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Glucocorticoids and the cell surface of human glioma cells: Relationship to cytostasis

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Summary The glucocorticoid hormones methyl prednisolone and dexamethasone were shown to be cytostatic, but not cytotoxic, at high cell densities for early passage and continuous cell lines from human glioma at 0.25 μM and above, in the presence or absence of serum. In the absence of serum both steroids at 2.5 nM increased the saturation density close to the level reached in serum. Examination of the iodinated glycoproteins of the cell surface by gel electrophoresis did not reveal any consistent change. However, gel exclusion chromatography of protease digests of the cell surface and of material released into the medium showed an increase in incorporation of ^{3}H-glucosamine in pronase digests after treatment with methyl prednisolone. Ion exchange chromatography showed that sulphated glycosaminoglycans, particularly heparan sulphate, increased and hyaluronic acid decreased in response to steroids, and there was increased retention of GAGs on the cell surface relative to the released fraction. It was concluded that glucocorticoid hormones modify the cell surface of human glioma cells and that this may contribute to enhanced cell intraction and lead to increased density limitation of cell proliferation.

Although glucocorticoids have been used for many years to reduce intracerebral pressure and post-operative brain swelling in cases of glioma of the brain, their effect on tumour growth is not clearly established. Glucocorticoids have been shown to inhibit growth of several different cell types *in vitro*, including glioma (Freshney *et al.*, 1980*a*), so it is possible that they may exert a direct cytostatic effect on the tumour.

Data from early passage cultures of anaplastic astrocytoma have shown that cytostasis is accompanied by a phenotypic modification of the cells, suggesting a shift towards a more differentiated state (McLean *et al.*, 1986). This could influence the rate of tumour growth and spread, as has been suggested for several other tumours such as neuroblastoma, melanoma and myeloid leukaemia (Freshney, 1985). However, it is not clear which element of the induced differentiated phenotype may have the greatest effect on growth limitation. Cytostasis would limit growth, but not necessarily spread. Induction of differentiated glial properties such as high affinity GABA or glutamate metabolism are significant markers of phenotypic change, but are unlikely, of themselves, to influence cell behaviour.

Amongst the several effects of glucocorticoids on glioma, and other cells, modification of the cell surface might be expected to have a more far reaching effect as it is at the cell surface that contact and, potentially, information exchange with adjacent cells occurs. Cell–cell recognition leads to contact inhibition of movement at high cell densities (Abercrombie & Heaysman, 1954), and cell contact may also induce, or at least facilitate, the expression of differentiation (Freshney *et al.*, 1980*b*).

Several cell surface and extracellular matrix glycoproteins and proteoglycans are involved in the complex interaction between cells, and it has been suggested that some of these may have direct effects on gene transcription and cell behaviour (Li *et al.*, 1987). Previous reports on transformed fibroblasts (Walker *et al.*, 1986), glioma (Freshney *et al.*, 1980*a*) and non-small cell lung carcinoma (McLean *et al.*, 1986) have shown that glucocorticoid treatment induces changes in cell glycoproteins and proteoglycans. Sialation is decreased in glioma (Freshney *et al.*, 1980*b*), making the surface more like that of normal glia, the cells apparently become more adhesive, both to the substratum and to each other (Guner *et al.*, 1977) and plasminogen activator is greatly reduced (McLean *et al.*, 1986).

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In the present report, the cytostatic effect of glucocorticoids has been confirmed with a large panel of cell lines from anaplastic astrocytoma. Cytostasis has been shown to be accompanied by a decrease in hyaluronic acid, and an increase in sulphated glycosaminoglycans, particularly heparan sulphate.

Materials and methods

Cell culture

Cell lines were initiated by collagenase digestion of biopsy material (Freshney, 1982) kindly provided by Prof. David I. Graham of the Institute of Neurological Sciences, Southern General Hospital, Glasgow and Dr Jeanne Bell of the Department of Neuropathology, Western General Hospital, Edinburgh. They were maintained in a 50:50 mixture of Ham's F10 and Dulbecco's modification of Eagle's Basal Medium (F10/DME) supplemented with 10% foetal bovine serum, except where specified, and with a gas phase of 2% CO_2 in equilibrium with 8 mM $NaHCO_3$.

Early passage lines were used between the 3rd and 10th passage. Approximately 3 generations occurred between each subculture. Those lines whose passage number exceeded 50 were assumed to be continuous. They were used from frozen stock within 3 months of thawing. Mycoplasma determinations were performed on all cultures at monthly intervals and only cultures shown to be mycoplasma free were used.

Cell lines prepared in this way have been shown to be glial and neoplastic by the presence of dexamethasone-inducible glutamine synthetase, high affinity uptake of γ-aminobutyrate, aneuploidy, plasminogen activator activity, a high saturation density and angiogenic activity. Lines MOG-G-CCM, MOG-G-UVW, and MOG-G-IJK$_t$ (cell line designations abbreviated in text to CCM, UVW, IJK$_t$), have been characterised previously by these criteria, although the early passage lines have not.

Cytostasis

Cultures were trypsinised and counted on a Coulter ZB at intervals during growth and the saturation density determined as the maximum cell density attainable under non-limiting medium conditions, by culturing the cells on a 15 mm coverslip in a 5 cm Petri dish. Saturation density was taken as that concentration where three successive counts gave no further

increase in cell number. Labelling indices were determined autoradiographically after labelling with 5-Me-^{3}H-thymidine, 4 μCi ml^{-1} (40 μCi mol^{-1}) for 24 h.

Electrophoresis of glycoproteins

Rinsed monolayers were labelled with Na^{125}I by the glucose oxidase/lactoperoxidase method and separated by electrophoresis in 6% polyacrylamide gels containing 0.2% SDS against molecular weight calibration standards. Radioactivity was determined in 1 mm slices. At least four replicate lanes were run for each sample.

Gel filtration

Cells were grown to 50% confluence, treated with 10 μg ml^{-1} glucocorticoid for 5 days, and then labelled for 16 h with 5 μCi ml^{-1} ^{3}H-glucosamine (2 Ci mmol^{-1}; Amersham International). After 3 days digestion in 1 mg ml^{-1} pronase (Sigma), unincorporated ^{3}H-glucosamine was removed by passing the sample through a Whatman C18 cartridge which does not retain free glucosamine. Macromolecular material was eluted with 50% acetronitrile containing 0.1% trichloroacetic acid, freeze dried and redissolved in distilled water, before applying to a TSK-G3000SW HPLC column. Fractions (0.1 ml) were collected and radioactivity determined by scintillation counting.

Ion exchange chromatography

Cells were grown to confluence in the presence and absence of glucocorticoids and then labelled for 16 h with 10μCi ml^{-1} $^{35}SO_4^{2-}$, or 5 μCi mol^{-1} ^{3}H-glucosamine. Unincorporated precursor and salt removed from the medium by treatment with a C18 cartridge, as above, and the reconstituted macromolecular material applied to a Whatman DE52 column and eluted with 0.145–0.8 M NaCl in 20 mM sodium phosphate buffer, pH 6.8. The identity of the peaks eluting in salt were confirmed by electrophoresis on cellulose acetate with standard GAGs.

Results

Cytostasis

A series of 8 early passage cultures from anaplastic astrocytoma were grown to the plateau phase of the growth cycle (10–20 days, depending on population doubling time). A reduction in saturation density in the presence of 10 μg ml^{-1} (25 μM) glucocorticoid was observed of just over 15% (16.4% ± 2.2) (Table I). One medulloblastoma, one Schwannoma, and one haemangioblastoma gave 8..6%, 12.5% and 7.8% reduction respectively, in saturation density, while one culture from normal brain (NOR) showed 52% reduction. One culture derived from a secondary carcinoma (CAM) (primary unknown), showed 31.6% reduction, but at the time of assay this culture showed little evidence of epithelial growth and was probably mainly reactive glia.

The non-astrocytic tumour cultures were not characterised, but similarly-derived cell lines from astrocytoma have been characterised previously (Frame *et al.*, 1984) and their glial and malignant origin confirmed by glutamyl synthetase activity, glial fibrillary acidic protein (CCM only), plasminogen activator activity and angiogenic potential.

The response of a separate series of early passage cultures to MP and DX was examined by saturation density and/or labelling index and complared to three continuous cell lines (Table II). Although differences were observed among the lines and, occasionally, between steroids, passage level had no consistent effect. The response of the continuous cell lines UVW, IJK$_t$, and CCM spanned the range of the other early passage lines.

Figure 1 shows dose response curves for one continuous line, IJK$_t$, in the presence and absence of 10% foetal bovine serum, and Figure 2 shows two early passage lines, BG-30 and BH-35 in the presence of 10% foetal bovine serum. (The early passage lines would not survive serum-free under the conditions used.) Greater than 90% reduction in saturation density was observed with IJK$_t$ in the presence of serum with both dexamethasone (DX) and methyl prednisolone (MP) at 10 μg ml^{-1} (25 μM). At 0.1 μg ml^{-1} (0.25 μM), ~60% reduction was observed with MP, and DX still gave >90% reduction. No significant reduction in saturation density was observed with either steroid at 1 ng ml^{-1} (2.5 nM).

In the absence of serum, IJK$_t$ cells reached a lower saturation density, ~25% of that in the presence of serum. The addition of 1 ng ml^{-1} (25 nM) MP increased the saturation density to above half that of cells in serum and 1 ng ml^{-1} (2.5 nM) DX to ~80% of cells in serum. At 0.1 μg ml^{-1} (0.25 μM) both steroids were cytostatic in the absence of serum, to a similar extent to that noted in the presence of serum, with DX more cytostatic than MP.

The early passage lines BG-30 and BG-35 were less sensitive than IJK$_t$, showing approximately 50% maximum inhibition at

Table I Early passage cultures from anaplastic astrocytoma and other tissues

Cell line	Pathology	*Plating efficiency* w/o DX	*Plating efficiency* 10 μg ml^{-1} DX	*Plating efficiency* % incr	*Saturation density* $\times 10^{-5}$ w/o DX	*Saturation density* $\times 10^{-5}$ 10 μg ml^{-1} DX	*Saturation density* $\times 10^{-5}$ % decr
Astrocytomas							
WCA	AA*	8.8	11.3	28.4	6.7	5.6	16.4
MRG	AA	6.7	8.8	31.3	8.9	7.3	17.9
SMO	AA	11.1	17.7	59.5	6.7	5 4	19.4
YMI	AA	12.8	17.5	36.7	8.9	7.7	13.5
ORW	AA	13.9	25.5	83.5	4.0	3.4	15.0
KML	AA	–	–	–	6.4	5.2	18.7
AMK	AA	–	–	–	8.2	6.9	15.8
MOS	AA	–	–	–	7.8	6.7	14.1
Mean		10.7	16.2	47.9	7.2	6.0	16.4
±s.d.		±2.9	±6.5	±23.4	±1.6	±1.4	±2.2
Others							
NOR	N	–	–	–	–	–	52
SPM	M	13.0	20.8	60.0	5.8	5.3	8.6
CRA	S	21.3	23.8	11.7	4.8	4.2	12.5
CAM	MC	19.4	13.5	−30.4	7.9	5.4	31.6
DDL	H	–	–	–	6.4	5.9	7.8

*AA: Anaplastic astrocytoma (Grade II or IV Kernohan & Sayre); H: Haemangioblastoma; M: Medulloblastoma; MC: Metastatic carcinoma; S: Schwannoma; N: Normal brain.

Table II Relative efficacy of methyl prednisolone and dexamethasone at different passage levels

Cell line	Pathology	Culture status	% Reduction of saturation density		% Reduction of labelling index	
			MP	DX	MP	DX
BG28	E*	Early passage	47	–	76	–
BG30	G	Early passage	52	58	73	74
BG35	AA	Early passage	48	42	–	–
ELL	AA	Early passage	51	61	–	–
BG103	A	Third passage	30	–	–	–
BG103	A	Tenth passage	41	40	–	–
BG105	A	Fifth passage	11	14	–	–
BG105	A	Tenth passage	11	14	–	–
UVW	AA	Continuous	3	44	–	–
IJK_t	AA	Continuous	93	96	–	–
CCM	AA	Continuous	–	–	40	14

*A: Low grade astrocytoma; AA: Anaplastic astrocytoma; E: Ependymoma; G: Glioma (unclassified).

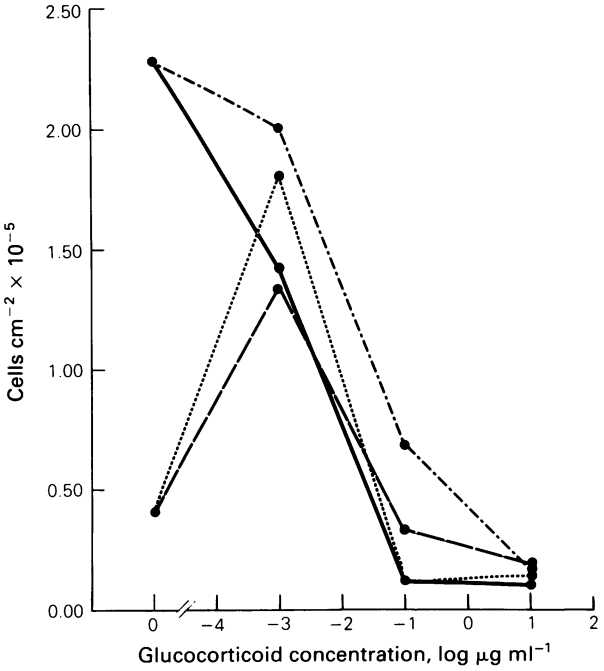

Figure 1 Effect of glucocorticoids on the growth of the continuous cell line IJK_t. Cells were seeded at 10^4 cells ml^{-1} (5,660 cm^{-2}) on 15 mm Thermanox coverslips in 24 well plates in the presence and absence of methyl prednisolone or dexamethasone at the concentrations indicated and then transferred to 50 mm petri dishes and grown for a further 5 days in the presence or absence of steroid. Serum was withdrawn from half the samples when confluence was reached at day 8. At day 13, cells were trypsinised and counted. —·— MP, with serum; —— DX, with serum; ——MP without serum; -----DX without serum.

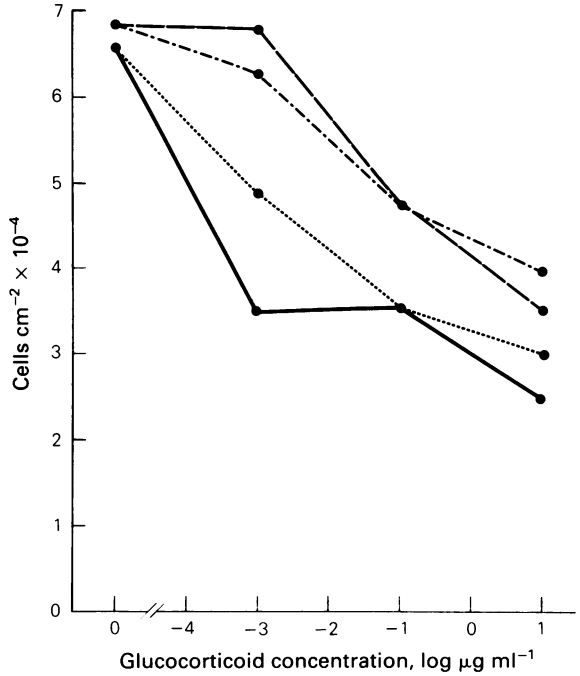

Figure 2 Effect of glucocorticoids on the growth of two early passage cell lines, BG30 and BG35. Cells were grown as in **Figure 1** except that all samples were grown in serum containing medium., -----BG30, MP; ——BG30, DX; ——BG35, MP; —·—BG35, DX.

10 $\mu g\,ml^{-1}$ (25 μM), BG-35 was less sensitive at 1 $ng\,ml^{-1}$ (2.5 nM), but showed similar inhibition at 10 $\mu g\,ml^{-1}$ (25 μM) (Figure 2). The smaller response in the early passage lines probably reflects the lower saturation density of controls.

As a reduction in saturation density could result from a change in cell volume or degree of spreading, cytostasis was confirmed by measuring the labelling index with ^3H-thymidine. Figure 3a shows a reduction in the labelling index of ~50% in 0.1 $\mu g\,ml^{-1}$ (0.25 μM) steroid, suggesting that labelling index may be a more sensitive measurement of cytostasis. This is confirmed in Figure 3b, with the early passage line BG-28. In this case, steroid (DX) was added after the cultures had entered plateau and it was possible to reduce the serum concentration to 1% without obvious deterioration of the culture. Saturation density was still reduced about 50% with steroid and cytostasis was observed, with the labelling index, to be about 65%.

When the continuous cell line CCM and the early passage lines BG-84 and BG-103 were cloned after treatment with steroid for 5 days, no decrease in plating efficiency was observed up to 25 μM. There was an enhancement in the plating efficiency of BG-103 cells pretreated with 25 μM DX, and it was only at 0.25 mM that pretreatment showed a reduction in plating efficiency of one of the lines, BG-84 (data not shown). This confirms previous indications that glucocorticoids are not cytotoxic to glioma even at quite high concentrations (Guner *et al.*, 1977).

11-α-epiydrocortisone, an analogue of hydrocortisone, was not found to be cytostatic to CCM cells and even increased saturation density (Table III), implying that the 11-β-OH group is required for cytostasis.

Cell surface glycopeptides

As previous data (Guner *et al.*, 1977) had suggested an increase in plating efficiency and demonstrated more

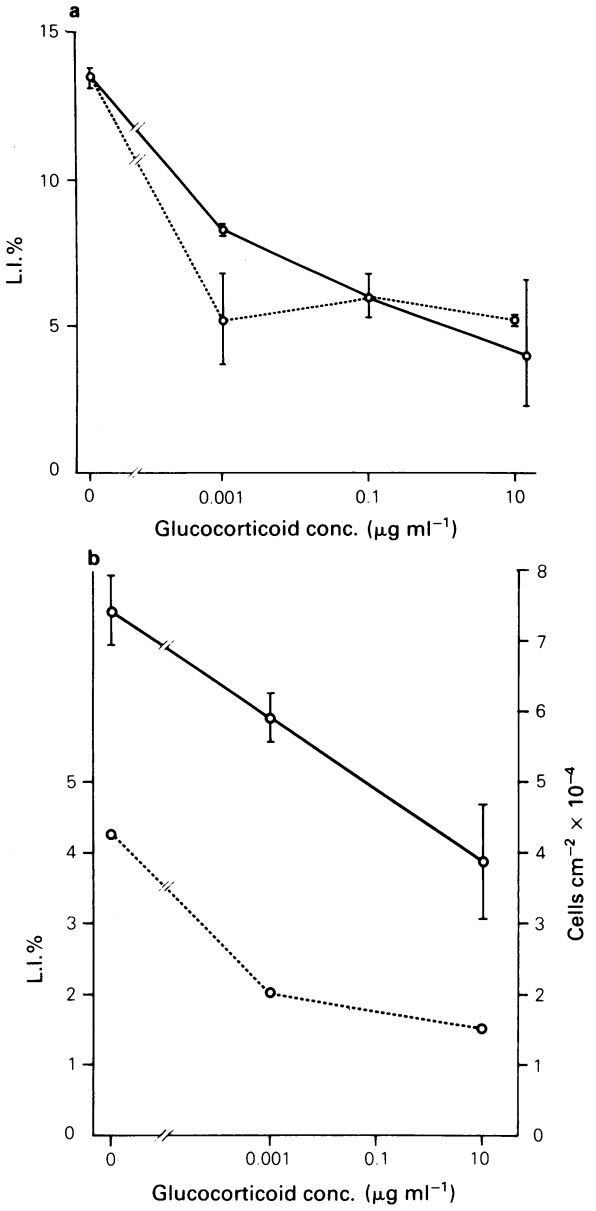

Figure 3 Effect of glucocorticoids on the labelling index and saturation density of two early passage cell lines, BG30 and BG28. 4 μCi ml^{-1} ^{3}H-thymidine (40 μCi mol^{-1}) was added at day 13 for 18 h and autoradiographs prepared as in **Materials and methods**. (a) Labelling index of BG30 with and without DX and MP at the concentrations indicated. —— MP, ----- DX. (b) Labelling index and saturation density of BG28: —— saturation density; ----- labelling index.

Table III Relative efficacy of 11-α-epihydrocortisone

	Concentration		
Steroid	*μg ml^{-1}*	*μM*	*Labelling index*
–	–	–	14.5±1.3
Methyl prednisolone	10	25	11.1±1.2
Dexamethasone	10	25	12.5±0.6
11-α-epihydrocortisone	1	2.5	18.7±1.1
11-α-epihydrocortisone	5	12.5	17.4±0.9
11-α-epihydrocortisone	10	25.0	14.8±0.42

compact colonies in the presence of DX, it was possible that at least part of the effect of steroid may be expressed via a modification of the cell surface. Confluent cultures of CCM and IJK$_t$, grown in the presence or absence of 10 μg ml^{-1} (25 μM) MP, were iodinated by the lactoperoxidase technique and the membrane glycoproteins separated by gel electrophoresis on 6% polyacrylamide containing 0.2% SDS. The radioactivity of sliced gels is present in Figure 4, and while significant differences are evident between treated and untreated cells, there is no similarity in the response between the two cell lines although cell proliferation of both is inhibited by steroids.

Changes in the 225 kD fraction would include alterations in fibronectin, although a slight increase (not statistically significant) was seen in IJK$_t$, CCM showed a significant decrease. In IJK$_t$ the low mol. wt peaks, 32, 48 and 83 kD were all decreased significantly by MP, and the high mol. wt peaks, 87 and 130 kD, were significantly increased. In CCM, although there was a slight increase in 87 kD material (not statistically significant), the major changes were in the significant reduction of the 20 kD and increase in the 63 kD fractions, together with the reduction in 225 kD previously mentioned. Hence there is no evidence for a consistent change in membrane glycoprotein with MP in these two cell lines.

Glycosaminoglycans

CCM cells were labelled with 25 μCi ml^{-1} (2.0 Ci m mol^{-1})

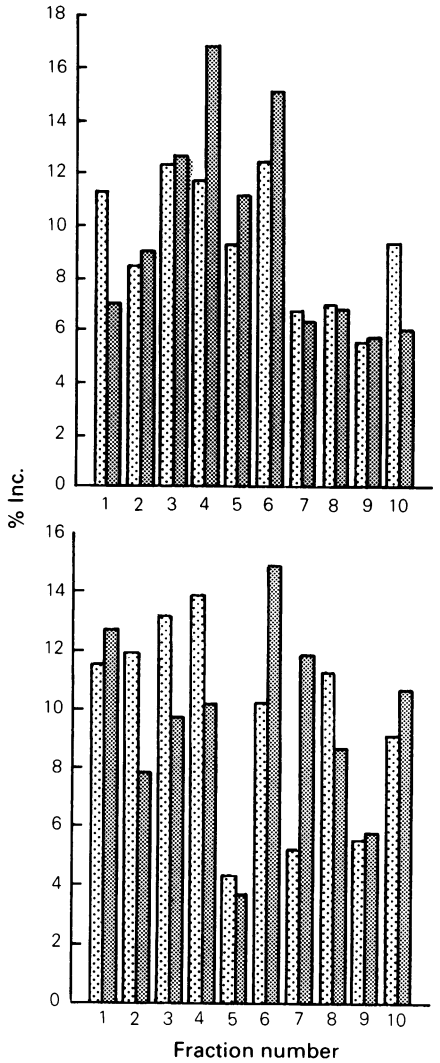

Figure 4 Electrophoresis of iodinated glycopeptides of the continuous cell lines IJK$_t$ and CCM-F4 (a clone of CCM). Each cell line was grown to confluence in a 75 cm^2 flask, exposed to 10 μg ml^{-1} (25 μM) DX or MP for 5 days post confluence and then iodinated as in the **Materials and methods**. *Light stipple*: control; *Dark stipple*: MP. *Numbering of bars*: 1:20 kD, 2:2.32 kD, 3:48 kD, 4:63 kD, 5:75 kD, 6:87 kD, 7:130 kD, 8:148 kD, 9:195 kD, 10:225 kD. *Top*: CCM-F4, significant differences in 20 kD and 225 kD ($P<0.05$) and 63 kD ($P<0.01$). *Bottom*: IJK$_t$, significant differences in 32 kD, 63 kD and 198 kD ($P<0.05$) and 48 kD and 130 kD ($P<0.01$).

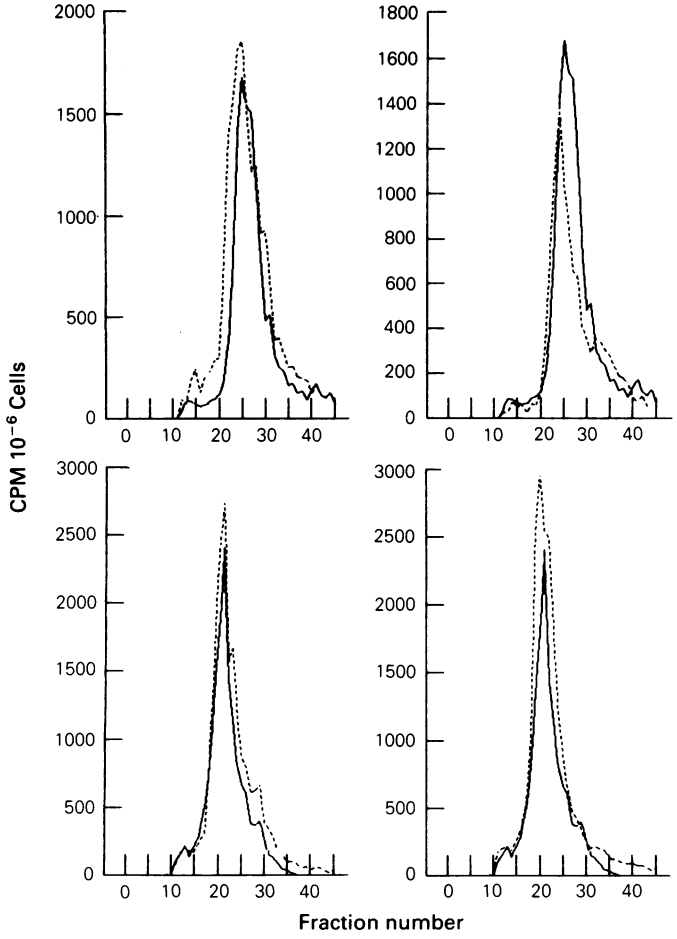

Figure 5 Gel filtration of conditioned medium and pronase digests of CCM cells grown to 50% confluence in 75 cm^2 flasks and exposed to 10 $\mu g\,ml^{-1}$ (25 μM) DX or MP for 5 days. During the last 16 h exposure to steroid, the cells were labelled with 5 $\mu Ci\,ml^{-1}$ ^{3}H-glucosamine (2.0 $\mu Ci\,mol^{-1}$). —— control; ----- 10 $\mu g\,ml^{-1}$ steroid. *Top left*: DX, medium; *Top right*: MP, medium; *Bottom left*: DX, pronase digest; *Bottom right*: MP, pronase digest.

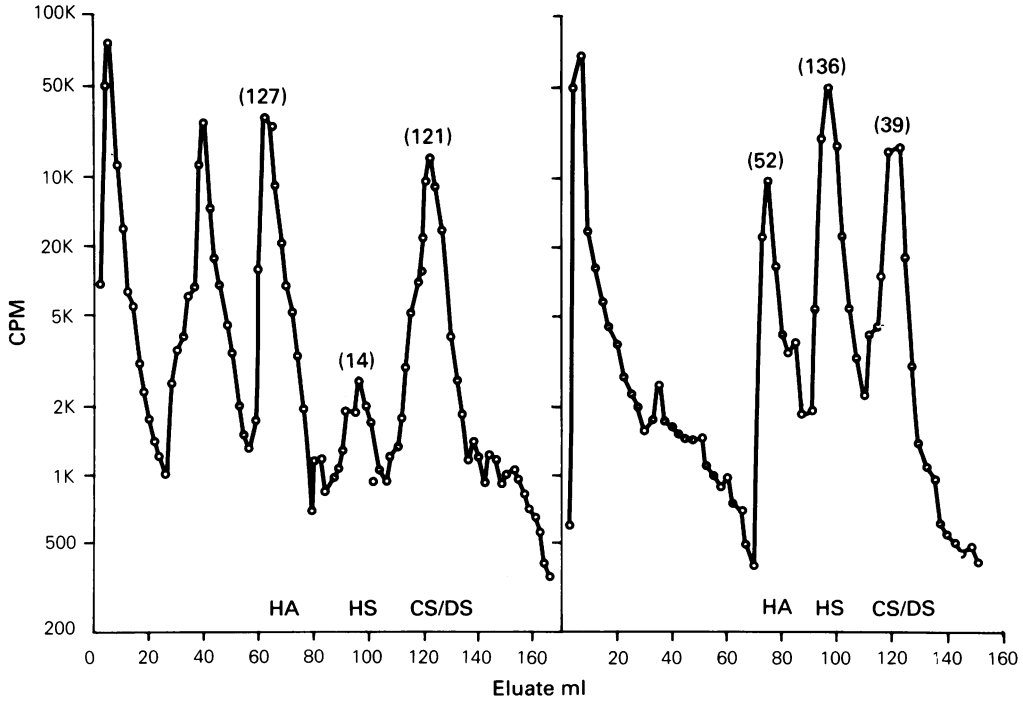

Figure 6 Ion exchange chromatography of conditioned medium of early passage cell line BG84. Cells were grown and labelled as for **Figure 5** and then applied to TSK3000SW HPLC as described in **Materials and methods**. *Left*: control; *Right*: 10 $\mu g\,ml^{-1}$ MP. Numbers in parentheses: Approximate integrated values of peaks ($\times 10^{-3}$).

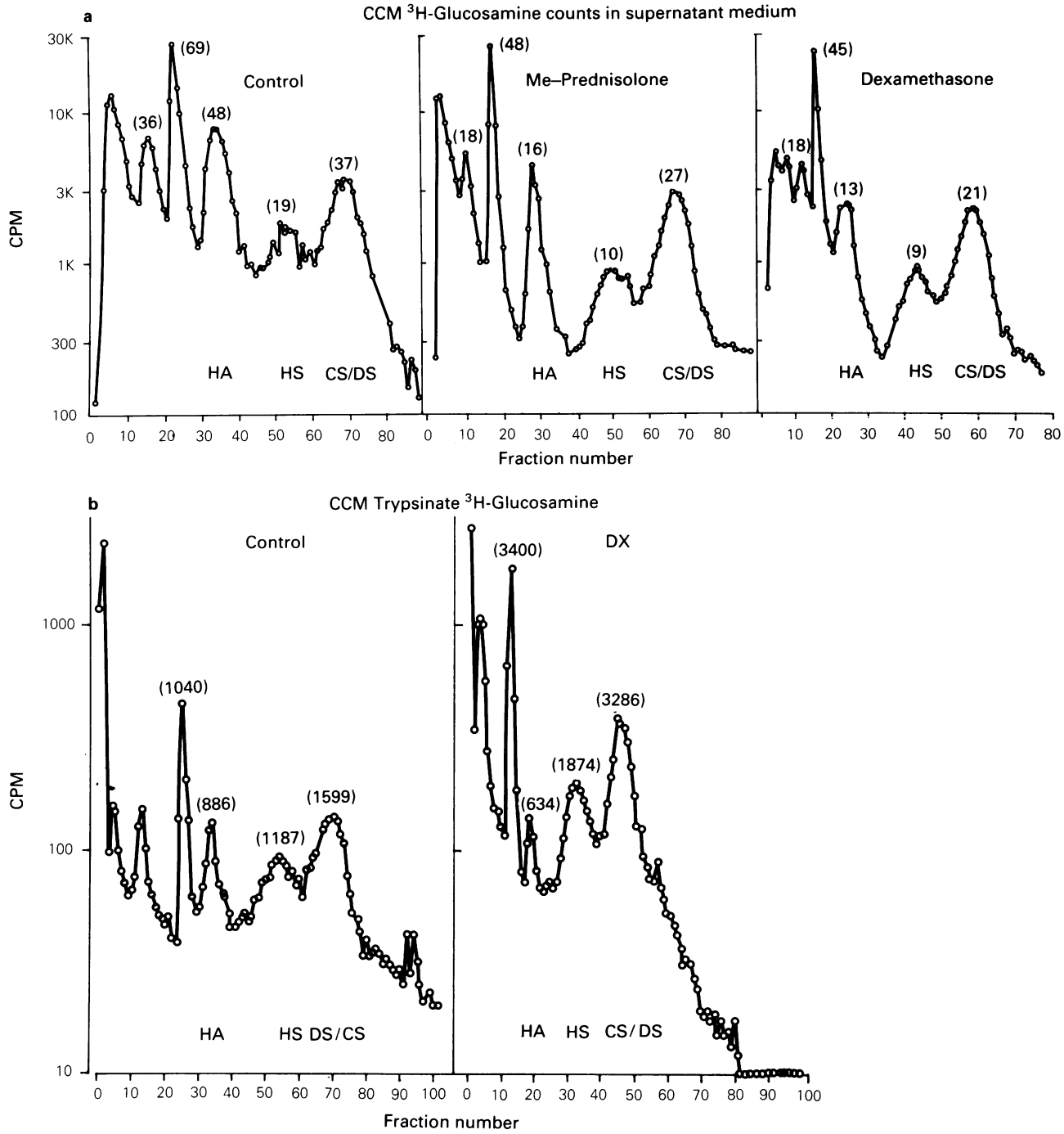

Figure 7 Ion exchange chromatography of conditioned medium and pronase digests of G-CCM. Prepared as in **Figure 6**. (a) Conditioned medium; (b) Pronase digest. HA: hyaluronic acid; HS: heparan sulphate; DS/CS: chondroitin SO_4, dermatan SO_4. Numbers in parentheses: Approximate integrated values of peaks ($\times 10^{-3}$ in (a)).

^{3}H-glucosamine (^{3}H-GLN) for 16 h and incorporation measured in material eluting from TSK-GB3000GW as described in the **Materials and methods**. Macromolecular material from the culture medium and pronase digests of whole cells were examined separately.

Activity in the medium of CCM increased slightly following treatment with 10 μg ml^{-1} (25 nM) DX (Figure 5a) and decreased with MP (Figure 5b).

Material in pronase digests increased slightly with 10 μg ml^{-1} (25 μM) DX and more significantly with MP (Figure 5c, d). There was no major change in molecular weight of the soluble or cell-associated material visible at this level of resolution.

Ion exchange chromatography of the soluble and cell-associated material gave one or two peaks of radioactivity excluded from the column, and one peak eluting immediately on initiating the salt gradient (Figures 6 and 7). All of these peaks showed absorption at 280 nm and were probably residual glycopeptides, in spite of the fact that both the trypsinate- and medium-derived material were digested with papain before loading on the column. Three peaks were eluted with NaCl, one around 0.3 M, one around 0.5 M and one around 0.6 M. By comparison with previous reports (Kraemer, 1971), and by electrophoretic analysis on cellulose acetate, these peaks were identified as hyaluronic acid (HA) at 0.3 M, heparan sulphate (HS) at 0.5 M, and a combination of chrondroitin sulphate (CS) and dermatan sulphate (DS) at 0.6 M (the last two not being separable by this method).

With CCM (Figure 7), all of the labelled material in the supernatant medium was decreased by treatment with 10 μg ml^{-1} (25 μM) MP, the greatest decrease being found in HA, and with DX more inhibitory than MP. In BG84, HA was reduced

more than 50% by 10 $\mu g\,ml^{-1}$ (25 μM) MP and one of the glycopeptide peaks disappeared completely (Figure 6). In this line, soluble HS was increased nearly tenfold by 10 $\mu g\,ml^{-1}$ (25 μM) MP.

Analysis of cell-associated material in CCM (Figure 7) showed a small reduction in HA (~25%) and an increase in both sulphated GAGs, ~60% in HS >100% in CS/DS. There was also an increase in cell-associated glycopeptide.

Discussion

Glucocorticoids are cytostatic to high density cultures of human anaplastic astrocytoma without being cytotoxic (Guner *et al.*, 1977; Freshney *et al.*, 1980a). Cytostasis has now been shown to be a general effect in all of the cultures examined, and was demonstrated both by cell number and labelling index. DX and MP have a similar effect, but their relative activity varies among cell lines; e.g. DX was more cytostatic for IJK_1, while MP was more cytostatic for CCM. Cytostasis is concentration dependent and requires >1 $ng\,ml^{-1}$ (2.5 nM) glucocorticoid. There is no effect with lower concentrations in the presence of serum, and in the absence of serum, 2.5 nM is stimulatory.

Glucocorticoid activity is dependent on the presence of an 11-β-OH group as 11-α-OH derivatives are inactive (Gower, 1979). Results from the present series showed that 11-α-epihydrocortisone was not cytostatic and may have been mitogenic, confirming that cytostasis resides in the same part of the molecule as glucocorticoid activity, but suggesting that mitogenic activity may not. It also suggests that glucocorticoids may exert both effects throughout their effective range, but that cytostasis predominates in most cultures at high steroid concentrations. Recent results with non-small cell lung cancer cell lines has shown several cell lines to be sensitive to the cytostatic effect of DX, but one that is not, NCI-H125, is stimulated, even at high cell densities (McLean and Freshney, unpublished observations). This effect was also observed in IJK (Morgan and Freshney, unpublished observations), the early passage cell line which eventually gave rise to IJK, via spontaneous transformation to a continuous cell line. In early passage cultures, cytostasis was not observed and saturation density was increased by DX, while after transformation the behaviour reverted to the more common pattern. As all of the lines that have been analysed have possessed similar levels of glucocorticoid receptor, it is possible that in some cases the receptor is modified and does not recognise the 11-β-OH moiety, while still responding to another, mitogenic component.

Previous results have shown that glucocorticoids are often mitogenic at low cell densities and enhance plating efficiency and clonal growth in both lung carcinoma and glioma (McLean *et al.*, 1986; Guner *et al.*, 1977). The colonies formed were more compact, suggesting greater cell–cell adhesion, and the enhanced plating efficiency may imply an increased cell-substrate adhesion. The latter may enhance clonal growth, while the former may increase cell interaction reactivating density limitation of growth at high cell density. If this were true then an alteration of the cell surface might be anticipated. While analysis of the cell surface has not shown any consistent alteration in glycoprotein, ^{3}H-GLN incorporation and subsequent chromatography have shown a shift from secreted to cell-associated GAGs and glycopeptides, and a reduction of the malignancy-associated HA and an increase in sulphated GAGs, particularly HS. An increase in HS as the predominant GAG and greater retention on the cell surface has been shown to be characteristic of normal brain derived cells when compared to glioma (Glimelius *et al.*, 1979).

Glucocorticoids have been shown to induce the differentiated phenotype in glioma cells *in vitro* (McLean *et al.*, 1986). This suggests that a modification of the cell surface may be an important component of the differentiated phenotype related to the reinduction of density limitation of growth.

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