8	Lung, liver
Heparin	4–16	3–4	Lung, liver

Illustrating the range in molecular weight and charge of proteoglycans, along with an example of where each proteoglycan is found in the body. The variation in "charge density" essentially resides in the sulphate content. Taken from Comper et al, 1978.

changes are prominent:-

i) Proteoglycan content of the cartilage decreases.

ii) Size of the proteoglycan subunits decreases.

iii) The proportion of keratan sulphate relative to chondroitin sulphate proteoglycans increases.

iv) 6-sulphation relative to 4-sulphation along the chondroitin sulphate chains increases.

v) The relative proportion of protein present relative to GAG chains increases.

These results stress that when comparing proteoglycan composition in tissues, it is important to take the maturation state and age of the tissue into account when reporting results. Unfortunately, determining the degree of maturation of the cells under investigation in vitro is not always possible as the parent cell lineage is seldom known. Some investigators have overcome this problem by either using chemically (Rabes et al, 1970) or virally transformed cell lines (Roblin et al, 1975), thereby producing both normal and transformed cells of similar age in terms of tissue maturation. However, it is questionable to what extent such transformants actually resemble the cells present in spontaneous tumour formation.

3.2. The relationship between cell proliferation and proteoglycan profile

Conflicting evidence exists about whether proliferation can influence proteoglycan production independently of cell shape. Extensive cell proliferation in vivo often takes place within a hyaluronic acid enriched environment (Toole, 1981). Greater quantities of cell-associated hyaluronic acid and lower quantities of sulphated proteoglycans have been found to be produced from both proliferating normal and transformed cells, compared to their corresponding resting cells (Glimelius et al, 1979). However, some investigators (e.g. Cohn et al, 1976) have not found this difference. Along with proliferation influencing the quantity of proteoglycans being produced, alterations in the actual structure of these molecules has also been noted. Heparan sulphate produced

by cells during the log phase, generally consists of simple, unbranched molecules while the same GAG produced during the stationary phase comprises a longer, more highly branched structure.

Heparan sulphate has been implicated in inhibiting cell proliferation as illustrated in the following example. Proliferation was drastically decreased when normal fibroblasts were plated onto confluent, glutaraldehyde fixed fibroblasts. Pretreating the latter substrata with heparitinase enabled the fibroblasts to continue proliferating (Culp et al, 1978). Kraemer and Tobey (1972) showed that a specific shedding of cell-surface associated heparan sulphate occurred immediately before Chinese hamster ovary cells divided thus implying that the heparan sulphate proteoglycan is associated with inhibition of cell proliferation. However it is not clear whether a change in cell shape was evident in the latter experiment.

Therefore, in order to compare proteoglycan production in any tissue, consideration must be given to the substrata involved, the cell's shape, state of maturation and whether the cells are in logarithmic or stationary phase of their growth cycle. These factors are important in normal tissues and need to be taken into consideration when making any correlation between proteoglycans and malignancy.

3.3. Proteoglycan production and cell shape.

A complicated relationship exists between cell shape and proteoglycan production (Folkman and Moscona, 1978). The cells synthesise and release the proteoglycans which are structural components of the extracellular matrix. Plating normal chondrocytes onto a substratum coated with increasing concentrations of poly-2-hydroxylethyl methacrylate (polyHEMA) caused cell spreading to be progressively restricted, while proteoglycan production was enhanced (Glowachi et al, 1983). Hence, upon inhibiting cell spreading the quantity of proteoglycans being synthesised was increased. Along with the substrata influencing the quantity of proteoglycans being

synthesised, the composition of these proteoglycans can also be affected, as shown by the following example. Human skin fibroblasts cultured on collagen gels produced two species of dermatan sulphate, one enriched in iduronic acid residues which binds specifically to collagenous fibres of the gel and the other enriched in glucuronic acid which is excreted from the cell in vitro. Both types of proteoglycans were also produced by cells grown on plastic, however in this environment they were both released into the growth medium. Net synthesis of dermatan sulphate was three fold higher on collagen gels than on plastic. In contrast, heparan sulphate synthesis was not influenced by the nature of the culture surface (Gallagher et al, 1983).

This relationship between cell shape and proteoglycan production is more complicated than depicted above as the proteoglycan composition of the extracellular matrix can have a profound effect on cell shape. In the presence of high concentrations of extracellular hyaluronic acid, the cells have a more rounded appearance, while in the presence of heparan sulphate proteoglycan they take on a more flattened appearance. Thus, an intricate system is set up where cell shape can influence proteoglycan production, which in turn can influence the composition of the extracellular matrix, which may affect cell shape etc. Hence, the relationship between cell shape, proteoglycan production and the composition of the extracellular matrix is complex.

3.4. The relationship between proteoglycan profile and cellular adhesion

Cellular adhesion has been found in vivo to be favoured by the presence of either heparan sulphate or low concentrations of hyaluronic acid and inhibited by high concentrations of hyaluronic acid. Biochemical analysis of cellular adhesive sites to the substrata showed that its proteoglycan composition altered in relation to age (Lark and Culp, 1984). High concentrations of heparan sulphate were found in the relatively newer sites, implying a relationship between the concentration of heparan

sulphate and the binding force of the cell. Cellular migration may therefore be favoured by the weaker binding forces present at the older adhesion sites due to lower heparan sulphate concentrations.

The above results may be explained in the following way. Hyaluronic acid possesses an unique ability among GAGs, to form aggregates due to the presence of multiple binding sites. Underhill and Toole (1980) found that these aggregates could be inhibited by either hyaluronidase (total inhibition was obtained in the absence of divalent cations such as Mg^{2+},Ca^{2+}) or high concentrations of hyaluronic acid. In addition to hyaluronic acid, chondroitin, (desulphated chondroitin-6-sulphate) and to a smaller extent dermatan (desulphated dermatan sulphate), also inhibited cellular aggregation. However, chondroitin-4-sulphate, chondroitin-6-sulphate, heparin and heparan sulphate had little or no effect on cellular aggregation (Underhill and Dorfman, 1978). The cell's binding affinity in vivo has been postulated as being much weaker in the presence of high concentrations of hyaluronic acid. Such conditions in foetal tissues have been found to be essential for cell migration (Toole, 1981). Hence, it may be that when the cell binds to the substrate through the hyaluronic acid receptors, by occupying these sites the tissues may be more prone to migrate due to the lack of adhesive forces to the substratum.

3.5. How can these alterations in proteoglycan composition affect growth control?

Proteoglycans are found to interact with the cytoskeleton, which, as discussed in the sections 1.3. and 1.4. in the introduction, can affect cell shape and gene transcription. Proteoglycans have been found to bind directly to collagen fibres and actin stress filaments within the cell, indicating a direct means of communication between structural elements of the stromal and cytoplasmic matrices. They may also act indirectly via other components such as cartilage link proteins, which have an affinity for collagen, laminin and fibronectin. The latter glycoprotein, as mentioned in section 6 in the introduction, can interact

directly with actin stress filaments. Thus there is a complex series of interactions where intercalated proteoglycans may interact with intracellular actin, or extracellular proteoglycans may interact with structural glycoproteins such as fibronectin, which in turn can interact with actin filaments (Hook, 1984; Woods et al, 1984). This results in an intricate system involving proteoglycans by which information can be sent from the extracellular matrix into the cell and vice versa (Figure 27).

4. THE RELATIONSHIP BETWEEN PROTEOGLYCAN PRODUCTION AND MALIGNANCY

4.1. Proteoglycan production in malignant tissues

Malignant glioma lines in comparison to their normal counterparts were found to produce greater quantities of hyaluronic acid and lower quantities of sulphated proteoglycans. In general higher concentrations of hyaluronic acid compared to sulphated glycosaminoglycans are associated with malignant tissues, hamster embryo fibroblasts and for most oncogenic lines derived by virus transformation by the Herpes Simplex Type-2 or Simian Virus-40. In comparison Chandrasekaran and Davidson (1979) found that the opposite relationship existed with breast tissue. By modifying the mode of proteoglycan extraction, Glimelius et al (1978) compartmentalised the differences between glioma and normal glia cell lines as illustrated in Table 10. Glimelius et al (1978, 1979) found that the majority of proteoglycans produced by normal brain cells in vitro were cell-associated, while in malignant cells a larger proportion of this proteoglycan was released into the surrounding medium. Proteoglycan production in terms of quantity and distribution, for both normal and malignant tissues, may be influenced by the way in which the cells are handled, but are neverless tissue specific. Various investigators have found that the net production and distribution of proteoglycans in vitro were relatively similar among normal cell lines, whereas malignant lines seemed to display more individual characteristics (Chandrasekaran and Davidson, 1979). Glioma cell lines in vitro produce higher concentrations of hyaluronic acid

Figure 27

A schematic diagram of how cell associated proteoglycans may alter the type of proteoglycans being synthesised by the cell. Adapted from Fransson, 1987. Abbreviations are:- RER, rough endoplasmic reticulum; SER., smooth vesicles; SV., secretory vesicles. The proteoglycans which are assembled in the golgi are either released by the cell where they can bind to membrane receptors or are intercalated into the cell membrane. The cell can alter its proteoglycan by endocytosing the surface proteoglycans, degrading them in the endosome and synthesising the new proteoglycans required. The above process may be controlled by the interaction of proteoglycans either directly or indirectly via the actin filaments which can in turn affect the array of nuclear tubules.

Figure 27

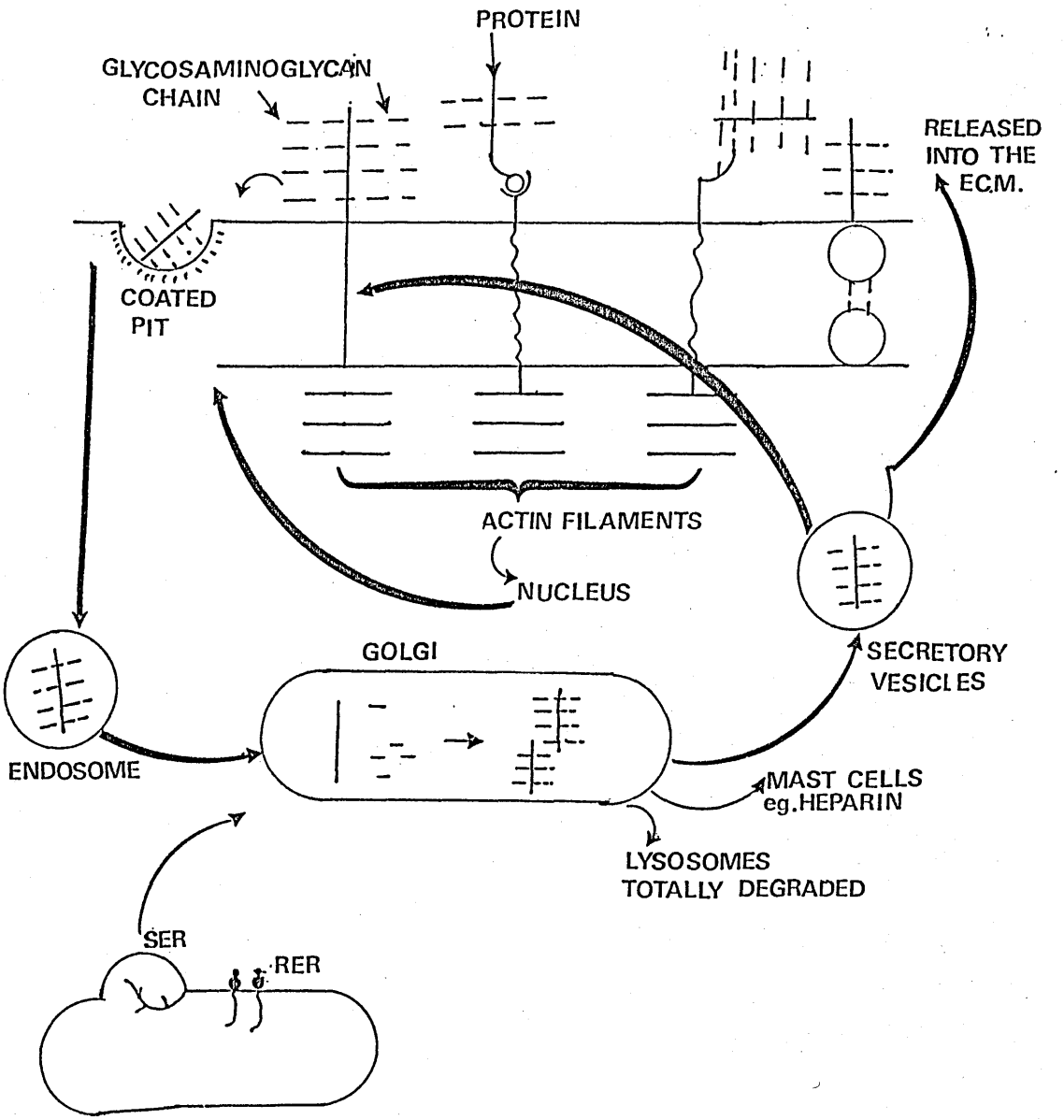

PROTEIN
GLYCOSAMINOGLYCAN CHAIN
RELEASED INTO THE E.C.M.
COATED PIT
ACTIN FILAMENTS
NUCLEUS
GOLGI
SECRETORY VESICLES
ENDOSOME
MAST CELLS eg.HEPARIN
LYSOSOMES TOTALLY DEGRADED
SER
RER

Table 10 Distribution of proteoglycans in glia and glioma cell lines as reported by Glimelius et al, 1979.

Compartment/ Enzymes or chemicals used	Glia	Glioma
EDTA resistent material i.e. matrix left on culture dish.	High conc. of Heparan sulphate	Reduced amounts of heparan sulphate
Medium	High conc. of dermatan sulphate.	Higher conc. of hyaluronic acid and reduced amounts of dermatan sulphate.
Trypsin susceptible	Mainly heparan sulphate, minor quantities of various GAGs.	Hyaluronic acid predominant, whereas chondroitin sulphate rather than heparan sulphate was the predominant sulphated GAG.
EDTA+Trypsin	Heparan sulphate was the dominant sulphated GAG.	Chondroitin sulphate rather than heparan sulphate was the predominant sulphated GAG.

and release a larger proportion of proteoglycans into the medium compared to normal glia.

4.2. The relationship between malignancy, proteoglycan production and cell shape

Cell shape, as seen in section 3.3 can dramatically influence the proteoglycan profile in normal cells, which in turn may affect the physiological behaviour of the cells. As discussed in the general introduction, malignant cells are thought to resemble immature foetal cells in terms of both cell shape and physiological properties. Therefore, it is reasonable to propose that malignant cells are only abnormal in respect that they are expressing foetal properties at an abnormal period of time. These processes themselves are not unique but appear to be not as tightly regulated. Such an effect was seen upon comparing proteoglycan composition of malignant and foetal cells. Both cell types were found, by a number of investigators, to contain larger quantities of hyaluronic acid compared to their normal mature counterparts (Satoh et al, 1973). Turley and Tretiak (1985) using B-16 melanoma metastatic cell lines found that hyaluronic acid was concentrated at the tumour-stromal interface. Hyaluronic acid has the ability to absorb large quantities of water, which results in the proteoglycans swelling. This may account for the more rounded appearance of both foetal and malignant cells, which in turn would reduce the quantity of the cell's surface in contact with the substrata. This may explain the reported lack of adhesion by both neoplastic and foetal cells. In support of this hypothesis is the finding that high concentrations of hyaluronic acid are essential for migration of immature cells to their site of differentiation (Toole, 1981).

High concentrations of hyaluronic acid have also been associated with the malignant cell's undifferentiated appearance and its ability to invade into surrounding tissues as illustrated by the following examples. High concentrations of hyaluronic acid were found to inhibit differentiation of chick somite cells as seen through the lack of morphological signs of chondrogenesis

(Toole, 1972). However, differentiation was found to occur if the concentration of hyaluronic acid was enzymatically reduced. Turley and Tretiak (1985), using melanoma cells, found a correlation between the cell's invasive potential and an increase in the concentration of hyaluronic acid.

Thus, the above hypothesis relating high concentrations of hyaluronic acid to the cells malignant behaviour is quite attractive, as it explains the tendency of malignant cell's to migrate from their site of origin, their more rounded, undifferentiated appearance and their ability to invade surrounding tissues. However, other investigators have either found the reverse to be true (Cohn et al, 1976) or no correlation at all (Davidson et al, 1975), but this may be a consequence of the type of cells used in their investigations.

4.3. The correlation between the extent of sulphation of glycosaminoglycan groups and malignancy

Malignancy has been correlated with either an over or under sulphation of proteoglycans (Kraemer, 1979; Hook, 1984). For example, the reduced binding affinity of rat hepatoma cells to a fibronectin-sepharose column has been correlated with the presence of undersulphated heparan sulphate (Robinson and Gospodarowicz, 1984). Disruption of the extracellular matrix *in vitro* has been associated with the presence of undersulphated proteoglycans as illustrated in the following example. Rat cells transformed by a temperature sensitive mutant of the Rous Sarcoma Virus were found to synthesise all the extracellular matrix components (fibronectin, laminin, heparan sulphate and slightly reduced quantities of procollagen), but were unable to lay down a matrix (Alitalo et al, 1982). Upon investigating the difference between normal and virally infected cells, the latter cells were found to produce undersulphated heparan sulphate. This proteoglycan has been implicated in matrix stabilisation (Gallagher et al, 1986). This may be due to multiple co-operative interactions between proteoglycans and collagen, fibronectin and laminin molecules. A good example of this is seen in rheumatoid arthritis, where

proteoglycan degradation (which is reversible) can lead to irreversible cartilage degradation (Handley et al, 1985). Hence, any abnormalities in proteoglycan structure may affect the structure of the extracellular matrix which can influence the physiological behaviour of the cell.

4.4. Correlation between the malignant cell's invasive capacity and proteoglycan degradation

A major characteristic of malignant cells compared to their benign counterparts, is their ability to invade surrounding tissues either through mechanical or enzymatic mechanisms (Barsky et al, 1983). This invasive property can be visualised *in vitro* by plating both malignant and normal cells onto a prelabelled matrix (Vlodavsky et al, 1983). Degradation of the extracellular matrix by the malignant cells, but not by their normal counterparts, was visualised by the appearance of radioactive material in the growth medium. This disruption of the structure of the extracellular matrix is thought to occur by activation of degradative enzymes such as plasminogen activators, collagenases and proteoglycanases. High levels of plasminogen activator (PA) have also been associated with a loss of anchorage regulation in primary rat embryo cells transformed by Simian Virus-40 (Pollack et al, 1974). Ossowski and Reich (1983) have associated a reduction in PA activity with a reduction in metastasis. Thus, the high levels of PA activity may be necessary for the cells to invade the surrounding tissues. Laug, 1983 and Frame et al, 1984 have shown that glucocorticoids can inhibit PA production in endothelial and glioma cells respectively. Thus, it could be postulated that this reduction in PA activity cause a reduction in the invasive capacity of malignant cells.

5. Aim of chapter

Interactions of cell associated proteoglycans with their microenvironment have been shown to be important for normal cell growth and development, and for the maintenance of differentiated functions. An intricate balance exists where proteoglycan

production is influenced by cell shape, state of proliferation and the degree of sulphation, which in turn can influence the physiological behaviour of the cell. It was asked whether the immature characteristics of malignant cells were related to their proteoglycan composition. This led to the hypothesis that induction of differentiation in malignant cells with drugs such as glucocorticoids may alter the proteoglycan composition of the malignant cells to resemble that of there mature counterparts, this would explain the re-expression of contact inhibition as observed in Chapter 2. This hypothesis will be investigated in this chapter by looking at the effect of glucocorticoids on proteoglycan composition and comparing the results with those of normal glia reported by Glimelius et al (1979).

Materials and Methods

1. Extraction of cell surface and extracellular matrix glycosaminoglycans (Gallagher et al, 1983)

Cultures of human glioma lines were grown in 75cm^2 flasks until 70% confluent. The cells were then grown in either the presence or absence of 2.5×10^{-5}M dexamethasone or methylprednisolone for 5 days. In order to ensure that the medium did not become the rate limiting factor of proliferation, it was changed every two days. The cells were then incubated with medium containing 5μCi/ml of [^{3}H] glucosamine (29Ci/mMol; NEN) for 48 hours. The glycosaminoglycans produced by the cells were subdivided according to the following extraction procedure.

(i) Medium extract

The medium was collected along with the two PBS washes of the cell monolayer.

(ii) Collagenase fraction

The cell monolayer was then incubated at 37^{0}C for 20 minutes with 3 ml of 0.05mg/ml of collagenase solution (grade IV, Boehringer) in Eagle's minimal essential medium containing serum albumin (0.1mg/ml). The enzyme supernatant was then removed and stored with two subsequent PBS washes of the cell monolayer.

(iii) Trypsin extract

The cell monolayer was then incubated with 3ml of 50 μg/ml trypsin (Worthington, 3x crystallised) and 2mM EGTA for 15 minutes at 37^{0}C and the reaction was terminated by the addition of 0.25mg/ml of soya bean trypsin inhibitor. The solution was then centrifuged and the trypsinate was collected. The cell suspension was then washed twice with PBS by centrifugation and these washes were then added to the trypsinate fraction.

(iv) Cell pellet

The pelleted cells were re-suspended in PBS and treated with papain as described below.

(v) Papain digestion

The medium, collagenase and trypsinate fractions were

dialyzed exhaustively against distilled water and the volume was reduced by using PEG 6000. These extracts were then dialyzed against 50mM sodium phosphate buffer pH 6.8 containing 1mM NaCl, 5 mM cysteine and 1mM EDTA. The cell pellet fraction was also suspended in this buffer, and incubated in the presence of 0.1mg/ml of papain overnight at 60^0C, followed by a further incubation of 50µg/ml of papain for 4 hours. These various extracts were then dialyzed against distilled water and concentrated using PEG 6000.

The various GAG groups in the above extracts were separated and identified at 4^0C using a DE52 column (25cm x 1cm) and the various GAG groups were identified according to the NaCl concentration at which they eluted. The experimental set-up is illustrated in Figure 28. The apparatus consists of two flasks with a side arm containing 250ml of 1M NaCl and 0.1M NaCl in 20mM sodium phosphate buffer, pH6.8. The side arms of the two flasks were connected together with a rubber tube containing a clip to initially separate the contents of both flasks. Both solutions were stirred continuously with a magnetic stirrer. The sample was loaded onto the column in phosphate-buffered saline (0.1MNaCl/10mM sodium phoshhate buffer, pH6.8), equilibrated in the same buffer and sufficient volumes of the equilibration buffer was passed through to remove the unbound material. The bound radioactivity was eluted by the NaCl gradient which was set up by opening the clip and pumping the solution, at a rate of 20ml/hour from the flask containing the lower NaCl concentration. Elution volumes of 2ml were collected using the automatic fraction collector. The salt concentration in the elution volumes was determined by comparing the conductivity reading with those from a NaCl standard curve. A 1ml aliquot of each sample was added to 10 ml of Ecoscint and read on the scintillation counter. The counts per fraction were then plotted against fraction number. The quantity of each GAG group present was estimated from the graph by adding up the absolute counts in each of the fractions making up the peak. The individual GAG groups were identified from the NaCl elution concentrations as stated by Glimelius et al 1983.

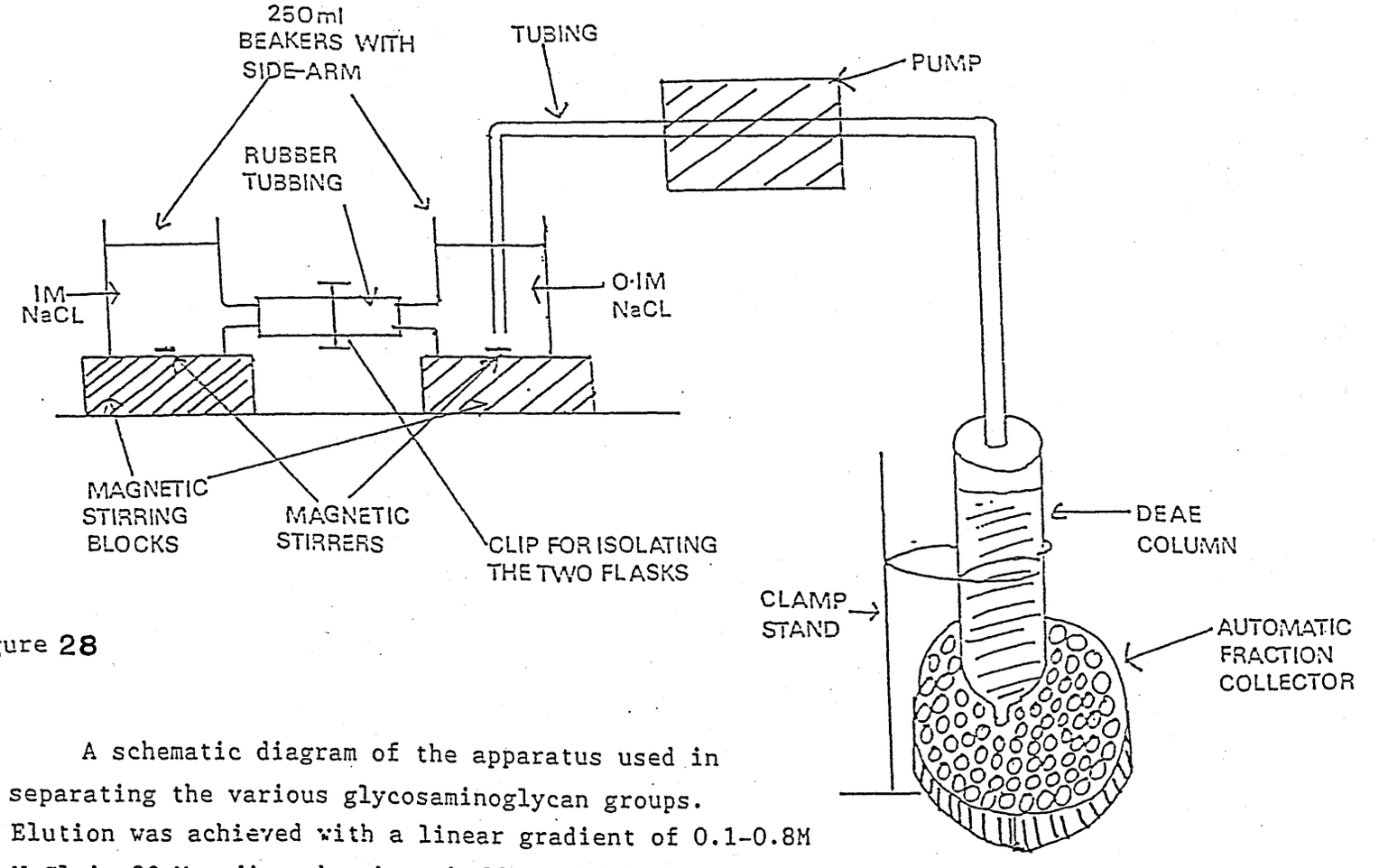


Figure 28

A schematic diagram of the apparatus used in separating the various glycosaminoglycan groups. Elution was achieved with a linear gradient of 0.1-0.8M NaCl in 20mM sodium phosphate buffer, pH6.8, at a flow rate of 20ml/hour.

2. Identifying individual GAG groups using a cellulose acetate electrophoretic technique (King, 1985)

In an attempt to identify the different peaks obtained from the DE52 column, each peak was collected, lyophilysed and resuspended in approximately 500µl of electrophoresis buffer. The cellulose acetate sheets (Cellogel), stored in 30% methanol, were soaked in electrophoresis buffer (0.05M phosphate buffer pH 7.2) for 5 minutes and the excess buffer was removed by blotting. 2µl of the sample was applied in 0.5µl aliquots, allowed to soak into the gel and then re-applied. These radioactive samples were then electrophoresised at 30mA for approximately 1 hour or as long as it took the Bromophenol Blue front to reach the other end of the gel. The gels were then stained with 1% (w/v) Alcian blue 8GX (Sigma) in 1:1 (v/v) ethanol/0.05M acetate pH5.8 for 10-15 minutes, and destained using 5% acetic acid/10% ethanol. To determine the GAG composition, each sample lane was cut into 1 cm^2 strips which were placed into counting vials. The strips were dissolved overnight using 1ml of 80% (v/v) acetic acid and were then counted in 10 ml of Ecoscint in the scintillation counter.

The quantity of hyaluronic acid present in the extract was taken to be the proportion of material susceptible to Streptomyces hyaluronidase (Sigma). 25µl of extract was treated with 10 units of S. hyaluronidase in a buffer consisting of 0.0375M sodium acetate/0.0375M NaCl pH5.4 and the incubation volume was 100µl.

The proportion of heparan sulphate present was taken to be the proportion of material resistant to treatment with chondroitinase ABC (Sigma) but susceptible to subsequent treatment with nitrous acid (HNO_2). 100µl samples of culture fluids were mixed with 100µl of 5.7M acetic acid and 100µl of 0.75M $NaNO_2$ and left at room temperature (22^0C) for 80 minutes. The reaction was inhibited by the addition of 100µl of 0.1M ammonium sulphamate. Controls were prepared by adding the test sample only after inactivation of the reaction mixture with sulphamate. Chondroitin sulphate was identified as the proportion of material resistant to HNO_2 and chondroitinase AC digestion. 100µl samples of extract was mixed with 0.1 unit (where 1 unit is that amount of enzyme

which converts 0.1µmol of substrate per minute), of chondroitinase AC (Sigma) in 300µl of 0.08M NaCl/0.06M sodium acetate/0.01M Tris HCl, pH8.0 containing 170µg of bovine serum albumin. The reaction mixture was incubated at 37^0C for 6 hours and was stopped by heating the mixture to 100^0C for 2 minutes. Finally, dermatan sulphate was estimated as the proportion of labelled material resistant to treatment with HNO_2 and chondroitinase AC (Sigma) but susceptible to chondroitinase ABC (Sigma).

3. Investigating the malignant cell's adhesion force to the substrata in the presence and absence of glucocorticoids.

Cells were grown to confluence in N25 flasks using growth medium, F10:DMEM (1:1), containing 10% foetal calf serum. The cells were then grown for a week in growth medium containing 1% foetal calf serum containing either $2.5x10^{-5}$M of dexamethasone or methylprednisolone. The cell monolayer was then dissociated using either EDTA or 0.25% trypsin. The cells were harvested in growth medium containing 1% foetal calf serum and this cell extract was then transferred into capillary tubes (Camlab) using a Gilson pipette. These capillary tubes were incubated in a humidified incubator at 37^0C, at various times these capillary tubes were inverted for 30 minutes and the number of cells adhering to the "top" surface were expressed as a percentage of the total number of cells in that field.

4. Investigating the ability of glioma cell lines to digest a prelaid extracellular matrix. (Vlodavsky et al, 1983)

The method used to lay down a prelabelled extracellular matrix is that reported by Vlodavsky et al. (1983). Normal endothelial cells were grown to confluence in 50mm petri dishes. Upon reaching confluence, the cells were fed every two days with growth medium containing [^{3}H] glucosamine. This overcame the possibility of the medium becoming exhausted of nutrients and label. The labelled extracellular matrix was then isolated by the following method. The cell monolayer was washed with PBS and then exposed to 0.5% Triton X-100 in PBS (v/v) for 30 minutes. The

latter treatment dissolved the cell layer leaving the underlying extracellular matrix intact. The remaining nuclei and cytoskeletons were then removed by a 2-3 minute exposure to 0.025M NH_4OH followed by four PBS washes. Malignant cells were then plated at a concentration of $2x10^4$ cells/ml (320 cells/cm^2) onto the above pre-labelled matrices. Experiments were carried out in medium containing either 10% and 1% FCS. 1ml samples of the medium were taken at various time points, added to 10 ml of Ecoscint and counted in the scintillation counter. The results were then expressed as the total quantity of label released by the cells compared to a control plate which only contained the appropriate growth medium.

RESULTS

1. Proteoglycan profile in relation to the presence/absence of glucocorticoids

Both dexamethasone and methylprednisolone were found in Chapter 2 to reduce malignancy associated characteristics, i.e. elevated saturation densities and plasminogen activator activity in glioma cell lines. In this chapter, the proteoglycan profile was investigated in the presence or absence of either dexamethasone or methylprednisolone and related to the cells' physiological behaviour. The proteoglycans were isolated according to the method described in the methods section. Pre-treating the cells with glucosamine resulted in all the carbohydrate groups of glycosaminoglycans, glycoproteins and glycolipids being labelled. If ^{35}S had been used, this would have labelled preferentially all the glycosaminoglycans groups with the exception of hyaluronic acid. As the latter glycosaminoglycan group is important in the present study, the former method was used in labelling the glycosaminoglycan groups and they were separated using a DE-52 column.

The glycosaminoglycan groups were found to elute at the following salt concentrations: hyaluronic acid at 0.26-0.3M NaCl; heparan sulphate at 0.4M NaCl, while dermatan sulphate eluted at 0.5M NaCl. An initial identification was carried out by collecting the eluted peaks, concentrating them and then treating each peak with various enzymes as described in section 2 in the Methods section. These solutions were then run through the column again in an attempt to determine whether the various peaks had been degraded. As this was a time-consuming process, the peaks were also identified on cellulose acetate gels. Standards were obtained, dermatan sulphate and chondroitin sulphate were found to comigrate, and could only be clearly identified by pretreating them with the enzymes described above. Unfortunately, hyaluronic acid purchased from Sigma produced a very diffuse band on cellulose acetate gels. After consultation with Dr. C.R.C. King, (Harrow), it was decided to use hyaluronic acid purchased from Life

Sciences, which was of a much purer quality. According to results published by King (1985), this glycosaminoglycan group should migrate in front of the sulphated glycosaminoglycans, however, this product could not be obtained during the duration of these experiments.

The proteoglycan composition of the medium, trypsinate, collagenase and cell pellet fractions of the cell was investigated in the presence or absence of either dexamethasone or methylprednisolone. The cells were pretreated with either dexamethasone or methylprednisolone for 6 days, then labelled with [^{3}H] glucosamine for 48 hours (for details see methods section). Figures 29 to 31 illustrates the data obtained from both the medium and trypsinate fractions of cell line G-CCM. The collagenase and cell pellet fractions were found to possess very low counts. As these counts were not much higher than the background of the scintillation counter it was decided that the errors involved in these results were too large, thus these fractions were not further considered.

2. Radioactivity eluting in the void volume

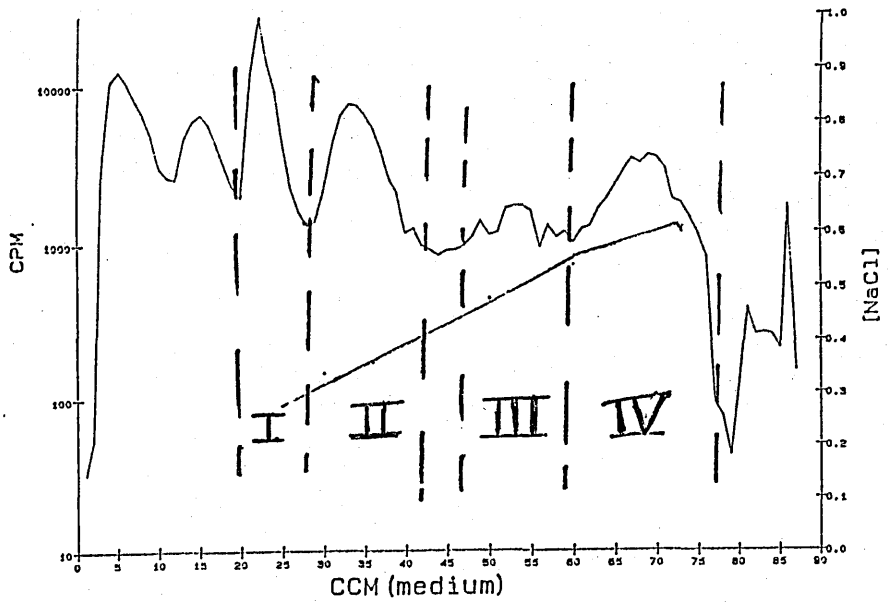
In each extract (medium and trypsinate) a proportion of the labelled material was eluted in the void volume before the gradient was applied, this was comprised in some cases of one or more peaks (Figures 29 to 31). These elution peaks were found to contain protein as determined from a Bradford assay and from its absorbance at 280nm. In the presence of dexamethasone or methylprednisolone, the quantity of the material eluting in the void volume was reduced. This material may consist of either glycoproteins or glycolipids as these molecules are also labelled with exogenous glucosamine. Alternatively, it may comprise of partially degraded proteoglycans or the protein cores of the sulphated proteoglycans.

3. Proteoglycan profile of the medium fraction

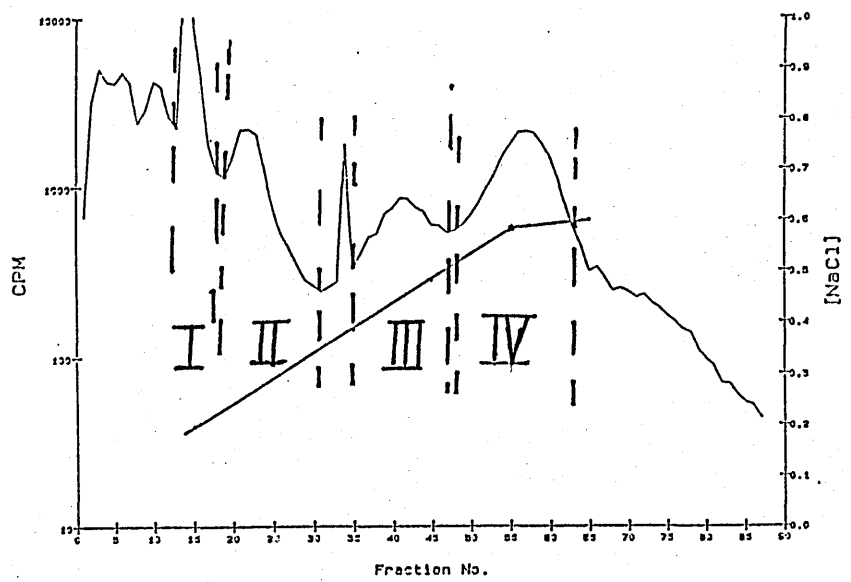
Figure 32 illustrates quantitatively the data from fractionating the medium of the cell lines as described in the

Figure 29

CCM (medium)
Control



CCM (medium)
Dx. (10ug/ml)



CCM (medium)
MP (10ug/ml)

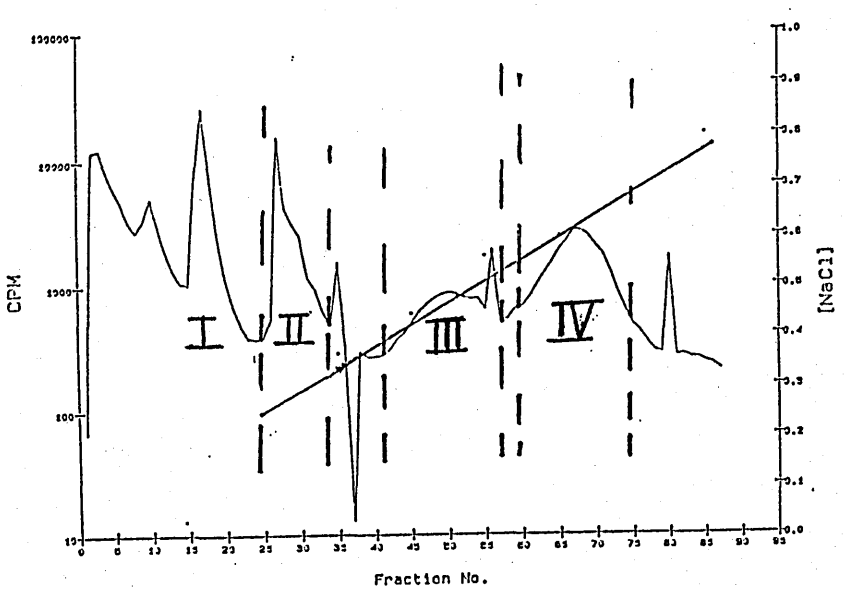
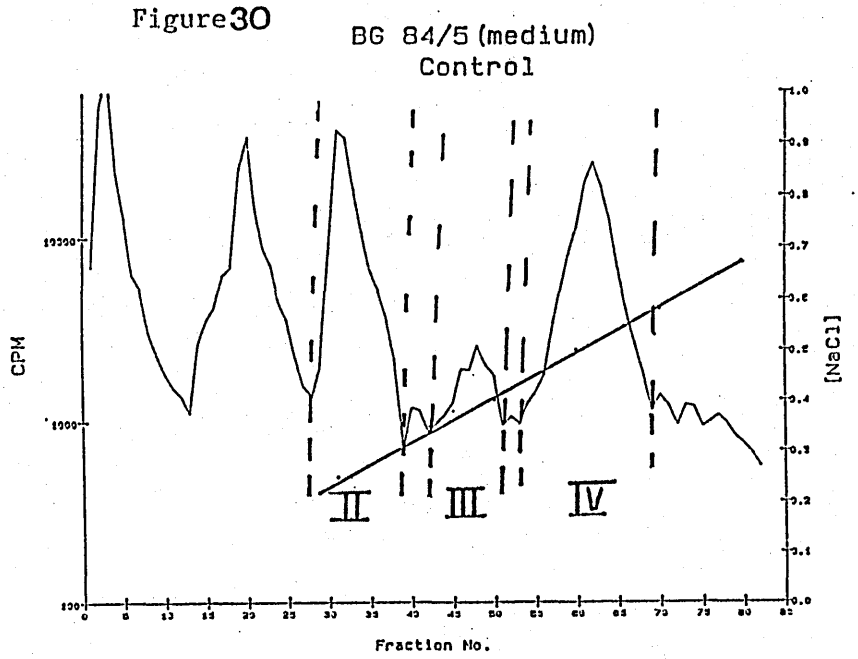


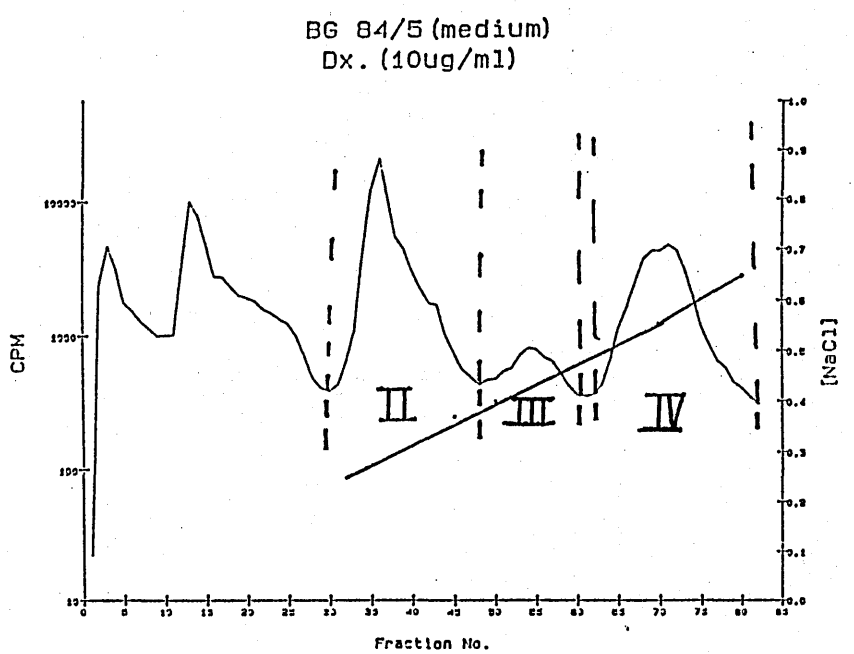
Figure29

Fractionation of radiolabelled glycosaminoglycans by chromatography on DEAE-cellulose.

The glycosaminoglycans in the medium extract of cell line G-CCM were elluted from a DEAE cellulose column with a linear gradient of 0.1-0.8M NaCl in 20mM sodium phosphate buffer. pH6.8. Peak II was identified as hyaluronic acid, peak III was thought to be heparan sulphate while peak IV was either dermatan sulphate or chondroitin sulphate.

Figure30


BG 84/5 (medium)
Control
CPM
[NaCl]
II
III
IV
Fraction No.


BG 84/5 (medium)
Dx. (10ug/ml)
CPM
[NaCl]
II
III
IV
Fraction No.

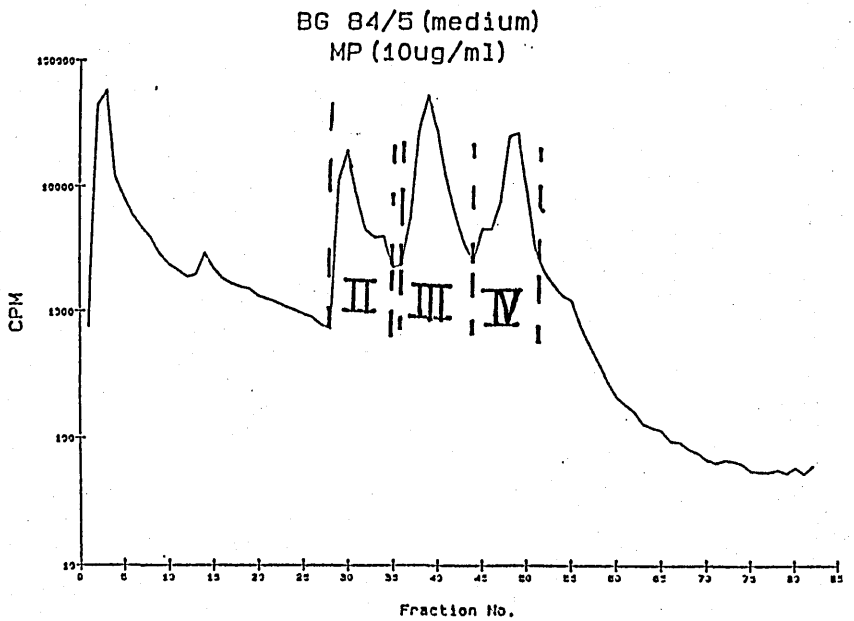

BG 84/5 (medium)
MP (10ug/ml)
CPM
II
III
IV
Fraction No.

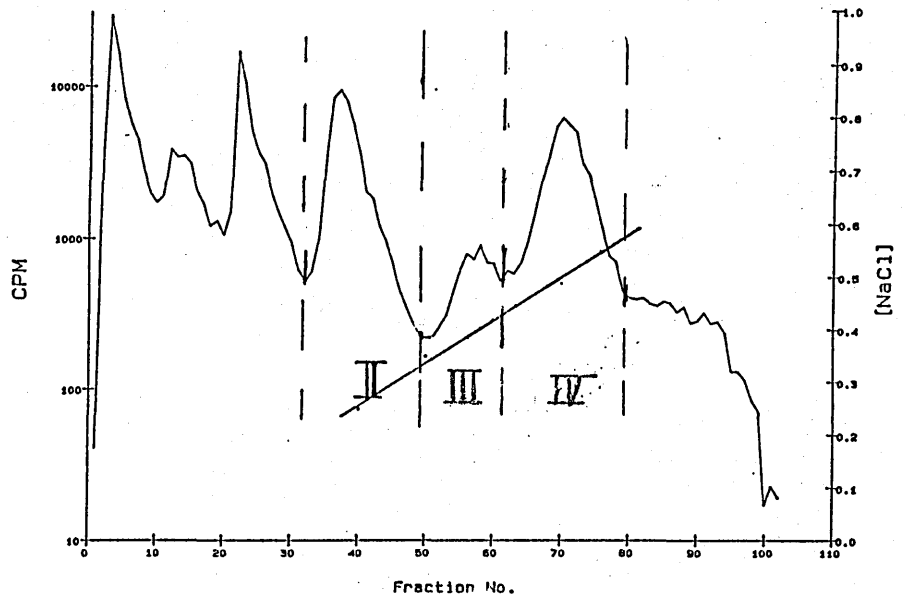
Figure 30

Fractionation of radiolabelled glycosaminoglycans by chromatography on DEAE-cellulose.

The composition of glycosaminoglycans in the presence and absence of either dexamethasone or methylprednisolone in cell line BG84/5 was determined using a DE-52 column with a linear gradient of 0.1-0.8M NaCl in 20mM sodium phosphate buffer, pH6.8. Peak II was identified as hyaluronic acid, peak III was thought to be heparan sulphate while peak IV was either dermatan sulphate or chondroitin sulphate.

Figure 31.

BG105/5 (medium)
Control



BG105/5 (medium)
Dx. (10ug/ml)

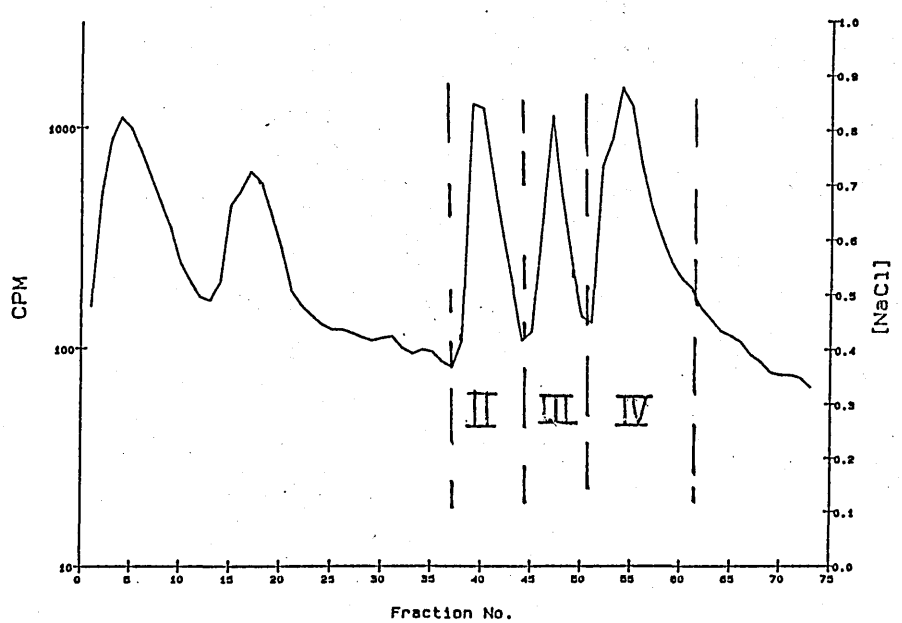
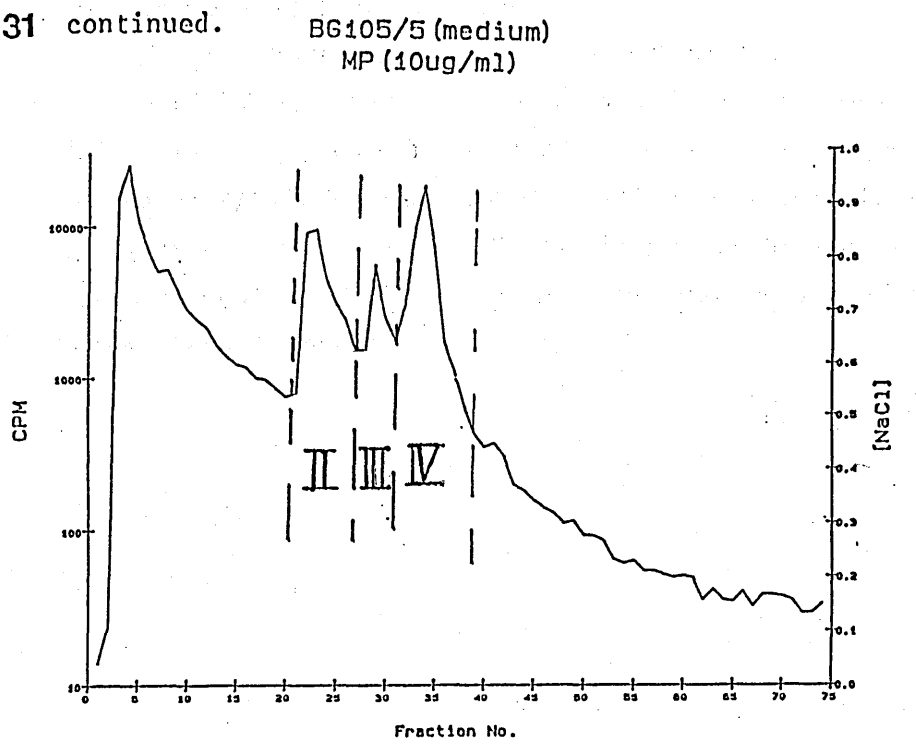


Figure 31 continued.



Fractionation of radiolabelled glycosaminoglycans by chromatography on DEAE-cellulose.

The glycosaminoglycans in the medium extract of cell line BG105/5 were elluted from a DEAE cellulose column with a linear gradient of 0.1-0.8M NaCl in 20mM sodium phosphate buffer. pH6.8. Peak II was identified as hyaluronic acid, peak III was thought to be heparan sulphate while peak IV was either dermatan sulphate or chondroitin sulphate.

methods section. Four peaks were isolated (numbered I, II, III and IV) as illustrated in Figures 29-31). Peak II was identified as hyaluronic acid, peak III heparan sulphate and peak IV dermatan sulphate or chondroitin sulphate according to their NaCl elution profiles. Peak I which absorbed at 280nm appeared at the beginning of the NaCl gradient and was not associated with the void volume peaks. As the relative proportion of the different glycosaminoglycan groups present was considered to be important in relation to cell's physiological activity, the quantity of the different glycosaminoglycans groups was expressed as a proportion of the total quantity of counts in fractions I-IV.

The data was expressed quantitatively by adding all the sample points (cpm/10^6cells) within one peak as illustrated on Figures 29-35 and expressing the results as a percentage of the total counts in that fraction. These results suggest that dexamethasone and methylprednisolone (at $2.5x10^{-5}$M) increased the quantity of peak I, decreased peak II (hyaluronic acid) and increased peaks III and IV. Dexamethasone was seen to have a greater effect than methylprednisolone. Thus, both methylprednisolone and dexamethasone were found to decrease the quantity of hyaluronic acid while increasing the quantity of sulphated glycosaminoglycans in the medium extract of cell line G-CCM.

In the medium extracts from the early passage cell lines only peaks II-IV were dominant after the addition of the NaCl gradient, as identified from their elution profiles. Thus the results were expressed as a percentage of the total counts in peaks II-IV. Once again, both dexamethasone and methylprednisolone were found to decrease the quantity of hyaluronic acid in the medium while increasing the relative quantity of sulphated glycosaminoglycans.

4. Proteoglycan profile of the trypsinate fraction

Figure 36 illustrates quantitatively the proteoglycan profile in the trypsinate fraction of both early passage and continuous cell lines in the presence and absence of either dexamethasone and methylprednisolone. This fraction represents the proportion of

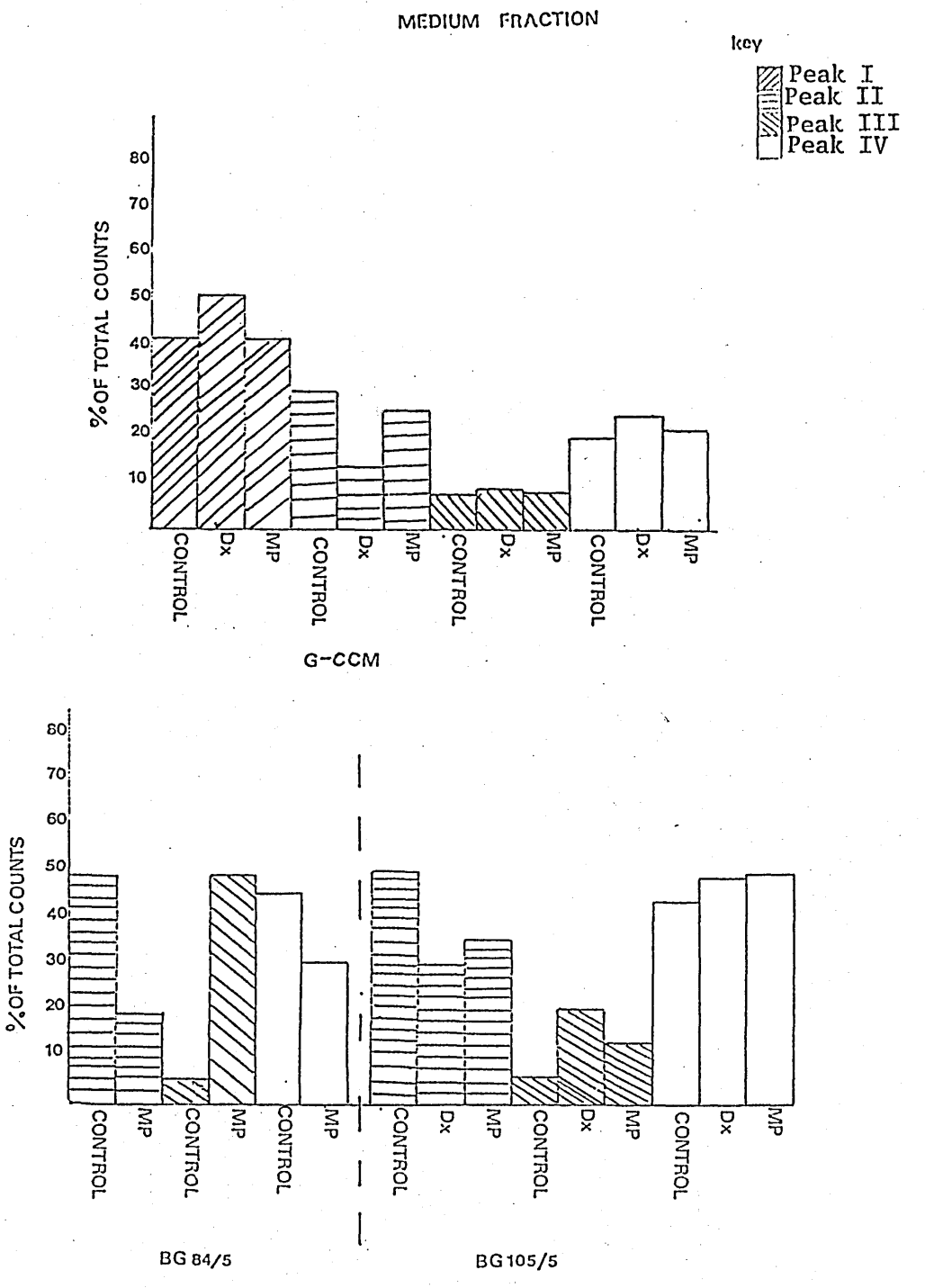
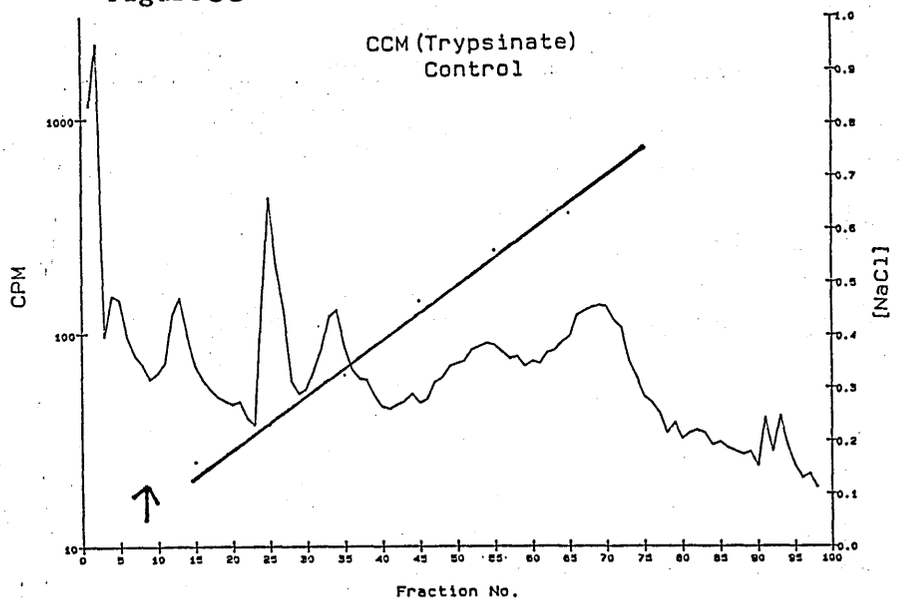


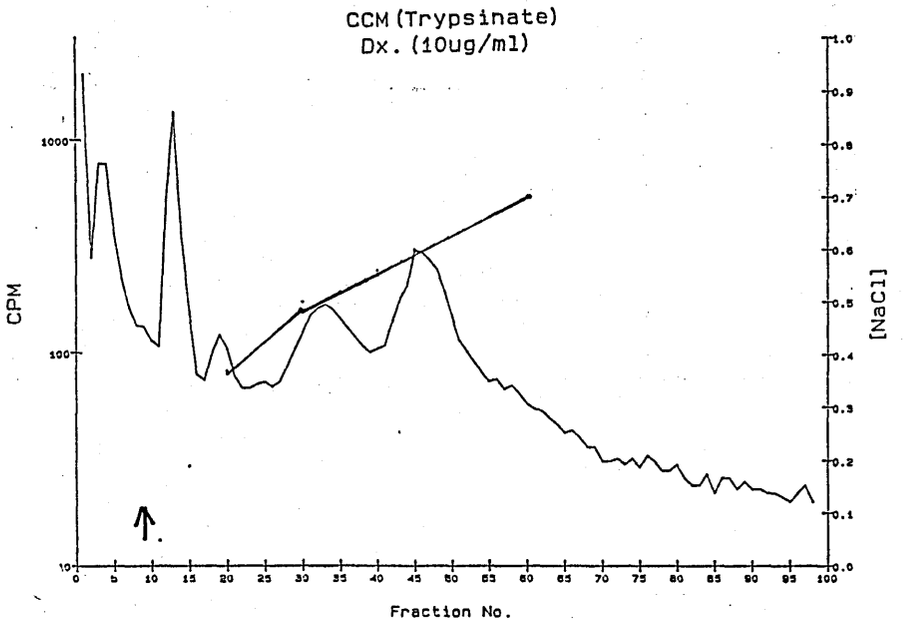
Figure 32

Illustrates quantitatively the area under the different glycosaminoglycan peaks in the medium fraction. The results were worked out by counting up all the radioactive counts in each peak and expressing them as a percentage of the total counts for peaks I-IV.

Peak I is unknown, peak II is hyaluronic acid, peak III is heparan sulphate, peak IV is either dermatan sulphate or chondroitin sulphate.

Figure 33


CCM (Trypsinate)
Control
CPM
[NaCl]
Fraction No.


CCM (Trypsinate)
Dx. (10ug/ml)
CPM
[NaCl]
Fraction No.

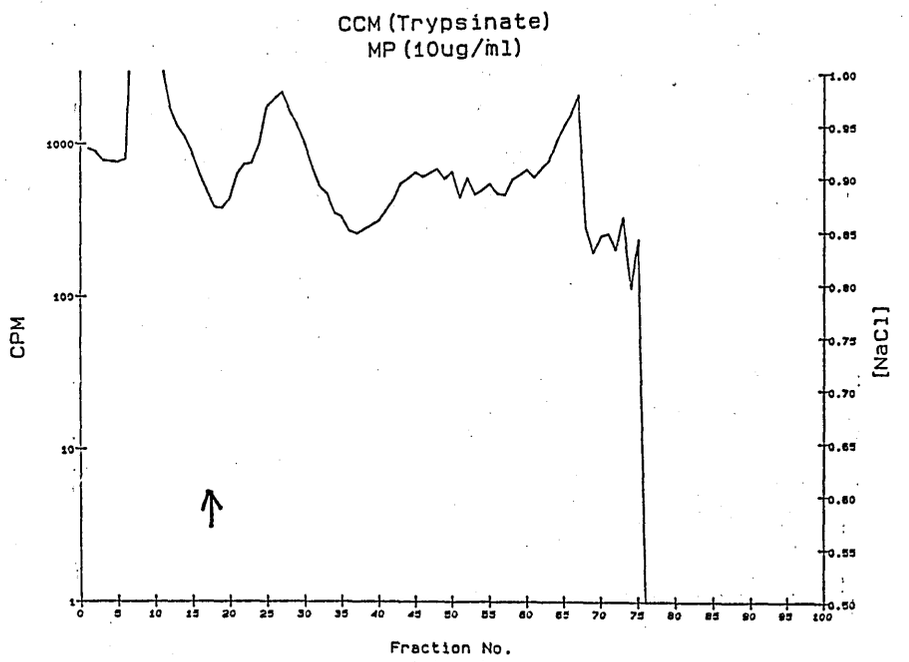

CCM (Trypsinate)
MP (10ug/ml)
CPM
[NaCl]
Fraction No.

Figure33

Fractionation of radiolabelled glycosaminoglycans by chromatography on DEAE-cellulose.

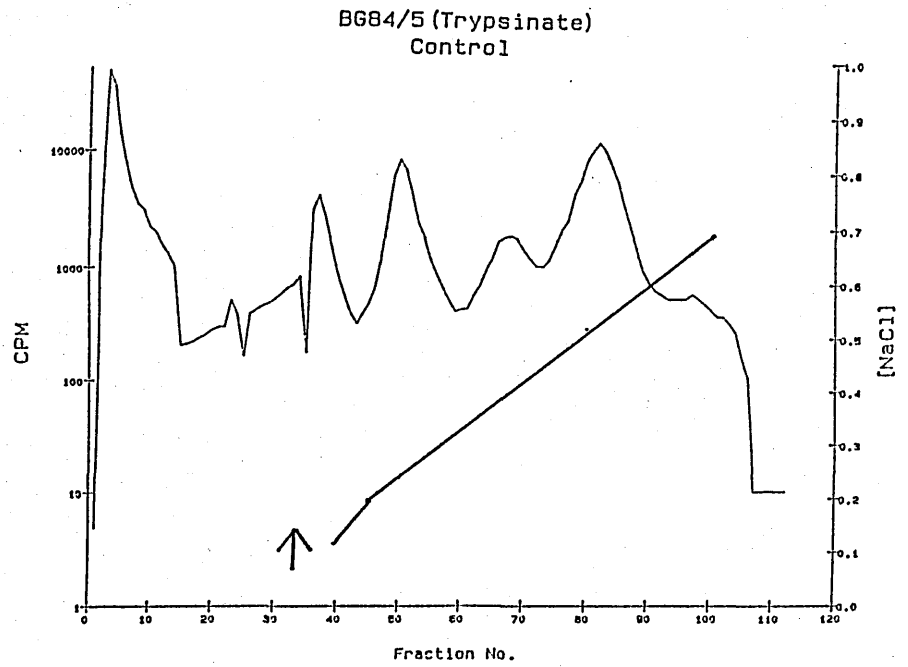
The composition of glycosaminoglycans in the presence and absence of either dexamethasone or methylprednisolone in the trypsinate extract of cell line BG-CCM was determined using a DE-52 column with a linear gradient of 0.1-0.8M NaCl in 20mM sodium phosphate buffer, pH6.8. Peak II was identified as hyaluronic acid, peak III was thought to be heparan sulphate while peak IV was either dermatan sulphate or chondroitin sulphate. The NaCl gradient is switched on at ↑.

Figure34

Fractionation of radiolabelled glycosaminoglycans by chromatography on DEAE-cellulose.

The composition of glycosaminoglycans in the presence and absence of either dexamethasone or methylprednisolone in the trypsinate extract of cell line BG84/5 was determined using a DE-52 column with a linear gradient of 0.1-0.8M NaCl in 20mM sodium phosphate buffer, pH6.8. Peak II was identified as hyaluronic acid, peak III was thought to be heparan sulphate while peak IV was either dermatan sulphate or chondroitin sulphate. The NaCl gradient is switched on at ↑.

Figure 34


BG84/5 (Trypsinate)
Control
CPM
[NaCl]
Fraction No.

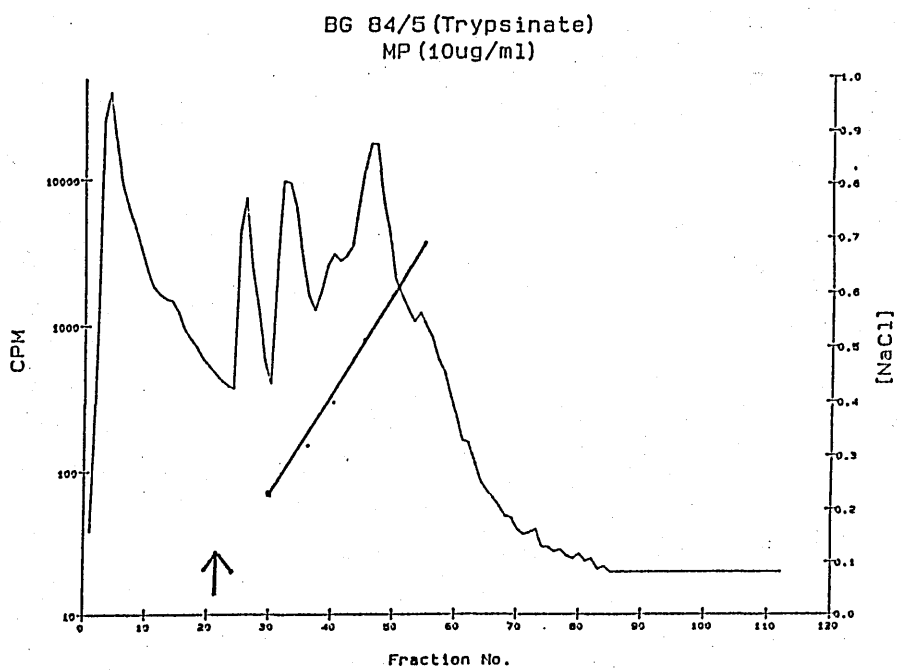
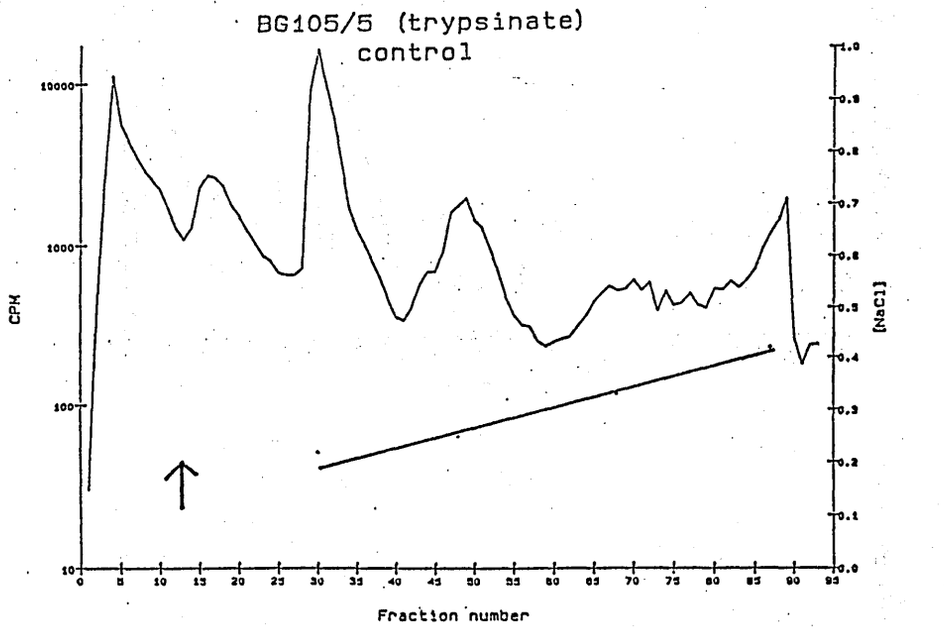
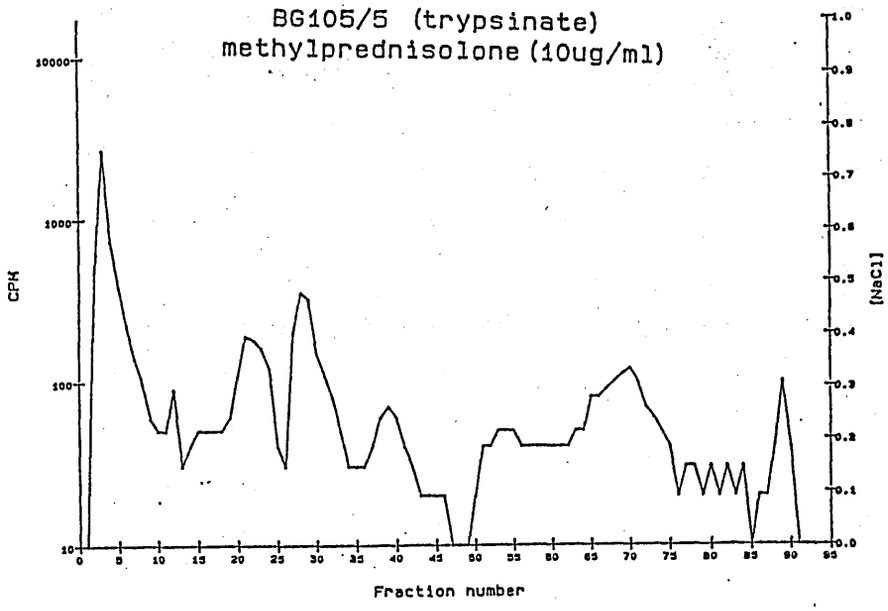

BG 84/5 (Trypsinate)
MP (10ug/ml)
CPM
[NaCl]
Fraction No.

Figure 35


BG105/5 (trypsinate)
control
CPM
[NaCl]
Fraction number


BG105/5 (trypsinate)
methylprednisolone (10ug/ml)
CPM
[NaCl]
Fraction number

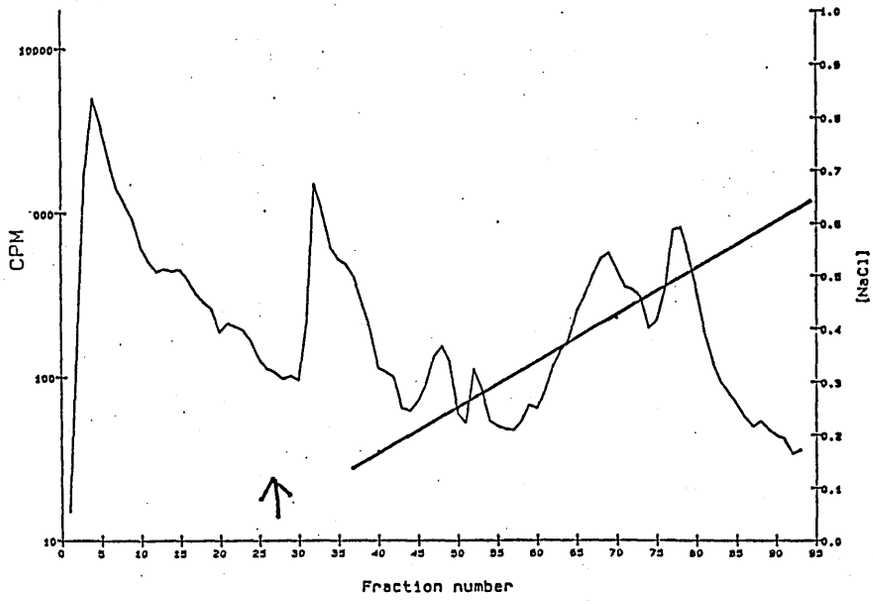

CPM
[NaCl]
Fraction number

Figure 35

Fractionation of radiolabelled glycosaminoglycans by chromatography on DEAE-cellulose.

The composition of glycosaminoglycans in the presence and absence of either dexamethasone or methylprednisolone in the trypsinate extract of cell line BG105/5 was determined using a DE-52 column with a linear gradient of 0.1-0.8M NaCl in 20mM sodium phosphate buffer, pH6.8. Peak II was identified as hyaluronic acid, peak III was thought to be heparan sulphate while peak IV was either dermatan sulphate or chondroitin sulphate. The NaCl gradient is switched on at ↑.

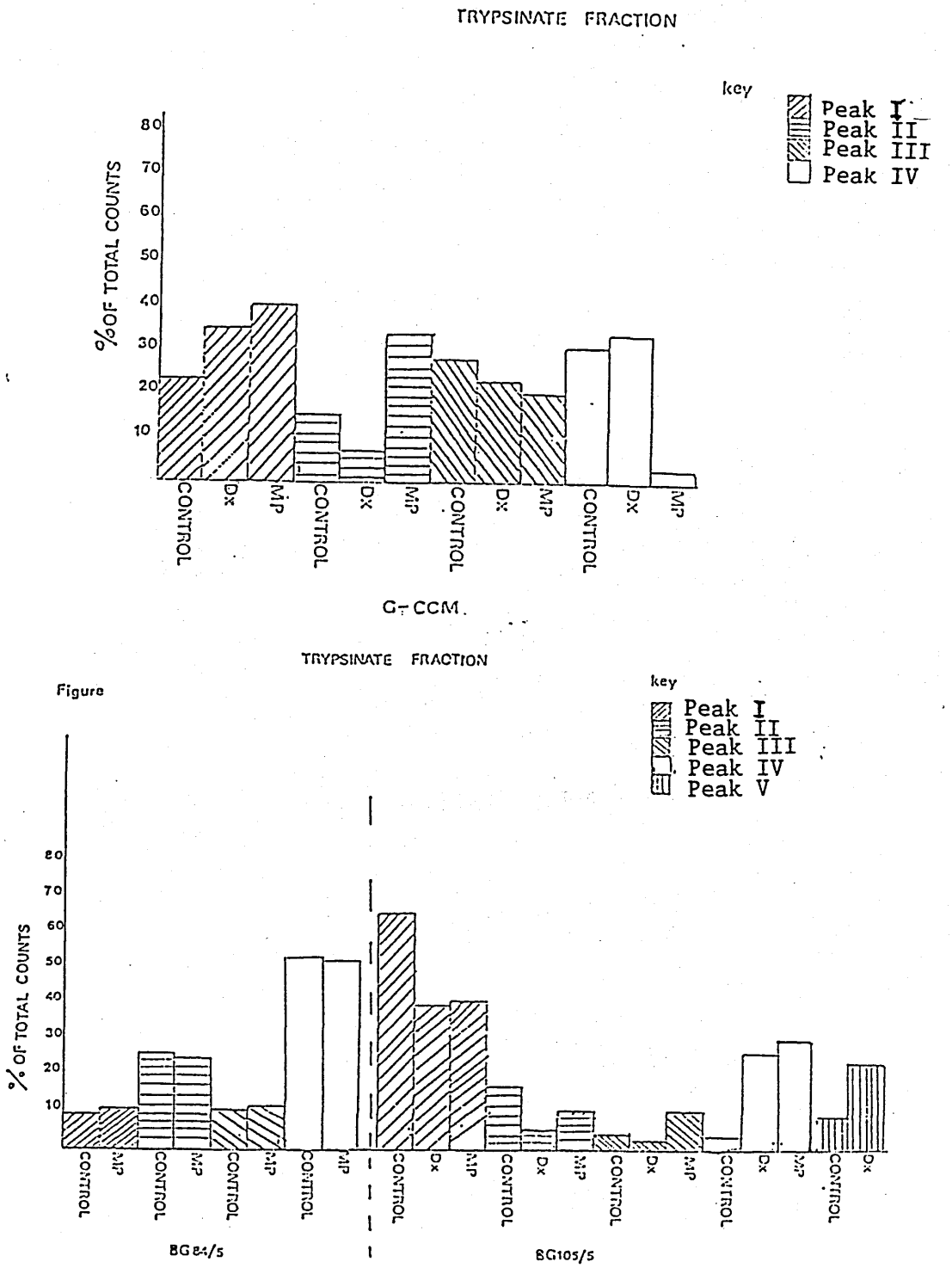


Figure 36

Illustrates quantitatively the area under the different glycosaminoglycan peaks in the trypsinate fraction. The results were worked out by counting up all the radioactive counts in each peak and expressing them as a percentage of the total counts for peaks I-V. Peak I is unknown, peak II is hyaluronic acid, peak III is heparan sulphate and peak four is either chondroitin sulphate or dermatan sulphate. Peak V is unknown.

cell-associated material. Compared to the medium fraction, the quantity of labelled proteoglycans present in this fraction was dramatically lower.

Five peaks were identified by the NaCl gradient in the medium extract of the early passage cell line BG105/5, (i.e. there was an additional peak at the end of the gradient (i.e. 0.7M) compared to the medium results). In this case the results are expressed as a percentage of the total counts in peaks I-V. The results suggest that in the presence of both dexamethasone and methylprednisolone, the absolute quantity of labelled proteoglycans increased in the cell lines under investigation. In the continuous cell lines the results suggest that dexamethasone increased the quantity of material eluting in peaks I and IV, while decreasing the quantity in peaks II and III, whereas methylprednisolone increased the quantity of material eluting in peaks I and II, while peaks III and IV were reduced. Hence, these results suggest that in the presence of glucocorticoids, the proportion of the proteoglycans bound to the cell surface were dramatically increased, hyaluronic acid decreased and heparan sulphate increased.

5. The effect of glucocorticoids on the malignant cell's adhesiveness

The question was asked whether the reduction in the concentration of hyaluronic acid by glucocorticoids would increase the adhesive force of these cells. This was tested according to the protocol in the methods section. Cells were grown either in the presence or absence of glucocorticoid, the monolayer was dispersed and the cells were transferred to capillary tubes. At various time points the capillary tubes were turned over for 30 minutes, then the number of cells adhering to the top surface was expressed as a percentage of the total number of cells present in that field. Preliminary experiments showed that upon dissociating cells with trypsin, compared to EDTA, the former cells took longer to adhere to the new surface (Figure 37). This may be related to the mechanisms through which EDTA and trypsin exerts their respective actions. Trypsin acts by removing surface

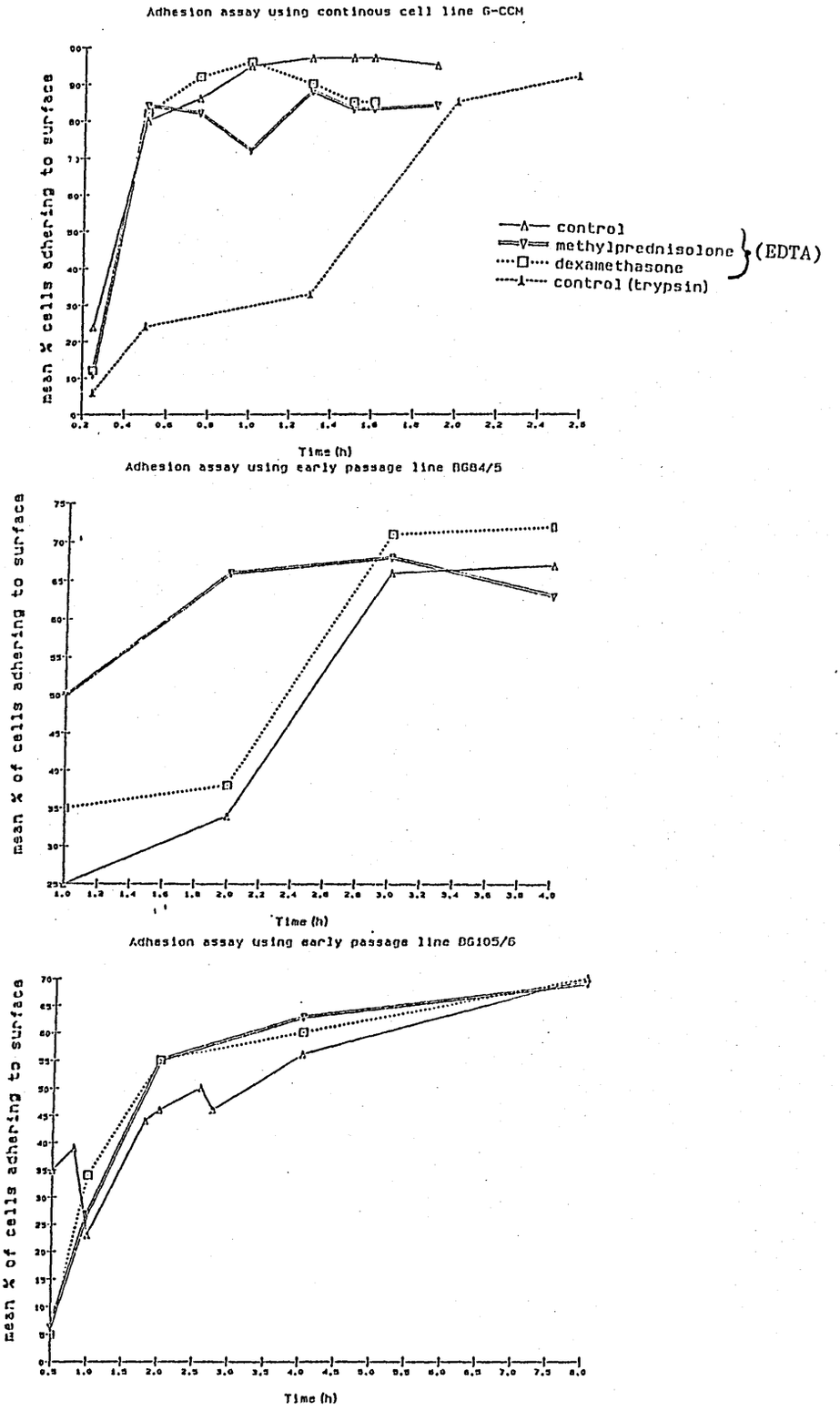


Figure 37

Illustrating the effect of glucocorticoids on cell adhesion. The cells were dissociated with either trypsin or EDTA and the time was monitored for them to adhere to the surface of capillary tubes. The method is described in the methods section. Each estimate consists of a mean or four readings, each consisting of more than a hundred cells.

glycoproteins, which presumably needs to be replaced before these cells can adhere to the substrata, whereas EDTA, a Ca^{2++} chelator, does not cause any structural damage. Cells treated with EDTA were seen to recover within 30 minutes, whereas, trypsinised cells took a couple of hours.

As the aim of the present report was to investigate the relationship between proteoglycans and cell behaviour, it was therefore decided to dissociate the cells in the present experiment with EDTA. The continuous cell lines were seen to recover much more quicker than the early passage cell lines from this treatment. This may be related to the ability of these malignant cells to adapt more quickly to new environments, a property which is required by malignant cells to migrate into surrounding tissues. However, the results obtained with the early passage cells, as discussed in the general introduction, are probably more representive of how tumours in vivo will react to glucocorticoids. The results obtained suggested that cell adhesion was increased, in the presence of glucocorticoids, most significantly in cell line BG84 within the first couple of hours, but further confirmation is required, this cell line had the greatest increase in heparan sulphate. However, this effect was most prominent in the early passage cell lines. The glucocorticoid was not found to produce any significant effect in the continuous cell line G-CCM.

6. Relationship between addition of exogenous proteoglycans and cellular proliferation

Throughout this chapter proteoglycans have been suggested to influence cellular behaviour. Therefore, it was proposed that the addition of exogenous proteoglycans may influence cellular proliferation. The effect of exogenous proteoglycans were investigated in terms of both labelling index (Figure 38) and cell proliferation (Figure 39). The exogenous GAGs investigated were not found to have any effect on cell proliferation. Hyaluronic acid (10µg/ml) and dermatan sulphate (10µg/ml) produced a slightly higher labelling index than that of the control group. This

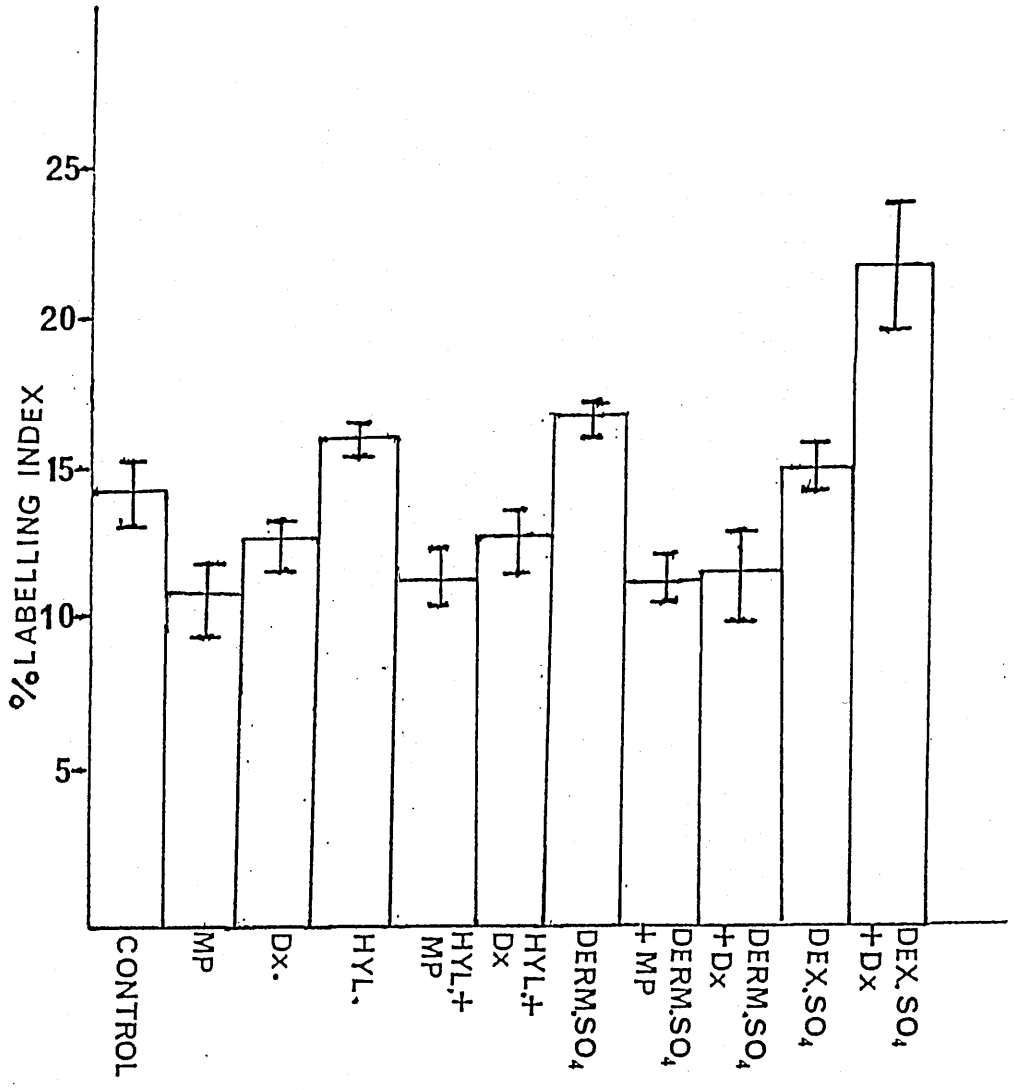


Figure 38

The effect of exogenous glycosaminoglycans in relation to labelling index in cell line G-CCM. Hyl, hyaluronic acid (10µg/ml, Derm SO_4, dermatan sulphate (10µg/ml), Dex. SO_4, dextran sulphate (10µg/ml), Dx., dexamethasone and MP, methylprednisolone, n=4. The experiment was carried out as described in the methods section.

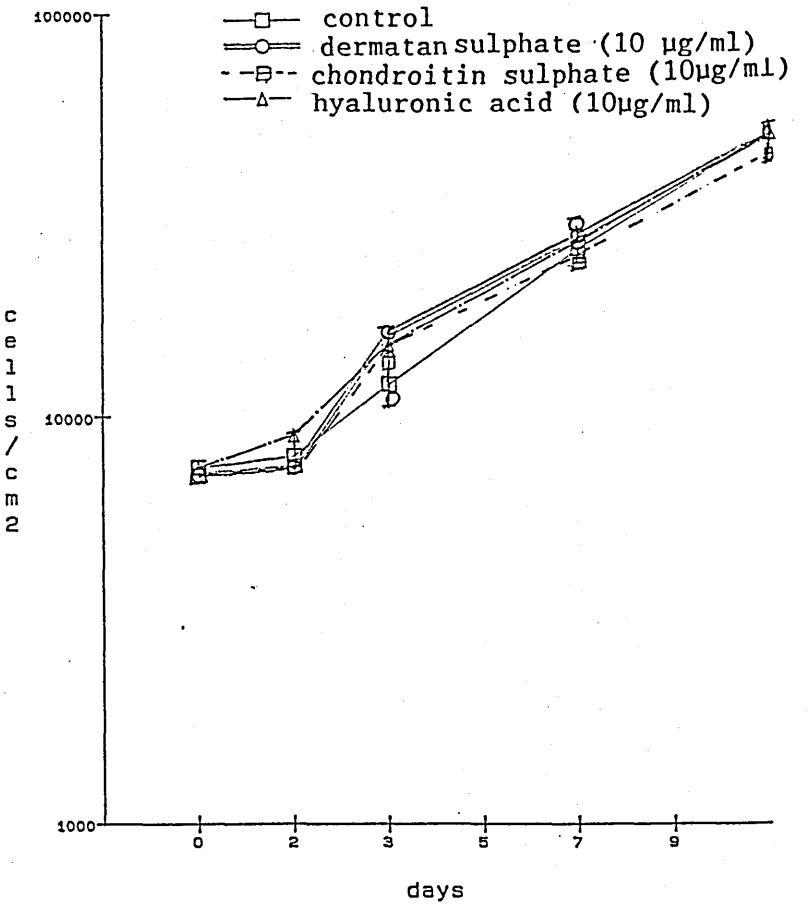


Figure **39**

The effect of exogenous glycosaminoglycans in relation to cell growth in cell line G-IJKt. Hyaluronic acid (10µg/ml, dermatan sulphate (10µg/ml) and chondroitin sulphate (10µg/ml). The results are means of four experiments. The experiment was carried out as described in the methods section.

effect could be diminished by 2.5×10^{-5}M of either dexamethasone or methylprednisolone. However, dextran sulphate in the presence of dexamethasone was observed to augment the labelling index. It was repeatedly found that the presence of either glucocorticoid reduced the labelling index even in the presence of exogenous glycosaminoglycans (with the exception of dextran sulphate) where the presence of dexamethasone augmented the labelling index. The presence of chondroitin sulphate, hyaluronic acid and dextran sulphate was found to have no effect on either saturation density or labelling index. Future work would involve investigating the effect of heparan sulphate on cell proliferation in vitro, as this is the most likely glycosaminoglycan to inhibit cell growth. Unfortunately we were unable to purchase any of this particular glycosaminoglycan.

7. Investigating the invasive properties of gliomas in vitro

The effect of dexamethasone and methylprednisolone on the cells invasive capacity was investigated. Plasminogen activator (P.A.) activity has been proposed to be correlated with the ability of malignant cells to invade surrounding tissues. It was seen in Chapter 2 that the glucocorticoids abolished the PA activity of the cell lines under investigation, so it was asked in whether this decrease was related to a reduction in the ability of the cells to invade an extracellular matrix. The ability of gliomas to degrade the extracellular matrix was investigated using the procedure described in the methods section. Using both 10% and 1% foetal calf serum, no detectable release of radioactive material was detected above the spontaneous release which occurred in the absence of cells. Thus, under these conditions the cell lines investigated did not seem to have the capacity to dissolve the extracellular matrix even though they possessed a high level of plasminogen activator activity.

DISCUSSION

The concept under investigation in this chapter was the effect of glucocorticoids on the composition of proteoglycans in malignant astrocytoma cell lines and whether this effect could account for the cytostatic activity of the glucocorticoids. The precise physiological functions of proteoglycans remain elusive, although they have been implicated as being involved in a number of different functions:-

(i) Cell-substrata adhesion (Lark and Culp, 1984). Heparan sulphate has been associated with promoting cell adhesion and in reducing cell proliferation (see section 4.2 of the introduction for further details).

(ii) As cell surface receptors (see section 2 of the introduction for full details).

(iii) In both inter- and intra-cellular communication as proposed from in vitro observations (see introduction for details).

(iv) Cell migration and differentiation. High concentrations of hyaluronic acid in foetal tissues have been found to be essential for cell migration and inhibit cell differentiation. The concentration of hyaluronic acid has to be enzymatically reduced before the cells are able to differentiate (see section 4 of the introduction.

(v) Selective filtration of blood plasma by the glomerular basement membrane of the kidney (Farquhar, 1981). This property was lost upon removing heparan sulphate from the basement membrane.

(vi) Stabilisation of the extracellular matrix. Undersulphated heparan sulphate has been associated with the breakdown of the extracellular matrix (section 4.3 of the introduction).

Malignant glia cells have been reported by Glimelius et al (1978 and 1979) to differ from their normal counterparts with respect to their proteoglycan composition. The malignant cells possess higher quantities of hyaluronic acid and lower quantities of sulphated proteoglycans than their normal counterparts. The proteoglycan composition between different normal cell lines was

quite reproducible, whereas a large variation was observed between different malignant cell lines. This latter variability may be related to the degree of malignancy, i.e the extent to which the various cell lines can escape from the normal mechanisms controlling growth and differentiation. This would result in the largest differences being between the more aggressive tumour cell lines and their normal counterparts.

In addition to the composition of proteoglycans altering in malignant tissues, their distribution was also affected. Malignant cells, compared to their normal counterparts, have been reported to retain a larger proportion of proteoglycans on their cell surface instead of secreting them into the medium.

It was asked whether the glucocorticoids were exerting their cytostatic effect through altering the proteoglycan composition to resemble that of normal cells. Many investigators have looked at the proteoglycan composition of normal and malignant cells, (Satoh et al, 1973; Glimelius et al, 1979). However, there has been little work carried out studying the effect of glucocorticoids on proteoglycan composition. Moczar et al (1985) have shown that hydrocortisone can increase the quantity of sulphated glycosaminoglycans by four fold. Thus, in the present investigation the effect of glucocorticoids on the composition of proteoglycans was investigated in astrocytoma cell lines.

In the present set of experiments the relative proportion of each proteoglycan peak was monitored in an attempt to see whether the composition of the various glycosaminoglycan groups changed in the presence of glucocorticoids. Both dexamethasone and methylprednisolone, (2.5×10^{-5}M), were found to reduce the proportion of hyaluronic acid present relative to sulphated proteoglycans in the glioma cell lines under investigation, while a larger quantity of the glycosaminoglycans were associated with the cell membrane. This results in the composition of the proteoglycans in the malignant cells now resembling closer that of normal glial cells as reported by Glimelius et al (1979). However, there was no apparent correlation between the increase in the quantity of cell-associated proteoglycans and a decrease in

the quantity of secreted material. This may be due to the fact that the radioactive counts obtained in the pronase extract were approximately ten times less than those in the medium extract, and such a small increase in the pronase extract may be within the statistical limits of the scale used in the former case. It was then postulated that the re-expression of density inhibition of growth by glucocorticoids as observed in Chapter 2 may occur through the above alteration in the proteoglycan composition.

How can alterations in proteoglycan composition relate to the physiological behaviour of the cells?

Elevated levels of hyaluronic acid are essential in foetal tissues to sustain cell migration, elevated levels of cell proliferation and inhibit cell differentiation (Toole, 1981). It has been proposed that cell migration may be due to the cell's more rounded appearance which may result in weaker binding forces to the substrate, as a smaller area of the cell is now in contact with the substrate. This alteration in morphological appearance of these tissues results from the proteoglycan's ability to absorb large quantities of water. Thus, the weaker binding forces would increase the ability of these cells to migrate. In contrast, heparan sulphate is thought to augment cell adhesion. High concentrations of hyaluronic acid have been shown to inhibit differentiation of foetal cells, whereas if the concentration of this proteoglycan is enzymatically reduced these cells are seen to differentiate. A reduction in cell proliferation has also been associated with a decrease in the levels of hyaluronic acid. Thus, the cell's overall physiological behaviour may result from a summation of the various physiological characteristics of the different proteoglycan groups and not due to the presence of one particular group.

It was therefore asked whether a reduction of the quantity of hyaluronic acid in malignant cells by glucocorticoids would cause the cells to bind more strongly to the substrata. An attempt was made to answer this question by using an adhesion assay. The preliminary results in the presence of EDTA (which is a Ca^{2+}

chelator and thus leaves the cell-associated proteoglycans intact) suggested, especially for cell line BG84/5, that the presence of glucocorticoids did increase the rate of cellular adhesion, but only in the first 60 minutes. However, cells which had been dissociated with trypsin (which removes surface glycoproteins), took longer to adhere to the surface of the capillary tubes compared to cells which had been dissociated with EDTA. These results suggest that glycoproteins are more important than proteoglycans in terms of cell adhesion. The present experiments only measure the "degree of adhesiveness" of the cells and not the actual force by which the cell binds to the substrata. As the concentration of sulphated proteoglycans are increased in the presence of glucocorticoids this suggests that cellular adhesion may be increased. Heparan sulphate has been documented in the literature to play an important role in cell adhesion (Gallagher et al, 1986), however, this proteoglycan may increase the force with which the cell binds to the substrata and not influence the initial adhesion itself. In order to answer this question an experiment would have to be devised where the actual force with which the cell binds to the substrate was investigated.

The extracellular matrix constitutes the normal barrier against the passage of cells and small particles from one area into another (Liotta et al, 1983; Bosman, 1985). It has been widely reported that malignant cells, compared to their normal counterparts, possess the ability to degrade proteoglycans present in the extracellular matrix (Laug, 1983; Kramer et al, 1981). This degradation may be due to increased levels of proteinase activities such as collagenases, proteoglycanases and plasminogen activators. Laug et al (1983) have found that proteoglycan degradation can proceed either through a plasminogen dependent or independent pathway. As the glioma cells under investigation have high levels of plasminogen activator activity (Figure 7), it was asked whether the presence of glucocorticoids would inhibit the degradation of a prelabelled extracellular matrix. Glioma cell lines were plated onto a prelabelled extracellular matrix laid down by normal endothelial cells, and the release of radioactivity

into the medium was measured. These experiments were carried out in growth medium containing either 10% or 1% Foetal Calf Serum, however, no radioactivity was measured except from the spontaneous release from the medium itself. Whether the presence of 1% serum was sufficient to prevent the degradation of the extracellular matrix, or whether these cells lacked the property to degrade the matrix requires further clarification.

The discussion up to now has implied that an alteration in proteoglycan composition in the presence of glucocorticoids may account for the re-expression of density limitation of growth observed in Chapter 2. So far however, the experiments do not demonstrate a direct correlation. In order to discover whether proteoglycans have a direct influence on saturation densities, a growth curve was carried out in the presence of exogenous proteoglycans. Addition of various exogenous proteoglycans (10µg/ml or 100µg/ml of either dermatan sulphate, chondroitin sulphate and hyaluronic acid) were found to have no influence on the growth curves of the glioma cell lines under investigation (Figure 39). Unfortunately, heparan sulphate, which has been implicated in inhibiting cell proliferation, could not be commercially obtained within the time limit of these investigations. The present findings were similar to those reported by Wright et al (1985) where they found that 100µg/ml of either chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate or hyaluronic acid had no effect on saturation densities on endothelial cells. In contrast, Ohnishi et al (1975) using Yoshida ascites hepatoma (AH66) found that 50-100µg/ml of acid mucopolysaccharides inhibited cell growth by approximately 84%. Whether these results were due to the presence of heparan sulphate was not established.

Thus the results in the present investigation imply that proteoglycans may be responsible, to a certain extent in influencing the physiological behaviour of the cell. They can achieve this by influencing either cell shape, proliferation or differentiation: this can result in an alteration in both the quantity and structure of proteoglycans synthesised by the cell,

which in turn can alter the composition of both cell-associated proteoglycans and those in the extracellular matrix. These changes can once again influence cell shape, proliferation and differentiation. Thus, an intricate system is set up between proteoglycan synthesis and the cell's physiological activity.

It was therefore asked how such a system may work in vivo. A possible explanation is that the cell may be able to control its physiological behaviour by altering the composition of cell-associated proteoglycans and those in the extracellular matrix as illustrated in the following example using foetal cells. High concentrations of hyaluronic acid are important for immature foetal cells to migrate to their site of differentiation. Upon reaching the site, in response to some external stimuli, the cells reduce the concentration of hyaluronic acid in their immediate vicinity, either through enzymatic degradation or by reducing the actual amount of hyaluronic acid being released from the cell, which then allows the cells to differentiate (Toole, 1981).

CHAPTER 5

FINAL DISCUSSION

As stated in the general introduction the underlying aim of this report was to investigate whether glucocorticoids, exert their cytostatic effect by altering the glycoprotein and proteoglycan composition associated with malignant glioma cells. Glucocorticoids have been widely used to reduce the oedema associated with brain tumours but recent evidence suggests that these drugs have a direct cytostatic effect on the tumour itself. The exact cellular mechanisms by which these effects are exerted remains unclear.

As glioma cell lines are comprised of a heterogeneous cell population it was important to prevent selection for one particular cell type, so the cells under investigation were used from frozen stocks for a limited period of three months. This resulted in all the experiments being carried out on cells of similar passage levels. If cell selection did occur then the results produced in vitro may not be representative of what would occur in vivo. Alternatively, by carrying out the experiments in a limited passage range in both early passage and continuous cell lines, any alteration in the concentration of specific proteins (as sometimes happens when a cell is passaged for a significant length of time in vitro) was prevented. A good example of where this is known to happen is with glia fibrillary acidic protein (GFAP) which is a specific marker for astrocytes. Cells taken from biopsy material were initially found to be GFAP+ve, whereas when they were grown for some time in culture they were seen to become GFAP-ve (Yates and Stephens, 1987).

Initially the cytostatic effect of glucocorticoids, which has been reported by a number of investigators (for example M^{c}Lean et al, 1986), was studied. Dexamethasone and methylprednisolone were found in Chapter 2 to reduce the saturation densities of both early passage and continuous cell lines in a dose dependent manner. This reduction in saturation densities was clearly seen

in the photographs in Figure 7, where the "piling up" effect was reduced. However the glucocorticoids were seen to exert a biphasic response, cell proliferation was augmented at low cell concentrations in the absence of serum while it was inhibited at high cell concentrations. The largest cytostatic effect (96%) was seen in the cell line which reached the highest saturation density i.e. cell line G-IJKt. A corresponding decrease in labelling index was observed in the presence of glucocorticoids. Assuming that the uptake of [^{3}H] thymidine was not affected by the presence of glucocorticoids, these results suggest that the proportion of cells undergoing proliferation had decreased. These observations imply that the decrease in saturation density was accompanied by a decrease in the number of cells proliferating at any period of time and was not due to cell death. A cytotoxic effect would have been suggested if no decrease in the labelling index results had been found in the presence of glucocorticoids. This hypothesis was confirmed by a clonogenic assay where the glucocorticoids were seen to have no cytotoxic effect, except at high concentrations i.e. 2.5×10^{-4}M. The latter concentration is much higher than would ever be used clinically. However, the glucocorticoids were seen to augment cell growth following subculture as observed by the increase in the number of colonies formed. In the present experiment the colonies formed in the presence of glucocorticoids were much more diffuse in appearance than the controls. These results are opposite to those found by Guner et al 1977, who showed that the size of the colonies in the presence of glucocorticoid were more compact in shape than the controls. It is not clear why such a difference should occur.

The cytostatic effect of glucocorticoids was found to be reversible since 24-48 hours after removal of the glucocorticoids the original properties of the cells were seen to be restored. As glucocorticoids are known to exert their effect mainly through cytoplasmic receptors (Alexis, 1987), it was therefore asked whether the largest effect occurred in the cell line which possessed the largest number of glucocorticoid receptors. This question was investigated with the help of Shelia Colven.

The various cell lines tested were found to possess glucocorticoid receptors but no clear relationship was found to exist between receptor number and reduction in saturation density. Cell line G-CCM was found to possess ten times the number of glucocorticoid receptors than the early passage cell lines, however a corresponding decrease in saturation density was not observed amongst these cell lines. It would be dangerous, as illustrated in the following hypothesis, to conclude that these results do not suggest the existence of such a relationship without further experimentation. As glucocorticoids readily bind to proteins it may be hypothesised that the concentration of "free" glucocorticoid (that is the proportion of the drug which produces the physiological effect by binding to the glucocorticoid receptors) may be too low to saturate all the receptors present in the cell lines under investigation. However, if only a fraction of all these receptors were occupied and if this number was less than the total number of receptors present in the early passage cell lines, this would result in any relationship between receptor number and reduction in saturation density being masked. Such a relationship could be investigated if the total number of receptors occupied was theoretically worked out. Knowing the partition coefficient, the concentration of "free" drug can be worked out for any one drug concentration. Thus, by plotting the concentration of "free" drug against reduction in saturation density any relationship existing between receptor number and saturation density would be obvious.

It may be said that if the glucocorticoids are exerting their action by making the properties of malignant cells more like those of normal cells, they may be reducing the saturation density of the malignant cells to a value comparable to that of their normal counterparts. This hypothesis was supported by the finding that the largest effect was seen in the cell line which reached the highest saturation density. If glucocorticoids were producing a standard effect in each cell line, a similar percentage reduction in saturation density would have been expected. However, as the extent of the reduction seems to be proportional to the saturation

density, this suggests that the glucocorticoids were reducing the saturation density to a certain value. This actual value seems to be reproducible within any one cell line, but differs among different cell lines. It may be that these differences would not be significant if a larger population of malignant cell lines had been investigated, or it may be that different types of cells may plateau at different values. In order to elucidate these points both the saturation and labelling index experiments would have to be repeated with a sufficiently large number of different cell lines in an attempt to see whether the results are statistically different from one another. If the values were all similar then it may then be assumed that the glucocorticoid is reducing the saturation density to resemble that of their normal, differentiated counterparts. Unfortunately, this is a difficult concept to investigate as in the majority of cases the parent material is not known. One way in which this assumption could be investigated would be to use either virally or chemically transformed glial cells, this would result in producing both malignant and normal cells of similar lineage. However, it is questionable whether cells resulting from such a transformation are representative of gliomas occurring in vivo.

As many of the side effects of glucocorticoids result from their glucocorticoid activity, it was asked whether a similar molecule which lacks activity would also possess the above cytostatic effect. As the glucococorticoid activity resides in the 11-β orientation, 11-α-epihydrocortisone was tested for cytostatic activity. This compound was found to be without cytostatic activity, if anything the results suggest that it may possess mitogenic activity. Thus, the present results suggest that both the cytostatic activity and glucocorticoid activity may be associated with the 11-β orientation.

It may be proposed that the glucocorticoids are acting by enhancing cell to cell contact by making the cells larger in size. This aspect was not dealt with in this report. It is a simple concept to investigate as cell size in the presence or absence of glucocorticoids could be investigated using a Coulter counter.

It was then asked how the above cytostatic effects are brought about at the molecular level. As malignant cells are able to overcome the normal constraints imposed on them by their environment, it was proposed that this behaviour may arise from a breakdown in the ability of these cells to communicate with their environment. Before this hypothesis can be investigated the mechanisms involved in normal growth control need to be considered.

An intricate system was seen to exist in section 1.A. where cell behaviour was influenced by the interaction between the cell surface and the extracellular matrix. It was proposed that information can be passed from the extracellular matrix through proteins, laminins and intermediate filaments to the plasma membrane (M^{c}Keon et al, 1986), where it is then sent to the nucleus through an intricate array of microtubules and filaments. The cell may respond to this information by affecting gene expression, at either the level of the nucleus (transcription) or the cytoplasm (translation) which in turn can influence the composition of proteoglycans being synthesised by the cell. Equally, the reverse relationship may occur, new gene expression could result in alterations in the cytoskeleton, cell shape and the extracellular matrix.
Thus, the co-operation of a number of different molecules are necessary for the passage of information between the cell and its environment.

It may be proposed that, for whatever reason, this method of communication may breakdown if the composition of one of the above components was altered. This is clearly seen from the following experimental evidence in terms of proteoglycans. Proteoglycans, in normal rat fibroblasts, are found in close association with actin filaments (Hynes, 1981; Gallagher et al, 1986) whereas in transformed rat fibroblasts the proteoglycans are found diffusely bound throughout the cells. This may suggest a breakdown in the relationship between actin and proteoglycans in malignant cells, which may lead to a breakdown in both intra- and inter-cellular communication.

If malignant cells arise from a breakdown in communication then it may be hypothesised that growth of malignant cells is not regulated. This has indeed been postulated but Westermark et al (1973) have shown that it is not the case. They have shown that malignant cells did respond to density inhibition, but this occurred at a much higher cell density than for their corresponding normal cells. Thus, it may be hypothesised that in malignant cells it is the mechanism which controls the cell concentration at which contact inhibition occurs which is at fault, and not the actual processes themselves. This would lead to the hypothesis that glucocorticoids may be exerting their cytostatic effect by "re-setting" the value at which contact inhibition occurs. However, the mechanisms through which this is achieved are not known.

It has been postulated that the above effects may result through an alteration of the composition of carbohydrates associated with either the extracellular matrix or the cell. Malignant cells have been reported to differ from their normal counterparts in terms of glycoprotein and proteoglycan composition. These molecules are thought to play an important role in cellular communication due to their position on the plasma membrane and in the extracellular matrix. Glycoproteins comprise 80% of all cell carbohydrates and are mainly situated on the periphery of the cell membrane. Proteoglycans differ from glycoproteins in that they consist of 90-95% carbohydrate by weight in the form of many long unbranched glycosaminoglycan chains, whereas glycoproteins contain a lower proportion of carbohydrate (1-60% by weight) in the form of numerous, relatively short (>15 sugar residues), branched oligosaccharide chains which often contain terminal sialic acid residues. In spite of their relatively small proportion by weight, the surface carbohydrates can cover the entire surface of the cell and thus form the first point of contact between the cell and its environment. Thus, it may be postulated that any alteration in the composition of the surface carbohydrates may lead to a breakdown in the communication between the cell and its environment.

The effect of glucocorticoids on the composition of intact glycoconjugates in astrocytoma cell lines was investigated with the help of Gillian Hunt. Both dexamethasone and methylprednisolone were found to alter the composition of intact glycoconjugates, however the actual alterations differed between the two cell lines under investigation. It has been reported by a number of investigators that malignant cells differ from their normal counterparts in the extent of sialation of the glycoproteins at the cell surface. Malignant cells in general are more highly sialated than their normal counterparts, this effect is thought to lead to an alteration in the cell's physiological activity. It was therefore considered necessary to investigate the effect of glucocorticoids on the composition of the extracellular glycoconjugates, as it is this material which is thought to play an important role in passing information from the environment to the inside of the cell. The glycoconjugates were isolated using gel exclusion chromatography, a quick method by which proteins can be separated according to their molecular weight. Once again both the cell lines under investigation were found to respond differently to glucocorticoids in terms of glycoprotein composition. Dexamethasone was found to increase the quantity of glycoconjugates present in both the pronase and medium fractions in cell line G-CCM and G-IJKt. However, methylprednisolone was found to only increase the quantity of glycoconjugates in the pronase fraction of cell line G-CCM and in the medium fraction of G-IJKt. It was felt that the resolution of the latter technique was not sufficient to identify a similar reduction in the proportion of the higher molecular weight glycoconjugates would be seen as reported by Van Beek et al (1978) using neuraminidase, and Freshney et al (1980b) using dexamethasone.

Only two cell lines were initially used in the latter experiments. These were chosen arbitrarily as the results in terms of cytostasis in Chapter 2 showed that the glucocorticoids produced a similar effect in all the cell lines under investigation, the only difference being the actual extent of the

reduction in either saturation density or labelling index. Before any conclusions can be made in terms of the effect of the glucocorticoids on glycoprotein composition, these experiments need to be repeated with a wide range of both early passage and continuous cell lines. The sensitivity of the above method was not regarded as being sufficient to see any slight change in glycoprotein composition which may occur. It was therefore concluded that a more sensitive technique was required before any conclusive results could be made as to whether the glucocorticoids may exert their cytostatic effect through altering the glycoprotein composition. If when carrying out these experiments on a larger population of cells there is still no common alteration in the glycoprotein composition, then it may be concluded that the glucocorticoids are not exerting their cytostatic effect through altering the glycoprotein composition.

The effect of glucocorticoids on the proteoglycan composition was then investigated. The proteoglycan composition on the cell surface and in the extracellular matrix, as seen in Chapter 4 can influence the cell's behaviour. High concentrations of hyaluronic acid have been associated with morphologically more rounded, undifferentiated cells which have the ability to migrate. In contrast high concentrations of heparan sulphate have been associated with a much flatter cells which do not possess the ability to migrate. It is plausible to suggest that the different proteoglycan groups may act synergistically in controlling cell behaviour. Thus as discussed in Chapter 4 it may not be the absolute quantities but the relative proportion of the proteoglycan groups present which are important in determining the physiological behaviour of the cell.

The different glycosaminoglycan groups were separated on the DE-52 ion-exchange column eluting with a NaCl gradient. As normal glial cell lines could not be grown with any success, the results obtained in the present investigation were compared with those reported by Gallagher et al (1979) for normal glia. The proportion of hyaluronic acid to sulphated proteoglycans was found to be reduced in the presence of glucocorticoids. The hypothesis

that glucocorticoids enable malignant cells to resemble their normal counterparts is supported by work carried out by Gallagher et al (1979). They reported that malignant cells possess higher quantities of hyaluronic acid than normal glia. Thus, the glucocorticoids are reducing the concentration of hyaluronic acid which makes the malignant cells resemble more their normal counterparts in terms of proteoglycan composition. In addition to altering the composition of proteoglycans, the glucocorticoids were found to alter the distribution of proteoglycans. The presence of both dexamethasone and methylprednisolone was found in the present investigation to increase the proportion of cell-associated proteoglycans, however there was no corresponding decrease in the quantity of proteoglycans released from the cell. This discrepancy may have been due to the fact that the number of radioactive counts in the trypsin extract was tenfold less than the amount secreted into the medium. Thus, the extent of this reduction may be within the sensitivity limits of the technique. Once again, glucocorticoids are seen to make the malignant cells resemble their normal counterparts. Malignant cells are reported in the literature (for example by Gallagher et al, 1979) to excrete a larger proportion of the synthesised proteoglycans into the medium in vitro. The above alterations were considered to be sufficient to account for the cytostatic effect of the glucocorticoids observed in Chapter 2.

The elevated proportion of hyaluronic acid in the malignant cells could account for the ability of these cells to grow on top of one another as shown in the photographs in Figure 7. High concentrations of hyaluronic acid were essential in foetal tissues for migration. The ability for cell migration was correlated in Chapter 4 to the cells' more rounded appearance, which resulted in a smaller area of their plasma membrane coming into contact with the substrate. This hypothesis is correct if the strength with which the cell binds to the substrate is proportional to the number of binding sites in contact with the substrate. Thus, the larger the number of attachment sites, the greater the force with which the cells bind to the substrate. The ability of the cell to

migrate may be a summation between the forces with which the cell binds to the substrate and those which stimulates the cell to migrate. Therefore, it may be hypothesised that the stronger the adhesive force then the less likely the cell is to migrate. The decrease in the tendency of malignant astrocytoma cells to migrate in the presence of the glucocorticoids is observed by the reduction in the "piling-up" effect as illustrated in the photographs in Figure 7.

It was asked whether a decrease in the concentration of hyaluronic acid brought about by the presence of glucocorticoids, would result in the cells adhering more strongly to the substrate. An experiment was devised to test this hypothesis. Cells were grown in the presence or absence of glucocorticoid, dissociated with EDTA in an attempt to prevent the removal of surface proteoglycans, and the time taken for them to adhere to a new surface was investigated. The results are illustrated in Figure 37 and show no distinct enhancement in cell adhesion of the glioma cell lines in the presence of glucocorticoids. These experiments investigate how quickly the cell binds to the capillary tubes but not the force with which it binds. As previously mentioned the ability of the cell to migrate may not be due to whether the cell can adhere to its environment or not, but may depend on the force with which it adheres. It would be interesting to devise an experiment where the actual binding force in the presence of EDTA is investigated and not just the rate of binding. It may be that glucocorticoids increase the force with which the cell binds to the substrate and do not affect the actual ability of cells to adhere.

Malignant behaviour has been described up to now in terms of a breakdown in cellular communication between the inside and the outside of the cell and between cells. However, it may also be alternatively described in terms of the state of cell differentiation.

Malignant cells, with the exception of virally transformed cells, have not been reported to produce any material which is not

found at some point in the development of normal cells. As previously discussed in Chapter 4 malignant cells resemble foetal cells in proteoglycan composition which can account for their ability to migrate and their more rounded appearance. Thus, the processes occurring are not abnormal but what is different is the time in the cell's life cycle at which this is occurring. It can be proposed that the malignant cells may only be abnormal in that they are expressing properties which are not normally found in that stage of a cell's life cycle. One way in which this situation may arise is if the total number of cells in any tissue is regulated by the "body" through monitoring the number of fully differentiated cells present. A reduction in the number of these cells would result in immature cells entering the maturation pathway until they become fully differentiated cells. This transition is a gradual process, thus a block occurring in the pathway would result in the presence of undifferentiated cells. As the transition from undifferentiated to fully differentiated cells is a gradual process, the block could theoretically occur at any point of this pathway. This would result in different tumour lines possessing different quantities of differentiated characteristics, which would agree with the heterogeneity found in different tumour cells and not in different normal cell lines (Yates et al, 1987). The immaturity of malignant cells is clearly seen in terms of proteoglycan composition. It may be postulated that by inducing the cells to differentiate this would push them past this "block". This does not however mean that the cells will become more differentiated than fully differentiated cells, but means that the immature cells can continue along the maturation pathway to the fully differentiated state.

Upon the cells differentiating the composition of the proteoglycans would alter to resemble that of normal mature cells which is reflected in an alteration in the physiological behaviour of the cell. McLean et al (1986) have shown that glucocorticoids can induce malignant glioma cells to differentiate as seen by the disappearance of P.A. activity and the appearance of differentiated characteristics such as glutamine synthetase

activity. In this investigation, both dexamethasone and methylprednisolone were found to abolish P.A. activity. Thus, the subsequent alteration in physiological activity may result in the more differentiated appearance of the cell. These alterations coincide with the results obtained in terms of cytostasis and proteoglycan composition.

Due to the cell's immature status, it is important to take the state of differentiation into account as the cell's abnormal properties need to be considered in terms of whether they are due to malignancy or whether they are merely due to an undifferentiated state. Glimelius et al (1979) have shown that the alteration in proteoglycan composition was not due to the malignant cells proliferative ability. The actual state of differentiation of the malignant cell needs to be compared with normal cells at the same stage in differentiation in order to determine whether the abnormal behaviour is due to the cells undifferentiated state. Differences in both the composition and quantity of proteoglycans have been found between the log and stationary phases of the normal cells cycle. Heparan sulphate produced by cells during the log phase generally consists of simple, unbranched molecules whilst the same glycosaminoglycan produced during the stationary phase consists of a longer, more highly branched structure. Thus care must be taken when relating an alteration in proteoglycan composition to malignancy as it has been suggested in the literature that the actual structure of the glycosaminoglycans groups may be related to their biological activity in relation to the cells physiological activity (Gallagher et al, 1986). This suggests that the proteoglycan composition (not only the type of proteoglycan produced but also its structure) associated with the cell may influence the cell's physiological activity.

It is difficult to say whether an alteration in proteoglycan composition results in cell differentiation or whether cell differentiation then results in an alteration in proteoglycan composition. It is felt that the former hypothesis may be correct as the results from foetal tissues show that these immature cells

are not able to differentiate unless the concentration of extracellular hyaluronic acid is reduced. It may be hypothesised that in malignant tissues it is the mechanism(s) which reduces the concentration of hyaluronic acid in the tissue which may be at fault as this would give rise to the properties associated with malignant cells. A large number of these cells may arise due to a "re-setting" of the process which determines at what stage contact inhibition occurs or it may arise from the "body's" inability to detect these undifferentiated cells.

Thus, the cell's properties in the presence and absence of glucocorticoids can be related to the proteoglycan composition. This implies that the glucocorticoids may be exerting their effect through altering the proteoglycan composition. The effects of exogenous proteoglycans were investigated in terms of saturation density and labelling index. It may be hypothesised that addition of exogenous heparan sulphate, at a concentration which would increase its relative proportion to hyaluronic acid, may have produced a similar cytostatic effect as that produced by the glucocorticoid. Such an experiment would clarify whether the alteration in proteoglycan composition is indeed responsible for the cytostatic effect of glucocorticoids.

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