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THE MODULATION OF THE PHENOTYPE OF HUMAN-NON-SMALL CELL LUNG CANCER

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FOR THE TITLE OF Ph. D.

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OCTOBER 1986

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TABLES AND FIGURES

SUMMARY

The effect of dexamethasone on human non-small cell lung carcinoma *in vitro* and *in vivo* was investigated. Dexamethasone caused a significant reduction in growth rate in most of the cell lines at normal passage densities with the exception of H125 which had an increased growth rate in the presence of dexamethasone. In both cases the effect was dose dependant. Clonogenicity as a monolayer was increased by dexamethasone in all cell lines investigated except H125. Cloning efficiency in agar of all the cell lines examined was reduced by dexamethasone in a dose dependant manner. Significant levels of glucocorticoid receptor were found in all cell lines investigated. PA activity was lowered by dexamethasone in all cell lines except H125 where it was raised though not significantly.

Some alterations in cell surface carbohydrates were found to correlate with alterations in cell proliferation. The cell lines WIL and H125 were examined for mucin/GAG secretion but no mucin secretion was found. Both cell lines secreted hyaluronic acid only. Dexamethasone increased hyaluronic acid secretion by H125 and decreased it in WIL. Pulmonary surfactant synthesis and secretion was increased by dexamethasone in the alveolar carcinoma A549.

Dexamethasone reduced the growth of the murine ROS tumour *in vivo*. The growth of WIL tumour was inhibited totally by dexamethasone in a reversible manner although pre-treatment of WIL cells *in vitro* with dexamethasone increased the frequency of successful tumour takes.

Apparently dexamethasone could increase differentiation and reduce malignancy associated properties a manner which suggested they were inter-related. The expression of the

differentiated phenotype is also correlated with a reduction in cell proliferation. Furthermore the *in vivo* response reflected the *in vitro* response.

GENERAL INTRODUCTION

Aims of Study

This study has two main aims. Firstly as an investigation into the biology of carcinomas, specifically lung, and secondly to investigate the possible utilization of dexamethasone in the chemotherapy of lung cancer. These two topics are intimately interlinked since the purpose of the first area of interest is to investigate the possible inter-relationship between malignancy and differentiation associated paramaters. It has been proposed that the induction of differentiation in tumour cells may be a rationale for cancer treatment.

Dexamethasone

Dexamethasone is a synthetic glucocorticoid (Fig 1). Glucocorticoids are synthesised in the adrenal cortex under the control of the anterior pituitary hormone adenocorticotrophichormone (ACTH) which stimulates their synthesis by increasing the conversion of cholesterol to pregnenolone, the rate limiting step in steroid hormone synthesis. As the name suggests a major function of the hormones is to control glucose levels. Basically, the glucocorticoids cause glucose levels to be maintained by stimulating the formation of glucose and diminishing its utilization. Increased excretion of nitrogen occurs as the glucose levels increase indicating that protein is converted to carbohydrate. However the glucocorticoids have other actions also some of which may be just as important as its metabolic effects. They do have some mineralocorticoid activity but this is of little importance physiologically. One of their most interesting effects is on lymphoid tissue where their administration causes a reduction in mass which may be associated with lympholysis. One of their best documented actions is inhibition of inflammation, As well as inhibiting oedema,

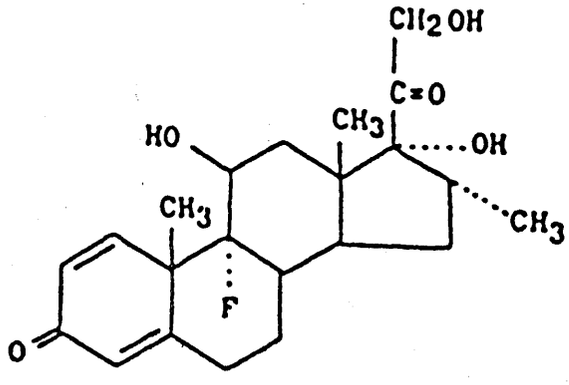


FIG.1 DEXAMETHASONE.

capillary dilatation and fibroblast proliferation and deposition of collagen. In the plasma 90% or more of cortisol is reversibly bound to protein under normal circumstances. The binding is accounted for by two fractions. One is corticosteroid binding globulin (CBG) and the other is plasma albumin. The globulin has high affinity but low total binding capacity; the albumin has low affinity but relatively large binding capacity (1).

The Need for New Chemotherapeutic Agents in the Treatment of Lung Cancer

The present role of cytotoxic chemotherapy in the treatment of lung cancer is very limited. A number of major problems exist that have to be answered. The first and most important of these is that some types of lung cancer do not respond to chemotherapy. This is due to one of two features, an inherent drug resistance in the neoplasm or an acquired resistance following treatment.

At present there are two approaches being used to circumvent this problem. One is to use two drugs in combination thus overcoming resistance. Another is to give the drug with an agent that will aid drug uptake, reduce drug efflux or act by some other means so increasing cytotoxicity.

However there are other avenues being investigated that do not involve the direct killing of the tumour cells. Inhibition of angiogenesis, the process by which tumours obtain the blood supply essential for their growth is one. Another is to inhibit invasion or metastasis and so making treatment by surgery easier.

A more novel approach is to induce differentiation and reduce the malignancy of the tumour. This and the aforementioned approaches have the added bonus that they are target specific, non-cytotoxic and have reduced side effects.

1. GLUCOCORTICOID MODULATION OF CELL PROLIFERATION AND CLONOGENICITY; RELATIONSHIP TO GLUCOCORTICOID RECEPTOR CONTENT

INTRODUCTION

1.1 Modulation of Cell Proliferation

It is well established that glucocorticoids modulate cell proliferation (2). In 1965 Ruhman and Berher noted that mouse fibroblasts *in vitro* had reduced growth rates when cortisone was present (3). Work since then has indicated that donor species, tissue origin, cell type, cell density and glucocorticoid concentration determine how cell proliferation is modified by glucocorticoids (4, 5, 6). Viral transformation can also alter the response to glucocorticoids. The human fetal lung fibroblast lines WI-38 and WI-26 are stimulated when hydrocortisone is present but when transformed with SV 40 their neoplastic counterparts show a reduction in growth.

Much time however has been spent on the effect of glucocorticoids upon material derived from neoplastic tissue especially human. Using *in vitro* techniques with cells at high density it has been found that lung alveolar carcinoma, A549, (7) and lymphoma cells (8) had reduced growth rates when incubated with dexamethasone. However mammary fibroadenoma cells (9) were stimulated by hydrocortisone. It should be noted however that it is very difficult to obtain cell lines from malignant breast cells and that often the cell type grown is derived from normal or benign tissue. Within this department dexamethasone was found to stimulate growth of early passage human gliomas at low cell densities while inhibiting growth at high densities though only with high concentrations of the drug (10, 11). It is important therefore that the density of the cells used in the experiments is taken into account when comparing results.

1.2 Modulation of Proliferation and Glucocorticoid Receptor Number

The cell type most widely investigated has been melanoma both *in vitro* and *in vivo*, human and non-human. This tumour type grows well in culture and many cell lines exist. Most investigations have attempted to correlate glucocorticoid presence with modulation of proliferation.

Bhakoo and colleagues using the murine B16 melanoma in mice found that cytosolic receptors with levels from 44 to 200 fmol mg^{-1} of protein and Kd's ranged from 2 to 43 nM. Administration of various glucocorticoids in different doses and regimes showed a singificant inhibition in mean tumour diameter and in animal survival times compared to controls. With higher doses of drug evidence of an increase in the number of pulmonary nodules was seen though there was no evidence of pulmonary metastases (12). Similar results were seen with the RPMI 3460 Syrian Hamster melanoma *in vitro*. The authors indicated that hydrocortisone and triamcinolone acetonide caused growth inhibition and that 1.0 μM progesterone overcame this effect indicating that the glucocorticoids action was through a specific receptor. More interestingly, limited exposure of the cells to dexamethasone failed to trigger the growth inhibition suggesting continuing presence of the drug is essential (13). The human malignant melanoma cell line NEL had glucocorticoid receptors with Kd 1nM and 247 fmol mg^{-1} of protein. A 30% inhibition in growth was seen with a continuous incubation of drug (triamcinolone acetonide) for 72 hours. Progesterone reversed this inhibition (14).

One of the best studies has been with Braunschweiger et al using rat, murine and xenograft tumour models (15). Glucocorticoid receptor was found with a range of 107-14 fmol mg^{-1} of protein. Following acute high dose injection of dexamethasone (10mg kg^{-1} every 6 hours, 4 doses) there was a reduction in glucocorticoid receptor and tumour labelling index. Furthermore the dexamethasone response was directly correlated to the saturable glucocorticoid receptor whether

assays
receptor were carried out in mice, rats, or athymic nude mice. The lung alveolar carcinoma previously mentioned, A549 (7) also had a glucocorticoid receptor Kd of 7.5nM and a level of 570 fmol mg^{-1} of protein. Dexamethasone at a concentration of 40nM caused a 44% inhibition of growth. Progesterone at 100-fold molar excess could only partially inhibit the action of dexamethasone. However, in a review of the literature Homo-Delarche found no clear correlation between the level of glucocorticoid receptor and the *in vitro* response in normal and neoplastic lymphoid tissue but *in vivo* attempts at correlation were more successful (16). Glucocorticoids can mediate their action on cells by permissive effects i.e. not directly through glucocorticoid receptor mediated effects. For example Gospodarowicz reported the potentiation of fibroblast growth factor induced proliferation by hydrocortisone in 3T3 cells (17). Much evidence also exists for permissive effects being mediated through the regulation of cells responsiveness to a second hormone by altering the number of the second hormone's receptor. Steroids have also been found to induce production of a growth stimulatory factor. WI-38 cells after steroid incubation produce a growth factor that acts upon Wl-38 cells in an autocrine fashion to stimulate growth and saturation density (18).

1.3 Steroid Receptors in Lung

A survey of the organs of 28 day old fetal rabbits found that the lungs had a higher concentration of glucocorticoid, 430 fmol mg^{-1} of protein, with respect to other tissues, the next highest being placenta with 260 fmol mg^{-1} of protein. There was no significant difference in the other tissues examined (19). However this study has the drawback that whole lung was used and so contains several tissue types. Further investigations using isolated fetal rat and human cells found glucocorticoid receptors in type II cells and fibroblasts (20).

Estrogens also directly alter fetal lung maturation (21). Low doses of estrone and estradiol when with maximal amounts

of glucocorticoid increase surfactant synthesis, while higher levels are inhibitory. Estradiol binds to fetal guinea pig (22) and adult rat lung cytosols (23). Human fetal and adult lung also bind estradiol (24, 25) but with slightly lower affinity. As with estrogens, androgens also bind to the cytosol from fetal rabbit lung (26) with no apparent sex difference in androgen binding.

A molecular examination of normal and neoplastic tissue revealed a significant number of specific receptors for glucocorticoids, estrogen and androgens with an affinity for hormone similar to those in other steroid responsive tissues (27). Glucocorticoid receptor was present in 82% of normal lung specimens and virtually identical to that reported for fetal and adult rabbit, rat and human lung. In normal lung glucocorticoid receptor resolved into two distinct binding components. with adenocarcinoma the receptor number was inversely proportional to the degree of tumour differentiation. Squamous cell carcinoma receptor levels were slightly lower With respect to normal tissue. Estrogen receptor was evenly distributed between squamous cell and adenocarcinoma cytosols with a slightly lower affinity, but higher content than normal lung. Androgen receptor resolved into two classes in squamous cell carcinoma similar to the situation with glucocorticoid receptor in normal tissue. The most undifferentiated squamous tumours had a lower androgen receptor content and higher affinity. In contrast there was no differentiation of androgen receptor content or affinity based on tumour grade in adenocarcinoma. Furthermore the receptor did not resolve into two distinct groups with respect to affinity.

Thus glucocorticoid receptors, and other steroid receptors are found in lung at higher levels than in other tissues making lung a good target for induction chemotherapy using dexamethasone.

1.4 Growth in Semi-Solid Medium

MacPherson and Montaginer (28) demonstrated that transformation of hamster BHK 21 cells by polyoma virus facilitated the growth of these cells in agar. Clonogenicity in agar was shown in correlate with tumour take in immuno-suppressed mice (29, 30).

METHODS

The methods stated here are the same as used in the rest of experiments unless stated otherwise.

1. Tissue Culture

1.1 Cell Maintenance

Monolayer cultures were fed with fresh medium every 4 to 5 days and routinely passaged by washing in PBS with 1mM EDTA followed by trypsinisation for 10 to 15 minutes at 37°C with 0.25% trypsin. The cells were resuspended in fresh medium and inoculated into new flasks or experimental culture dishes. Experimental cells in 24 well plates, or 50 mm Petri dishes were incubated at 37°C in a humidified atmosphere with 2 or 5% CO_2.

Medium is F10:DMEM (1:1) with 2mM glutamine and the appropriate percentage of serum. Sodium bicarbonate was also added, the amount depending on the gas mixture being used.

1.2 Cell Counting and Viability

Single cell suspensions were counted electronically using a Coulter Counter (Model ZB1). Cell viability was determined by exclusion of 0.1% trypan blue. In cloning experiments colony size was determined using an Artek colony counter. The conversion of colony size to cells per colony was done by counting the number of cells in a colony of known size.

1.3 Cell Freezing

Frozen stocks of all cell lines were maintained in liquid nitrogen. Cell suspensions of greater than $1x10^6$ cells ml^{-1} in medium containing 10% DMSO were frozen with an approximate cooling rate of 1^oC $minute^{-1}$.

1.4 Staining

Monolayer cell preparations were washed with PBS, fixed in methanol for 10 minutes and stained with 10% Giemsa or 0.25% crystal violet. Excess stain was removed with tap water and the stained preparations air dried.

1.5 Mycoplasma Staining

Cultures fixed with 25% acetic acid in methanol were monitored by incubating with the fluorescent DNA stain Hoescht 33258 at 0.05 $\mu g ml^{-1}$ for 15 minutes at room temperature. The detection of extranuclear DNA by fluorescence microscopy was indicitave of the presence of mycoplasma infection (31).

1.6 Cloning Experiments

1.6.1 Monolayer

In these experiments log phase cells were freshly trypsinized and inoculated in 60mm petri dishes at a concentration of 100 cells per ml^{-1}. After 14 days incubation unless otherwise stated the clones were fixed, stained and counted by eye or an electronic colony counter.

1.6.2 Suspension

Cells were inoculated at $10^4 ml^{-1}$ in a top layer of 0.5% agar over a 1% agar underlay. Colonies were counted using an inverted microscope after approximately 30 days. Agar cloning was carried out in 6 well plates. In all cloning experiments the serum concentration was 10%.

1.7 Glucocorticoid Receptor Assay

Plataeu phase cells were trypsinized and washed with PBS twice and then resuspended in 2ml of assay buffer. The buffer was10mM HEPES, 1.5 mM $MgCl_2$ and 10% glycerol (pH 7.4). Dithiothreitol (final concentration 0.25 mM) was added prior to addition of buffer to cells. To 150 µl of cell suspension 50 µl of ligand was added. The 'hot' ligand was (1, 2, 4) (n)-(3H) triamcinolone acetate (30 Ci $mmol^{-1}$). The cells were incubated at 20°C for 2 hours. Varying concentrations of dexamethasone were added. The remaining cell suspension was retained for DNA measurements (32). Protein content was measured by the Bradford assay using bovine serum albumin as the standard (33). The bound ligand was separated from the free using a Millipore filtration system apparatus as described by Laing et al (34). The bound ligand is retained on the Millipore filter and the free ligand passes through. The filters are then counted in a scintillation counter. Another aliquot is then placed directly onto a clean filter and counted; this gives the total ligand available for binding. The results are calculated using a computer programme (35) using Scatchard plots (36).

1.8 Cell Lines Used

The origin and type of cell lines used are displayed in Table 1. The pathology of the tumours is that found at initial development of the cell line. All the cell lines have been passaged at least 50 times and can therefore be regarded as continuous cell lines.

RESULTS

1.1 Clonogenicity and Dexamethasone

The effect of dexamethasone on the clonogenecity of four cell lines was investigated as a simple assay for cytotoxicity. Three of the cell lines had an increased plating efficiency in the presence of dexamethasone while the other was

TABLE 1 ORIGIN, CELL TYPE AND PATHOLOGY OF LUNG CELL LINES USED

Cell Line	Origin	Cell Type	State of Differentiation
A549	ATCC Maryland	Type II Alveolar	Alveolar, poorly differentiated Mucin +ve.
L-DAN	Medical Oncology Glasgow	Large cell/ Squamous	Large cell, anaplastic/squamous poorly differentiated.
SKMES	ATCC Maryland	Squamous	Squamous, moderately differentiated.
H125	NCl Bethseda	Adenocarcinoma	Adenocarcinoma, well differentiated.
H23	NCl Bethseda	Adenocarcinoma	Adenocarcinoma, poorly differentiated.
WIL	Haddow Laboratories Sutton	Adenocarcinoma	Adenocarcinoma, moderately differentiated, mucin +ve.

unaffected. The results for WIL are not shown. The clonogenicity of A549 and SKMES was maximally stimulated at a concentration of 2.6nM. Dexamethasone levels up to and including 26 μm were not cytotoxic although dexamethasone levels of 0.26 mM and higher abolished colony formation. In Figure 2 typical results are shown. The clonogenicities of A549 and SKMES were stimulated 2 and 3 fold respectively at a concentration of 2.6nM.

The cell line H125 did not respond to dexamethasone with respect to clonogenicity.

From these results it is clear that dexamethasone can alter the cloning ability of certain human lung cancer cell lines. This indicates that at the concentrations used dexamethasone was not cytotoxic. H125 does not respond to dexamethasone in these experiments.

1.2 Glucocorticoid Modulation of Proliferation at Regular Passage Densities

Growth curves were carried out for each cell line and terminl cell density and population doubling time determined from the data. Cells were in oculated in 24 well plates at a density of 5×10^3 cm-2 (10^4 cells ml^{-1}). Dexamethasone was added 24 hours after in oculation at a concentration of 26μM unless otherwise stated. Medium was changed every other day and replicate samples trypsinized and counted. Cells reached confluence within 10 days of innoculation.

A typical growth curve is shown in Figure 3. An increase in doubling time during late exponential growth and a decrease in terminal cell density can be seen. The effect of dexamethasone upon terminal cell density of SKMES was found at non-physiological concentrations of glucocorticoid (10 μM), significant reductions were seen with levels normally found in vivo (10nM; Figure 4). With a dexamethasone concentration of 26nM a 50% reduction in terminal cell density was seen.

FIGURE 2 EFFECT OF DEXAMETHASONE UPON CLONOGENICITY

Cells were cloned at 100 cells ml^{-1} (5mls) for 16 days. Cultures were then fixed and stained and colonies containing more than 16 cells counted. Results are the mean of triplicates.

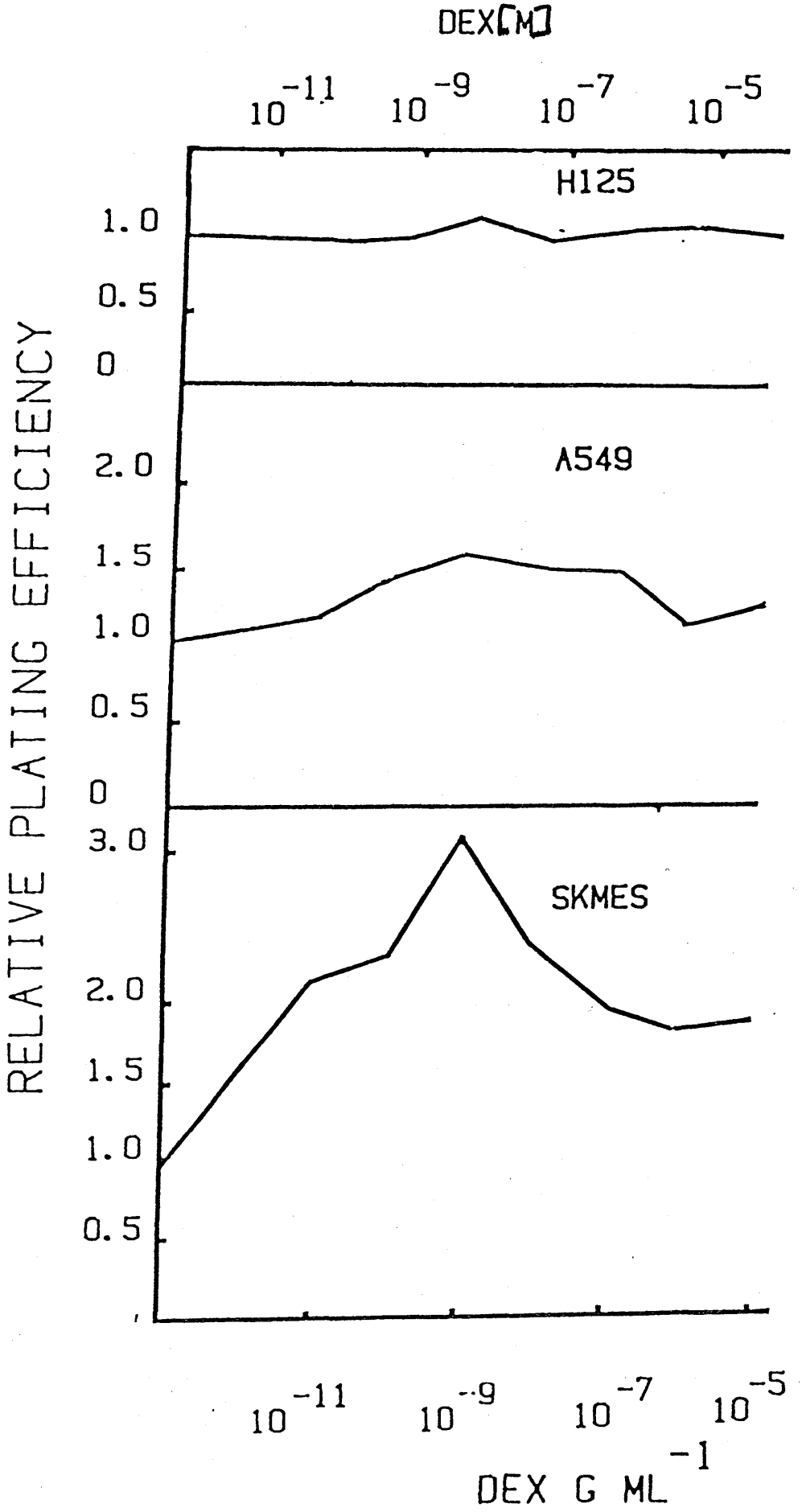

DEX[M]
10^-11
10^-9
10^-7
10^-5
H125
1.0
0.5
0
A549
2.0
1.5
1.0
0.5
0
SKMES
3.0
2.0
1.5
1.0
0.5
RELATIVE PLATING EFFICIENCY
10^-11
10^-9
10^-7
10^-5
DEX G ML^-1

FIGURE 3 EFFECT OF DEXAMETHASONE UPON GROWTH OF SKMES AT REGULAR PASSAGE DENSITIES

Cells were inoculated at 10^4 ml^{-1} in 24 plates with or without dexamethasone. Cells were counted every other day. Results are the mean of triplicates.

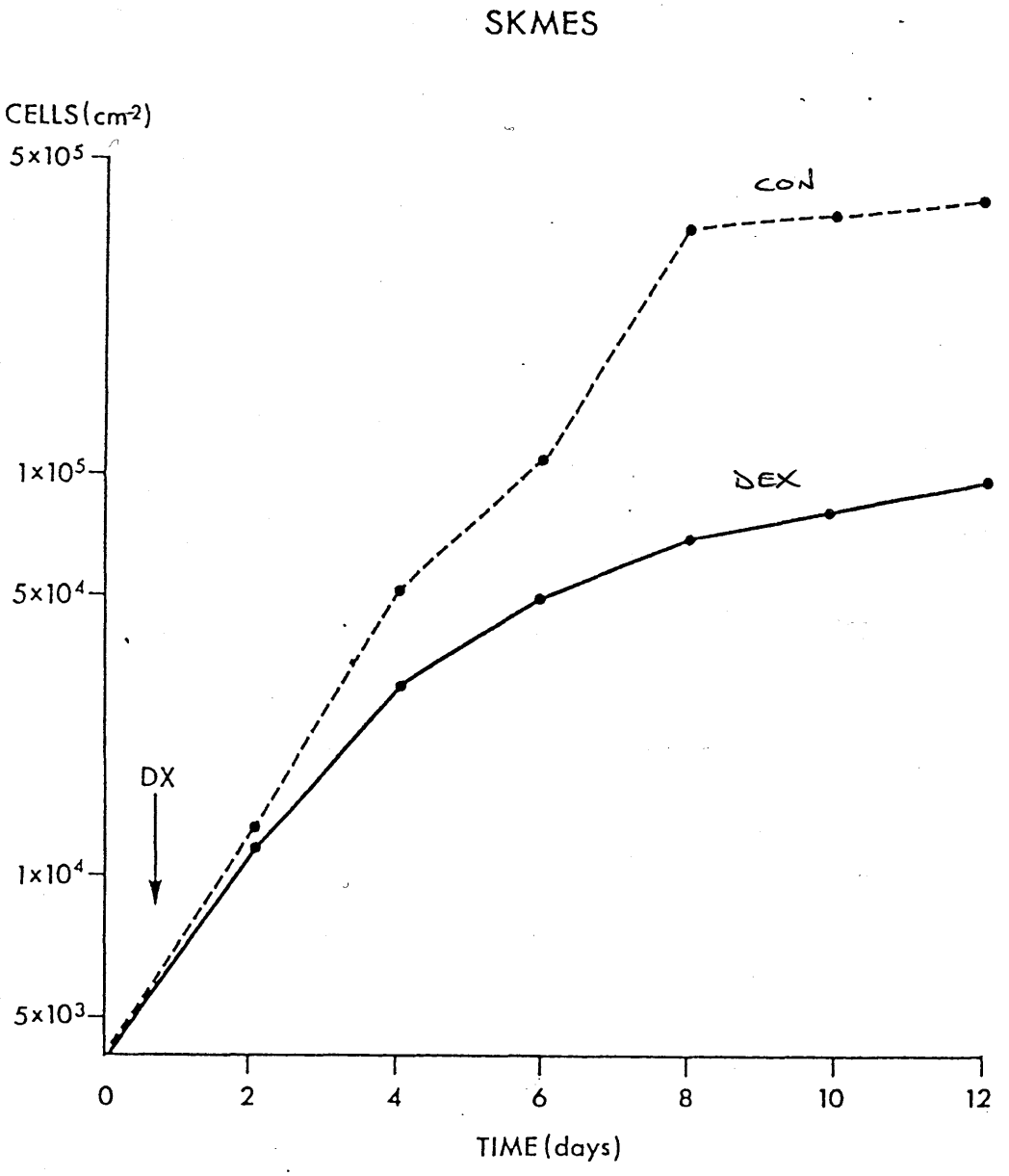

SKMES
CELLS (cm-2)
5x10^5
1x10^5
5x10^4
1x10^4
5x10^3
CON
DEX
DX
0
2
4
6
8
10
12
TIME (days)

FIGURE 4 EFFECT OF DEXAMETHASONE UPON TERMINAL CELL DENSITY OF SKMES

Late log phase cultures of SKMES were incubated with varying concentrations of dexamethasone. After 6 days the cells were trypsinised and counted. Mean $\pm$ s d (n=3).

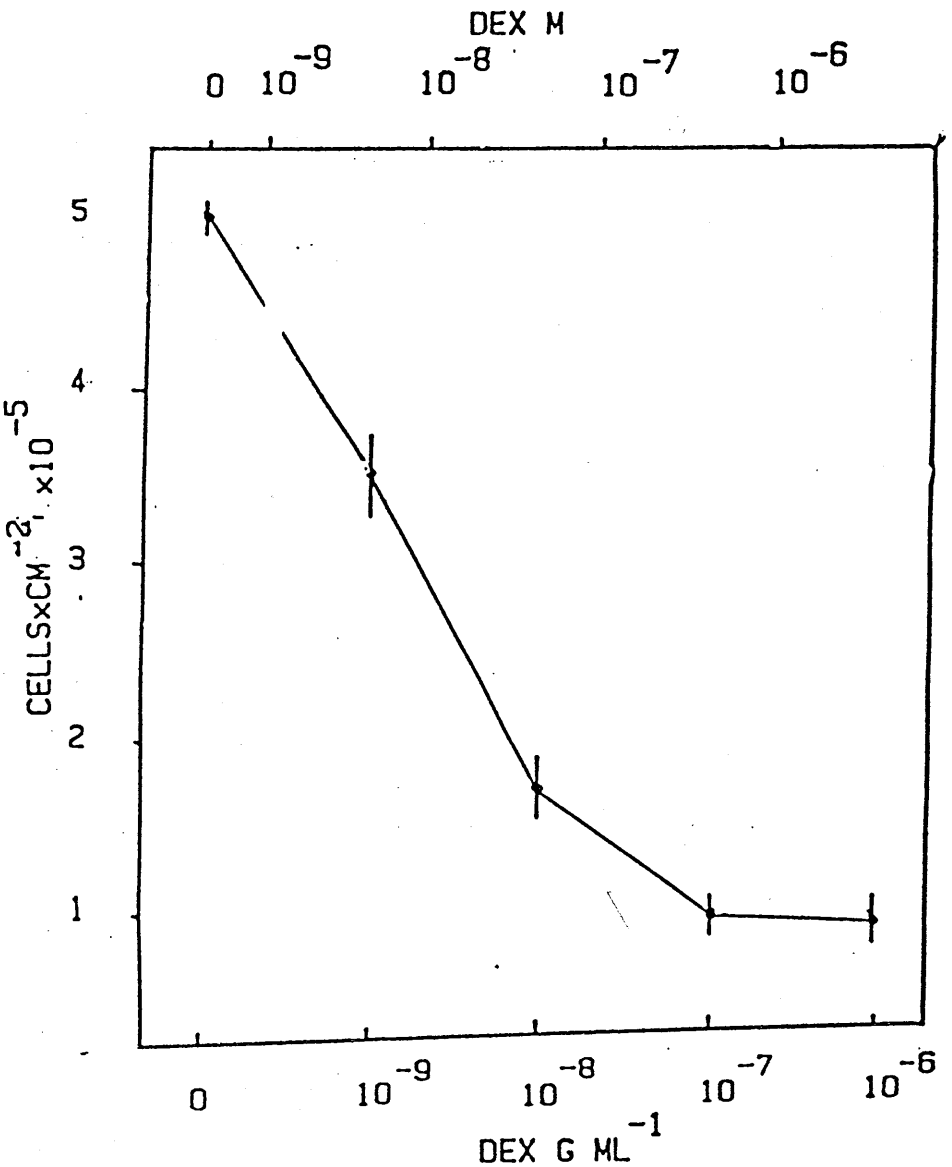

DEX M
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
5
4
3
2
1
CELLSxCM^{-2}, x10^{-5}
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
DEX G ML^{-1}

Table 2 presents the data for of all of the cell lines investigated. A549, L-DAN and WIL all respond in the same way with an almost 2-fold increase in doubling time. The terminal cell densities of the cell lines are decreased also; 4-fold, 6-fold and 6-fold respectively. However the two remaining cell lines, H23 and H125, had different responses to the presence of dexamethasone. The cell line H23's terminal cell density and doubling time was unaffected by the glucocorticoid. The growth rate of H125 was increased significantly by dexamethasone as was the terminal cell density.

Dexamethasone had a profound effect upon the proliferation of human lung carcinoma cell lines. All but two of the cell lines investigated had a significantly reduced terminal cell density and increased doubling time. Of the other cell lines investigated H23 was unaffected while H125 had an increased terminal cell density and decreased doubling time.

1.3 Glucocorticoid Modulation of Proliferation at Low Cell Densities

The effect of dexamethasone upon cell growth at clonal densities (approximately 25 cm^{-2}) was investigated using cloning experiments as in section 1.2. Cells were plated at a concentration of 100 cells ml^{-1} (5mls) in 5cm petri dishes in the presence of various concentrations of dexamethasone. At various time points the experiments were stopped, the cultures fixed, stained with crystal violet (0.25%) and sized electronically. In Figure 5 an experiment using the cell line WIL is shown from a culture 8 days post-in oculation. As can be seen at higher drug concentrations (26 uM) there is a general increase in colony size. If the incubation is allowed to continue for 15 days very similar results were seen with an increase in colony size as the concentration of dexamethasone increased (Figure 6). Converse results were obtained with the cell line H125. The presence of dexamethasone reduced the growth rate of H125 at clonal densities (Figure 7). For all of these experiments the effects were found to be dose dependant.

TABLE 2 EFFECT OF DEXAMETHASONE (26uM) ON DOUBLING TIME AND TERMINAL CELL DENSITY

	DOUBLING TIME (HRS)		TERMINAL CELL DENSITY (CELLS cm^{-2} 10^{-4})	
	+ DEX	CON	+ DEX	CON
A549	45.2 ± 1.3	26.7 ± 1.15*	6.1 ± 0.9	24.7 ± 0.5*
L-DAN	59.6 ± 1.6	30.4 ± 1.4*	5.3 ± 1.5	34.1 ± 2.4*
SKMES	54.2 ± 4.3	27.5 ± 1.6*	6.5 ± 2.4	42.7 ± 1.7*
H125	27.4 ± 2.3	41.0 ± 3.75*	17.9 ± 2.1	10.5 ± 0.8*
H23	33.8 ± 2.4	33.2 ± 1.9	19.3 ± 1.2	20.8 ± 1.1
WIL	46.4 ± 2.7	27.4 ± 1.65*	5.2 ± 1.5	35.0 ± 0.8*

* Significant at the 5% level

n = 4 mean ± standard errors.

FIGURE 5 EFFECT OF DEXAMETHASONE UPON GROWTH OF WIL AT CLONAL CELL DENSITIES AFTER 8 DAYS.

Cells were inoculated at 100 cells ml^{-1} (5mls). After 8 days cultures were fixed and colonies sized electronically.

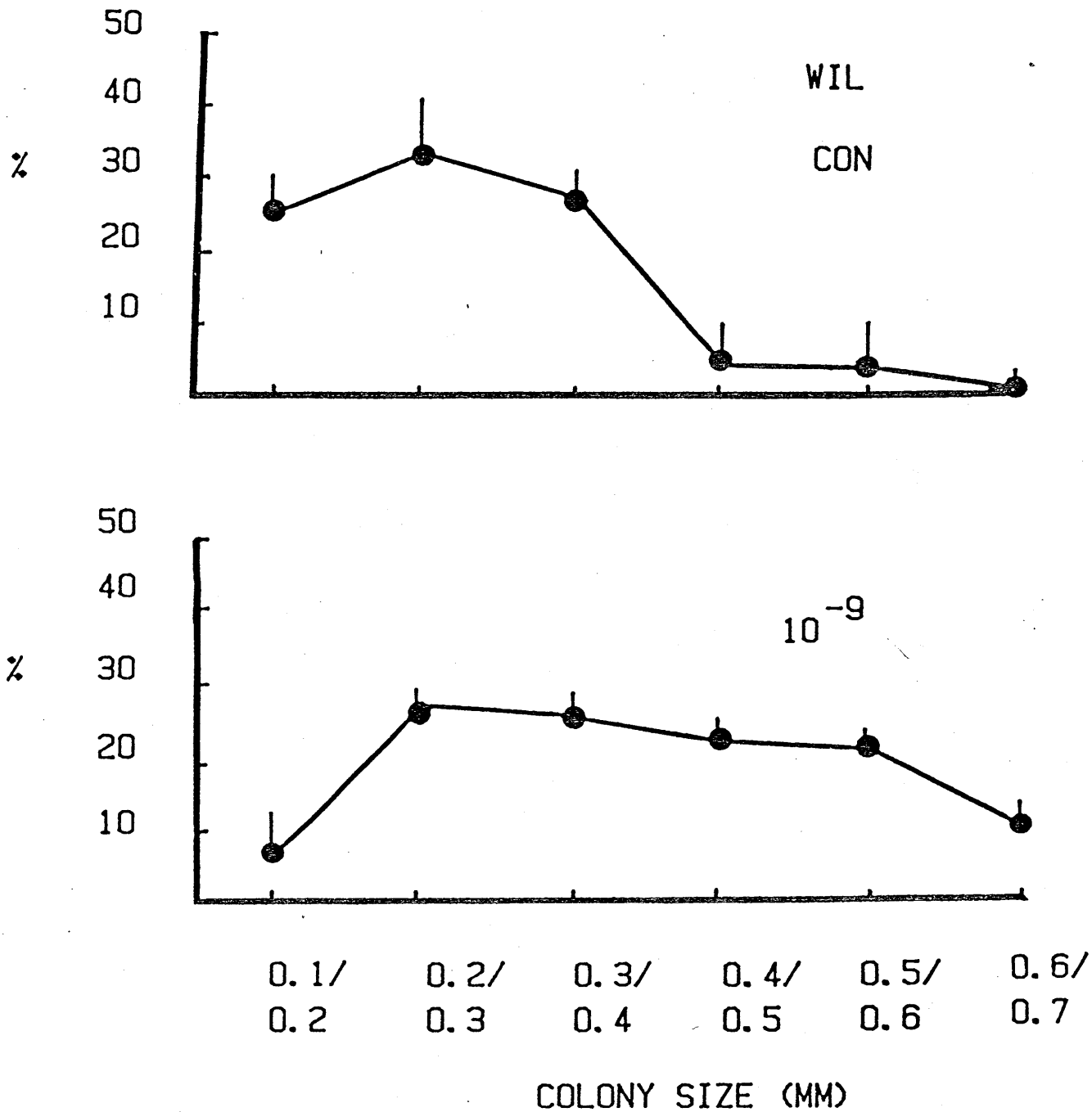

50
40
%
30
20
10
WIL
CON
50
40
%
30
20
10
10^{-9}
0.1/ 0.2
0.2/ 0.3
0.3/ 0.4
0.4/ 0.5
0.5/ 0.6
0.6/ 0.7
COLONY SIZE (MM)

FIGURE 6 EFFECT OF DEXAMETHASONE UPON GROWTH OF WIL AT CLONAL CELL DENSITIES AFTER 15 DAYS.

Cells were inoculated at 100 cells ml^{-1} (5mls). After 15 days cultures were fixed and colonies sized electronically.
Mean $\pm$ s d (n=3).

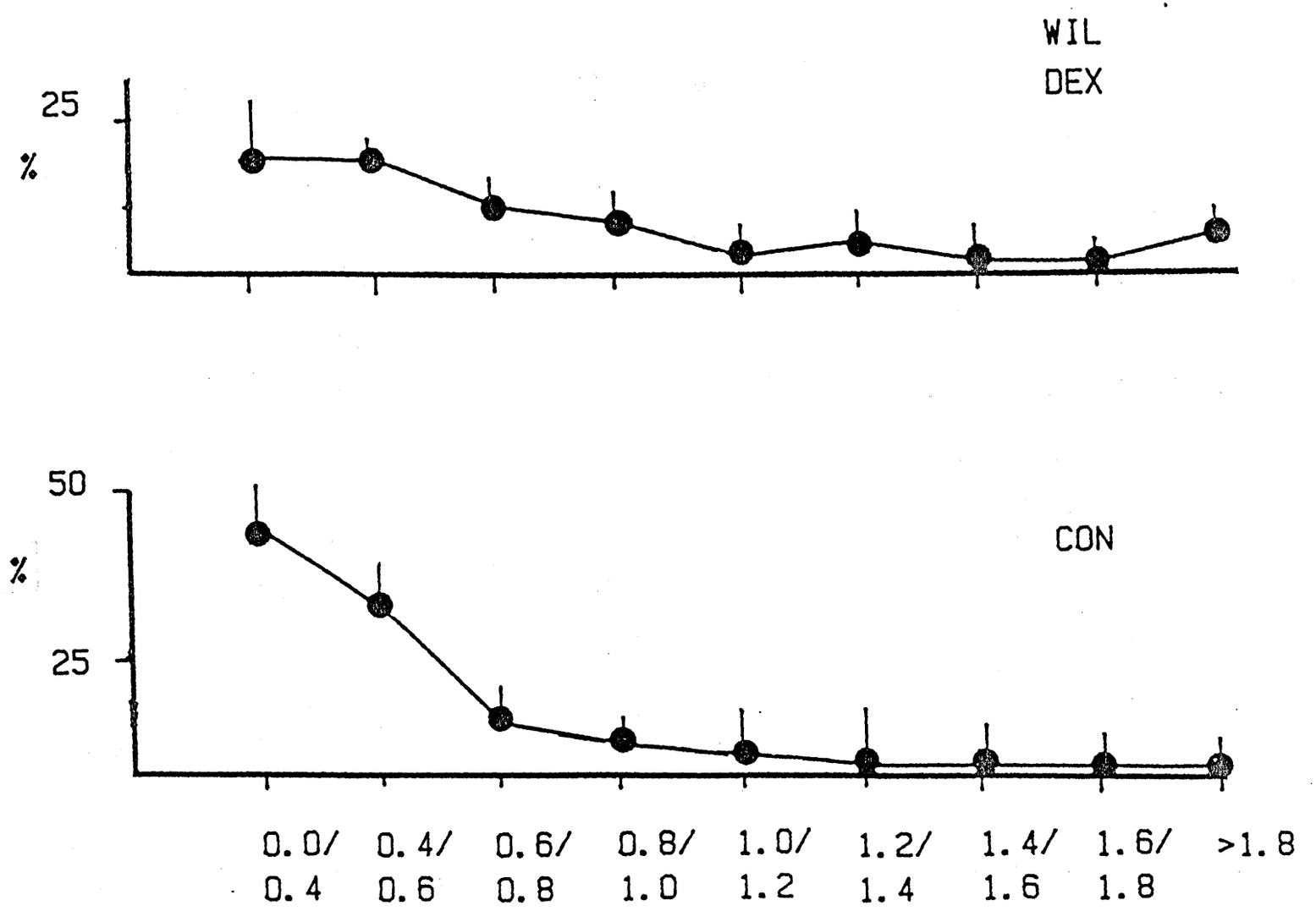
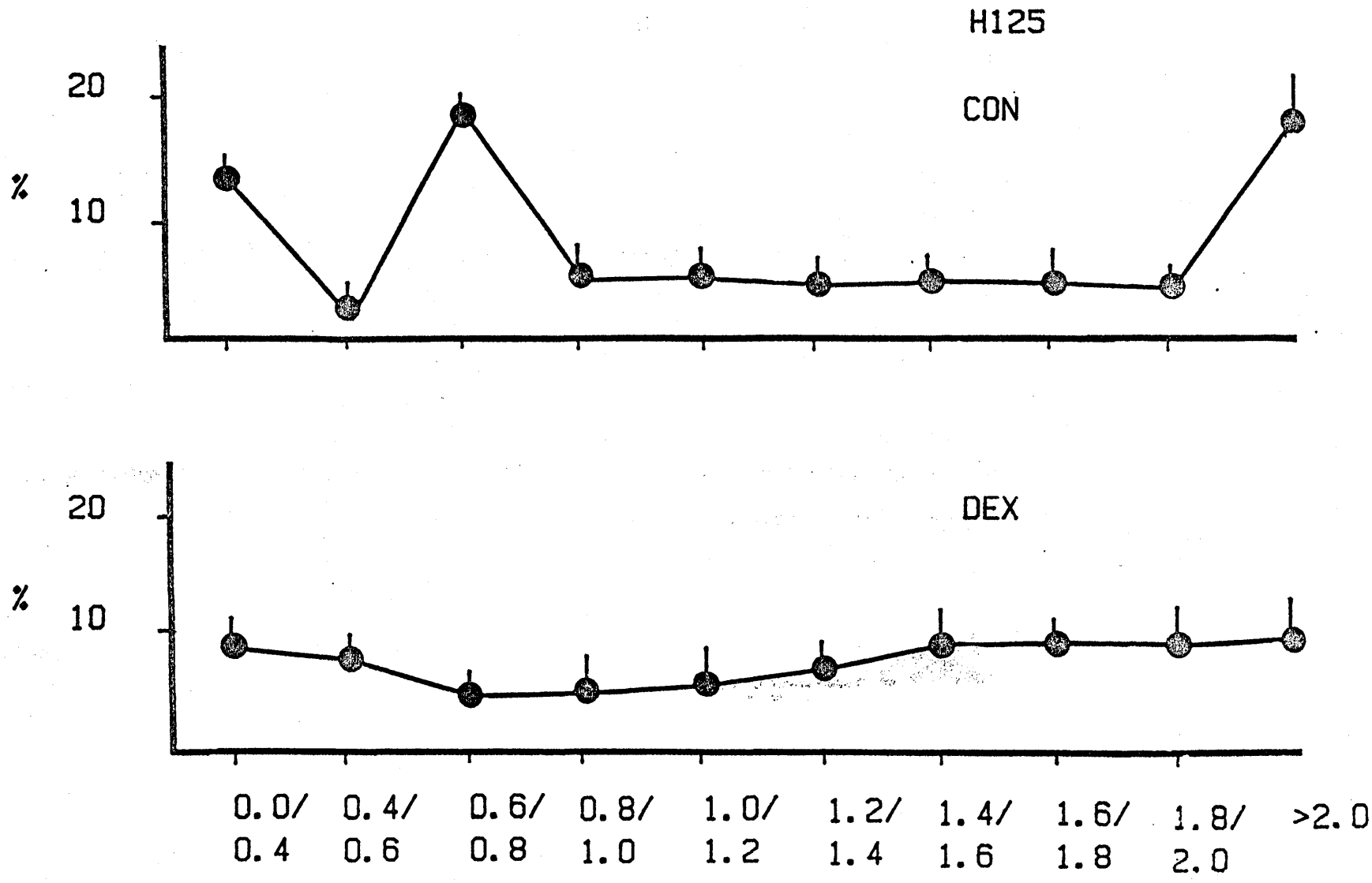

WIL
DEX
25
%
50
%
CON
25
0.0/ 0.4
0.4/ 0.6
0.6/ 0.8
0.8/ 1.0
1.0/ 1.2
1.2/ 1.4
1.4/ 1.6
1.6/ 1.8
>1.8

FIGURE 7 EFFECT OF DEXAMETHASONE UPON GROWTH OF H125 AT CLONAL CELL DENSITIES AFTER 15 DAYS.

Cells were inoculated at 100 cells ml^{-1} (5mls). Ater 15 days cultures were fixed and colonies sized electronically.
Mean $\pm$ s.d (n=3)


H125
CON
20
%
10
20
%
10
DEX
0.0/ 0.4
0.4/ 0.6
0.6/ 0.8
0.8/ 1.0
1.0/ 1.2
1.2/ 1.4
1.4/ 1.6
1.6/ 1.8
1.8/ 2.0
>2.0

These results indicate that at clonal cell densities the effect of dexamethasone on the cell lines was the opposite of that seen at normal cell densities. In summary, at clonal cell densities the proliferation of WIL is increased while that of H125 is decreased.

1.4 Glucocorticoid Receptor Assay

Cells were assayed for glucocorticoid receptor as stated in Methods. All the cell lines were found to have significant amounts of glucocorticoid receptor (Table 3). The levels are very similar to those obtained for lung tissues, both biopsies (19, 20, 21, 27) and cell lines (7). There was no obvious correlation between the effect of dexamethasone and receptor concentration. The unresponsive cell line with respect to proliferation, H23, had measurable glucocorticoid receptor number, as did H125. Overall, besides A549, the cell lines had more or less similar levels of receptor.

1.5 Effect of Dexamethasone on Clonogenecity in Agar

As stated earlier ability to grow in agar correlates with transformation. Thus a reduction in clonogenicity in agar is indicative of a reduction in the expression of the malignant phenotype. Dexamethasone was found to reduce colony formation in all three lines investigated. The results are shown in Figure 8. As can be seen the biggest reduction was achieved with WIL. At a concentration of 26 μM clonogenecity was inhibited by 97%. This inhibition was dose dependent. A most interesting result in these experiments was that with H125. Again a dose dependant reduction was seen.

DISCUSSION

Dexamethasone was found to have varied effects on proliferation. The effect on proliferation depended on cell density and cell type. Interestingly, if a cell's growth rate was reduced by dexamethasone at regular passage density then it was increased at low cell density by the

TABLE 3 GLUCOCORTICOID RECEPTOR CONTENT OF CELL LINES

Cell Line	fmol per Sample	fmol 10^{-6} Cells	fmol mg^{-1} Protein	fmol μg^{-1} DNA	nM K_D
WIL	4772	130	309	2117	1.15
A549	6130	190	703	2797	3.36
H125	1070	44	116	1098	1.0
H23	578	26	123	729	N.D.
SKMES	1736	70	184	3535	0.82

FIGURE 8 EFFECT OF DEXAMETHASONE UPON AGAR CLONING.

Cells (20,000 ml^{-1}, 2mls) were suspended in 1% agar over a 2% base. After 30 days the cultures were examined for colonies containing greater than 16 cells.
Mean $\pm$ s.d (n=6)

RELATIVE CLONING EFFICIENCY
0.01
0.1
1.0
0
10-9
10-5
WIL
A549
H125
DEX G M-1

drug. The converse was found for the cell line H125 which had an increased growht rate and terminal cell density in the presence of dexamethasone. Why is there a difference at clonal compared to normal cell densities? It may be that at clonal cell densities the in vitro system may mimic wound repair i.e. the reformation of a confluent sheet of cells. At or approaching confluence growth rate would be reduced as a confluent sheet of cells is obtained. If dexamethasone has a role in wound repair then the results obtained with WIL are in line with this hypothesis. In the case of H125, the cell has the opposite response to dexamethasone and thus then explains the results seen. There was no correlation between response to glucocorticoid and the presence of glucocorticoid receptor. Receptor was found in significant amounts in all cell lines examined and the levels found were similar to those reported in the literature. Dexamethasone was found to reduce the cloning efficiency in agar of the three cell lines investigated, thus indicating a reduction in malignancy.

As stated earlier dexamethasone reduced the clonogenecity of WIL, A549 and H125 in soft agar. This is interesting since in previous experiments H125 responded in a different fashion to the other cell lines investigated (an increased growth rate and terminal cell density in the presence of dexamethasone). This may be explained by the fact that properties other than transformation affect growth in agar. A simple reduction in growth rate may reduce growth in agar. In the case of H125 this is an attractive proposition since stated earlier (section 1.4) the growth of the cell line is inhibited at low cell densities. The cell lines A549 and WIL have increased growth rate at low cell densities. The possibility that the reduction in clonogenicity is due to tumour cell kill can be discounted since the clonogenicity of these cell lines is not reduced when examined as a monolayer (section 1.2).

2. PLASMINOGEN ACTIVATORS AND NEOPLASIA

INTRODUCTION

Plasminogen activators (PA) are a group of serine proteases found in nearly all tissues and many body fluids. At present the best known function of PA is its involvement in the catalyzation of inactive plasminogen to plasmin, which digests fibrin clots. The PA group of enzymes is divided into 3 types, circulating PA (blood), tissue PA (tPA), and urinary PA (uPA) commonly known as urokinase.

2.1 Elevation of PA Levels and Alteration in PA Type

It is known that PA plays a major role in malignant transformation and the spread of cancer but it is important to remember that PA also has a major role in many non-neoplastic and non-pathological processes (e.g. mammary gland involution involves tPA). Hence it is interesting to note that some normal biological processes may have counterparts in neoplasia e.g. mammary gland involution versus invasion and uterine blood supply versus tumour angiogenesis.

The PA found in tumour cells has a higher than normal amount of urokinase-like activity compared to that of normal tissue. This was ascertained by monitoring the inhibition of PA activity by antibodies to urokinase. By this approach it was found that in human lung tumours greater than 90% of PA activity is of the urokinase-like type compared to 50% in normal tissue (38). Similarly in breast carcinomas 80% of PA activity is of the urokinase like type compared to 62% in normal breast (39). However separation of tumour PA activity into urokinase and non-urokinase like is over simplistic. Medium from a human lung tumour cell line has been reported to have a PA activity associated with a protein of molecular weight 1-2 million daltons which possessed reactivity in all antibodies against urokinase (40). Urokinase has a molecular weight of 70 k daltons. Furthermore 6 different

proteases with PA activity were secreted by MCF-7 breast cancer cells. All but one of these was immunologically related to urokinase (41).

Compared with normal tissues a variety of human cancers show elevated levels of PA. These include carcinoma of colon, lung, breast, cervix, prostate and melanoma (39, 39, 42, 43). Lung tumour explants release 40 times more PA activity into the medium than did normal lung. In contrast tPA extracted with Triton-X-100 was only about 3 times greater than in malignant tissue compared to normal lung (44). This was taken to indicate the importance of secreted PAs in examining differences in activity between normal and malignant cells. In summary, PA of malignant carcinoma is more likely to be urokinase-like and released by the cells.

2.2 The Role of Tumorigenesis, Invasion and Metastasis

Unkeless et al (45) were first to show that cultured chick embryo fibroblasts transformed with Rous Sarcoma Virus (RSV) had increased cell associated PA. It was also proven that the increased PA activity was due to transformation not virus infection per se by using temperature sensitive mutants of RSV. Tumour promoters such as phorbol myristate acetate can also increase levels of PA in both normal and RSV transformed fibroblasts along with morphological changes not associated with a rise in PA (46).

The aforementioned data indicate that transformed cells have elevated PA levels as compared with their normal counterparts. However the most important question is still unanswered. Does PA have a role in tumorigenesis, invasion, or metastasis?

Indirect evidence for these processes does exist. In some transformed cell lines secretion of PA has been shown to correlate with growth of the cells in soft agar (47), their ability to form a tumour in immunosupressed animals

(48) and their increased rates of migration (49). Utilizing tumorigenic and non-tumorogenic melanoma, PA activity was found to correlate with tumour take in mice. A highly tumorigenic clone $B_5$59 if grown in the presence of bromodeoxyuridine (Br dU) lost its ability to grow in mice, had an altered morphological appearance and could no longer digest fibrin. These effects were reversed when Br dU was removed (50). Thus in this system at least PA production was directly related to tumorigenicity. A more comprehensive study with early passage human gliomas and normal glia found that PA production was inversely related to the expression of differentiation (51).

In contrast to these results linking PA with tumorigenicity Nicolson et al (52) found no significant differences in PA activity between B16 melanoma cells with different metastatic potentials.

Using human specimens a relationship has been found between fibrinolysis and intravascular growth in epidermoid lung carcinoma[53]. More recently Markus and co-workers (54) found a significant correlation between tPA levels and local invasion in colonic carcinomas. However the same workers were unable to demonstrate a relationship between PA levels and local invasion in either lung (38) or breast cancer (39).

More direct evidence that PA enzymes are involved in metastasis has come from experiments involving the administration of urokinase to rabbits bearing V2 carcinoma. It was shown that there was increased growth and metastasis of the tumour in the presence of enzyme (55). The most impressive data for the involvement of PA directly in the spread of cancer has come from <u>in vitro</u> work by examining the ability of the human carcinoma HEp3 to cross the chorio-allantoic membrane and metastasis into the chick embryo lung (56). An antibody specific for human urokinase either prevented or strongly inhibited metastasis to the lung but had no effect on tumour growth. The authors concluded that PA is essential during the early stages

of metastasis.

Thus there are a number of reasons to suggest that the PA/plasmin system may be important in tumour invasion. Firstly, as mentioned earlier, PA is involved in normal physiological events associated with invasion, morphogenesis and tissue reconstruction. Secondly due to the catalytic nature of PA and the high level of plasminogen found in extracellular spaces, large amounts of plasmin may be produced locally. Thirdly, PA appears to be able to digest directly components of the basement membrane (57). Furthermore there is strong evidence that collagenases have a role in invasion and metastasis and it has been shown that plasmin activates procollagenase (58).

2.3 Alteration of PA Activity by Chemicals

If PA is involved in neoplasia then an agent that lowers the enzyme's activity may be useful in the treatment of cancer. A reduction in PA would indicate an increase in differentiation. Rifkin examined the suppressive effects of various glucocorticoids on human embryonic lung cells *in vitro*. Inhibition was reversible and the growth of the cells was not affected. Dexamethasone and B-methasone had the most potent effect almost totally abolishing PA activity (59). Dexamethasone regulation of PA was also investigated in tumour and embryonic cell lines. This study indicated that dexamethasone could inhibit, stimulate or have no effect upon PA activity (60). A human lung adenocarcinoma cell line had reduced PA activity when incubated with dexamethasone (61).

The mechanism of dexamethasone inhibition of PA activity has been elucidated with rat hepatoma cells (62). Extracts of dexamethasone treated cells were mixed with extracts of control cells and a drop in expected PA activity was seen indicating that the glucocorticoid induced an inhibitor of protease activity. A dexamethasone-resistant variant produced no inhibitor.

Contrary to the above results and hypothesis, elevated PA has been indicated as a marker of differentiation in normal human bronchal epithelial cells (63). At a concentration of 0.4pM Transforming Growth Factor β (TGF-β) was found to induce differentiation and cause a concomitant increase in PA. However squamous differentiation is a metaplastic event and not strictly "normal" differentiation in the bronchus.

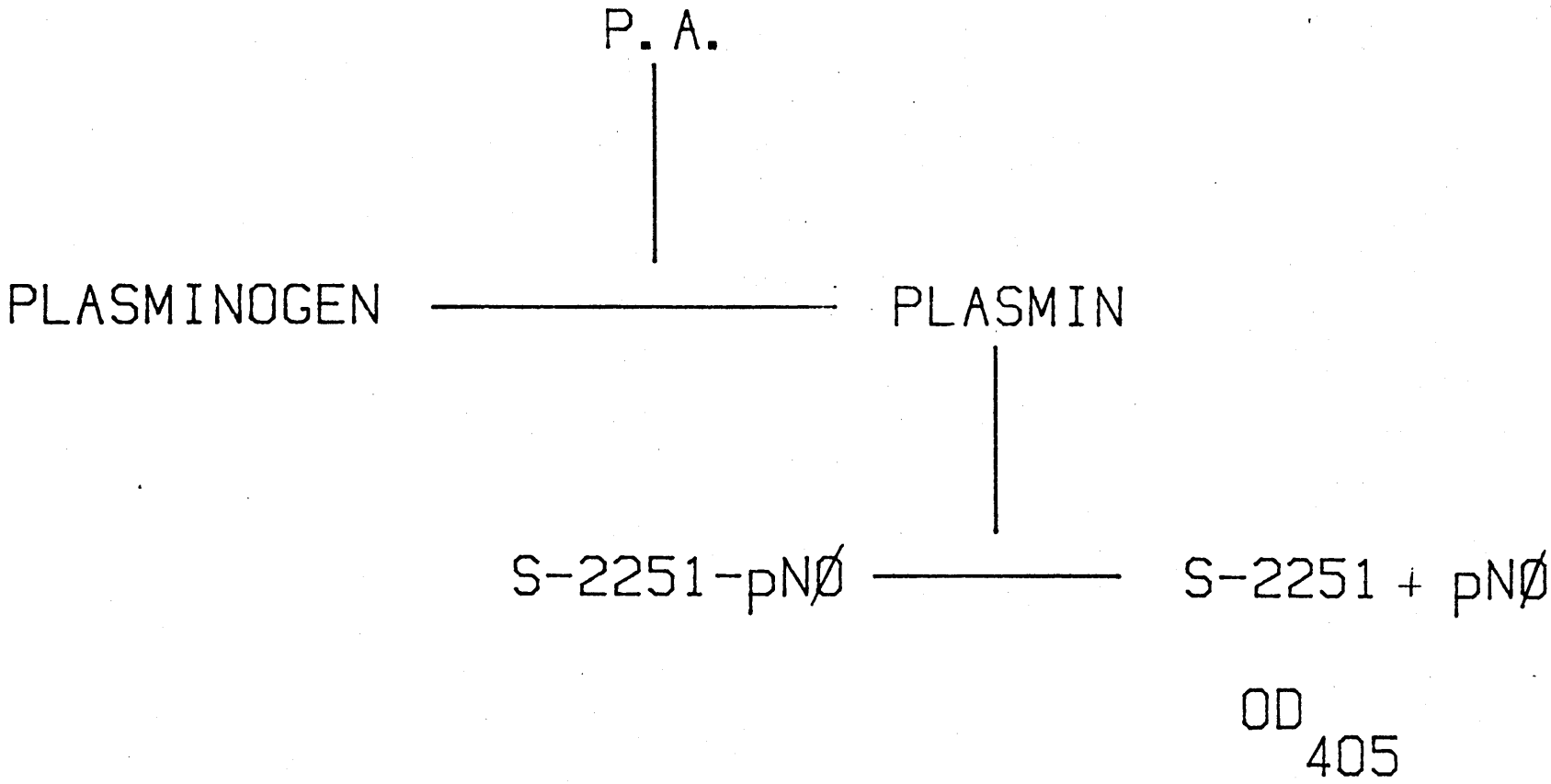
In conclusion, with certain reservations PA activity, particularly soluble uPA, may be regarded as a malignancy-associated marker for many tumours including non-small cell lung carcinoma.

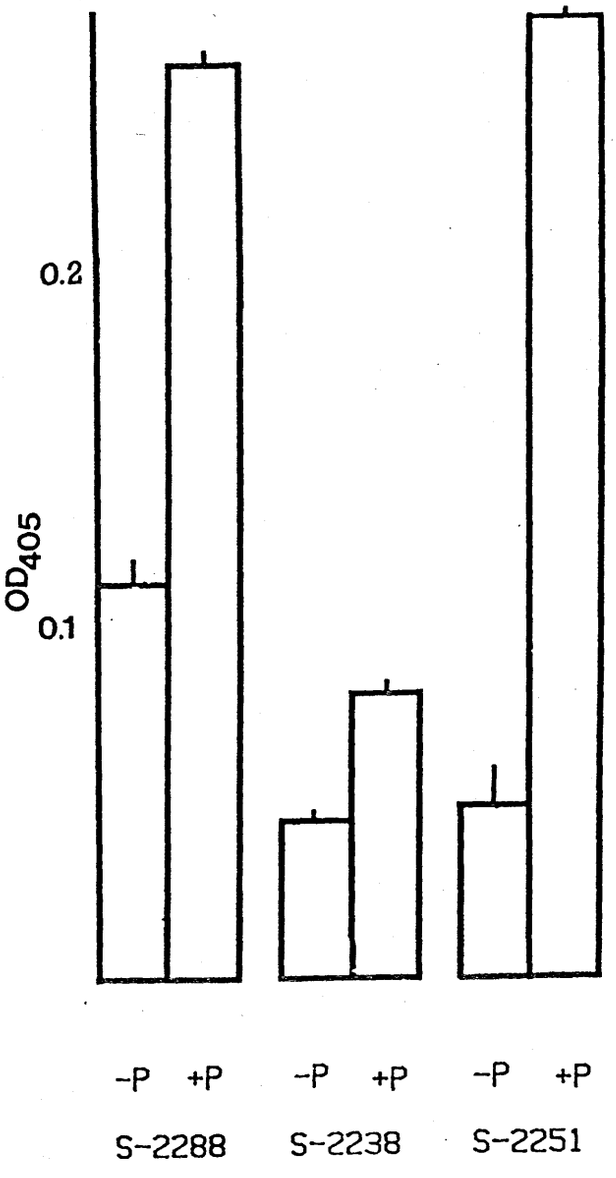
METHODS

2.1 Measurement of Total Plasminogen Activator

A modification of the chromogenic assay developed by Whur et al (64) was used to measure the PA activity. Cells were grown to confluence in 24 well plates before the assay was carried out. The assay is a two step process resulting in an amido-lytic reaction releasing p-nitroaniline from the synthetic chromogenic substrate. Plasmin cleaves the substrate which in the majority of the forthcoming experiments is S-2251, H-D-valyl-L-leucyl-L-lysine-p-nitroaniline dihydrochloride, and the p-nitroaniline released has an absorbance at 405nm. The absorbance at 405nm is then a direct measure of PA. This process is schematically presented in Figure 9. To remove inhibitors within the serum the medium was aspirated and the cells washed twice with Hanks' Balanced Salt Solution (HBSS) without phenol red. Reaction mixture was then added to the cell monolayers. The reaction mixture consisted of 1mM chromogenic substrate, 1C.U. ml-1 of plasminogen, and 0.15 mg ml^{-1} poly-D-lysine. All the reagents were dissolved in HBSS. The assay was terminated after 2 hours with the addition of 5% acetic acid. Two controls are needed one for endogenous PA activity within the reagents and the other for general protease activity associated with the cells. The proteolytic enzyme urokinase, which also activates plasminogen, was used as a standard. PA activities were expressed as Ploug Units 10^{-6} cells (PU 10^{-6} cells). Experiments were carried out to ascertain what was the best substrate to measure PA ac tivity. The results are shown in Figure 10. The substrates S-2288, S-2238, S-2251 were incubated with 0.5 PU ml^{-1} of urokinase for two hours with or without poly-D-lysine (0.15 mg ml^{-1}). As can be seen S-2251 is the best agent although only marginally better than S-2288. From these data it was decided that S-2251 was the best substrate for the cells under study.

FIGURE 9 PLASMINOGEN ACTIVATOR ASSAY.




0.2
0.1
OD_{405}
-P +P -P +P -P +P
S-2288 S-2238 S-2251

The incorporation of poly-D-lysine in the reaction mixture increased the rate of the reaction by almost 5-fold with substrate S-2251.

2.2 Measurement of t-PA Activity

The measurement of t-PA activity was carried out using a commercially available kit. Fibrinogen was added to activate any t-PA present along with acidification of the assay to remove the effects of any inhibitor present. A standard curve was used to measure t-PA activity utilizing the substrate S-2251 (Flow/Kabivitrum).

RESULTS

2.1 Effect of Dexamethasone upon PA Activity

A number of drugs and hormones were tested for their effect on PA activity in the cell line SKMES. The most dramatic reduction was seen with the dexamethasone at a concentration of 26μM (10ug ml^{-1}). To examine time dependent reduction of PA SKMES cells were plated at a density of 10^5 cells cm^{-2} for 24 hours and dexamethasone (26μM) then added and the cells incubated for the appropriate time. Control cells were seeded at the same density at the same time and underwent the same manipulation but received no dexamethasone. The results are shown in Figure 11. Within 25 hours there was a 90% reduction in PA activity in SKMES and by 75 hours this reduction was 95%. Thus the greater part of the reduction was seen within 25 hours of incubation with drug. Reduction of PA in SKMES was dose dependant (Figure 12). Cells were incubated with dexamethasone for 3 days before measuring PA. There was a significant decrease at 26μM, a concentration close to the expected physiological level. On the basis of these results it was decided to use dexamethasone at the maximum non-toxic concentration (26μM) and to have an incubation period of 3 days.

Of the other cell lines tested 4 had reduced PA activities

FIGURE 11 TIME DEPENDENT REDUCTION OF PA ACTIVITY IN SKMES BY DEXAMETHASONE.

Post-confluent cultures of cells were incubated with or without dexamethasone (26μM) for the appropriate time. Results are expressed as a percentage of parallel cultures containing no drug.
Mean $\pm$ s d (n=3)

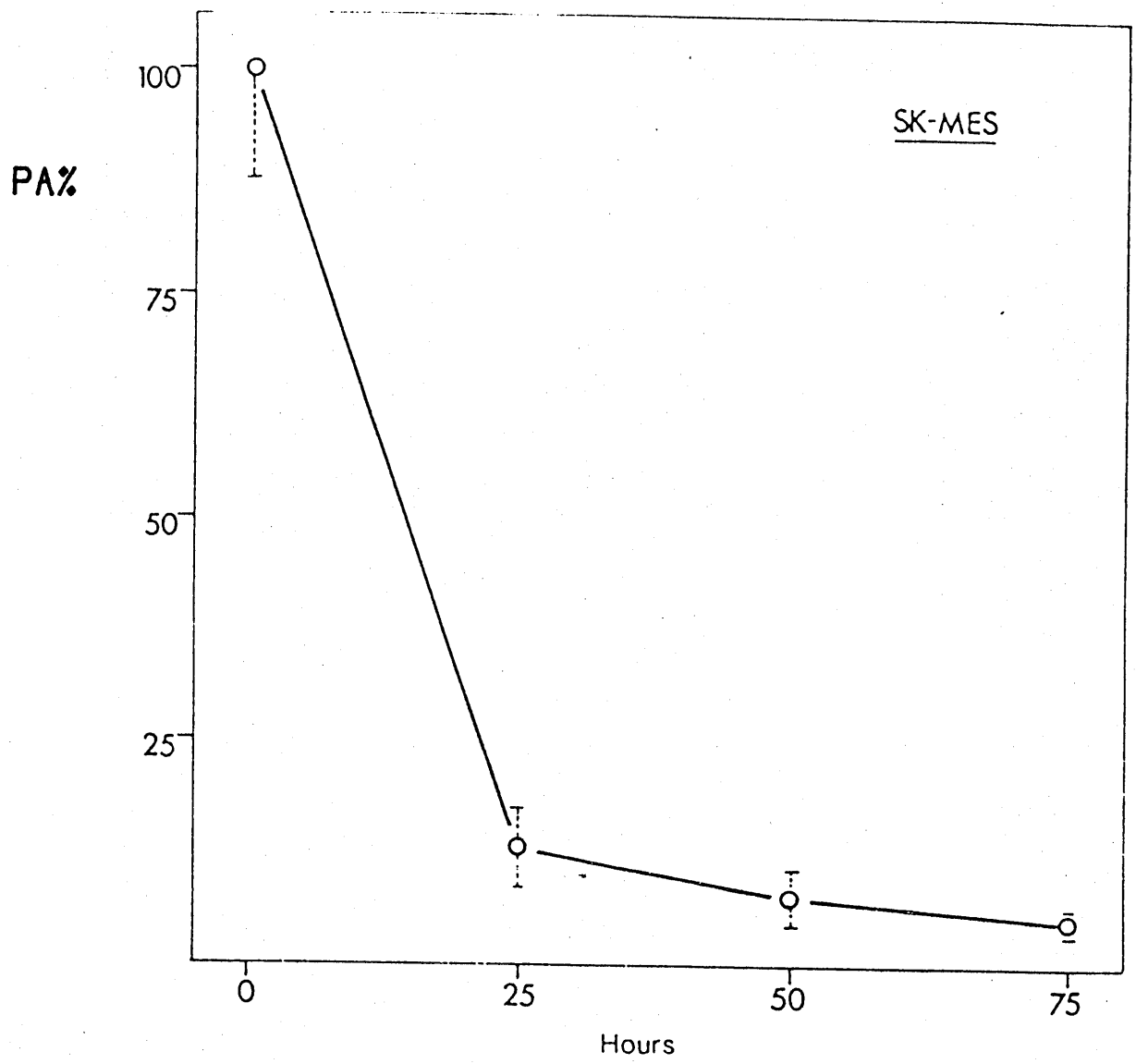
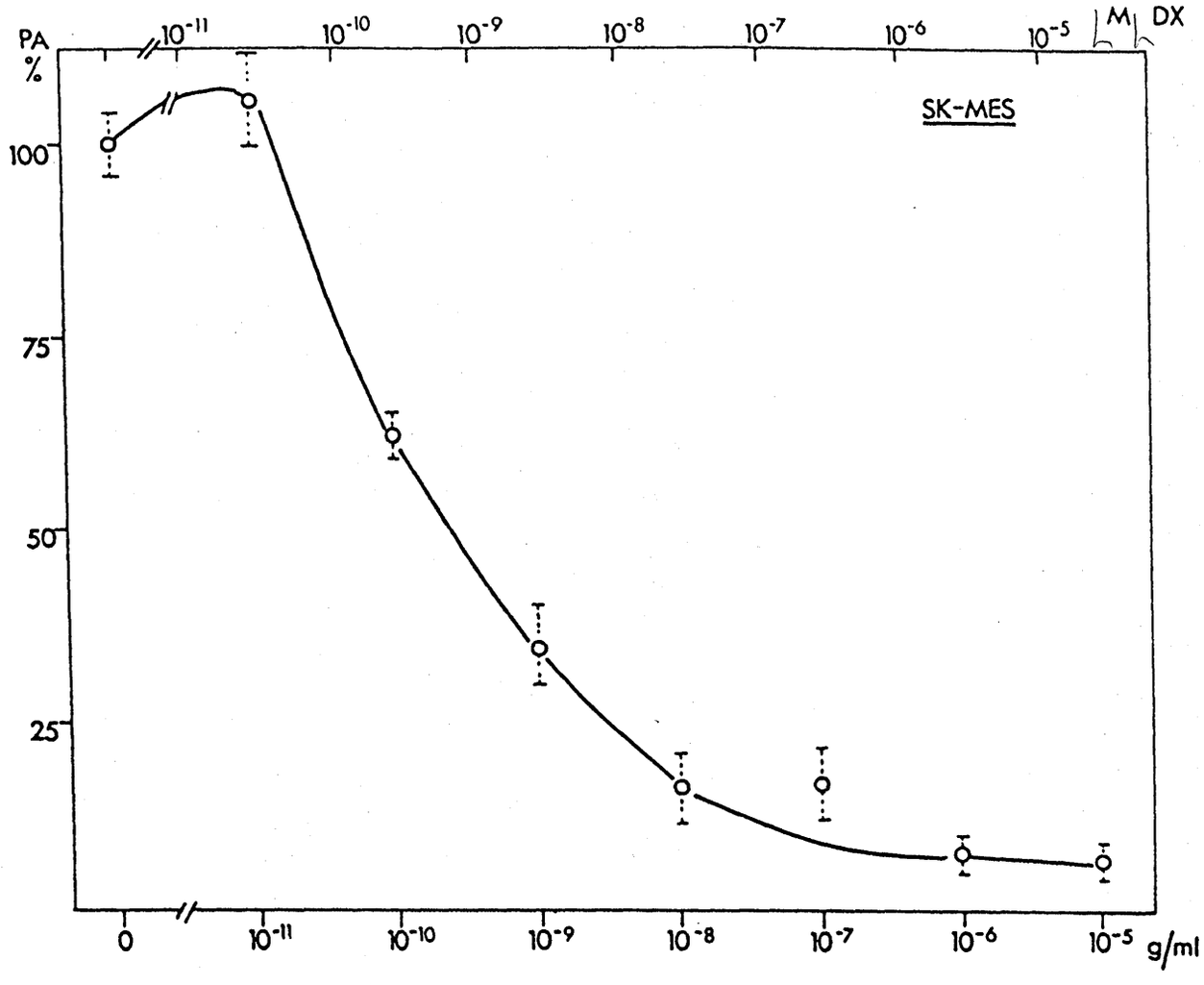

PA%
100
75
50
25
SK-MES
0
25
50
75
Hours

FIGURE 12 DOSE DEPENDANT REDUCTION OF PA ACTIVITY IN SKMES BY DEXAMETHASONE.

Post confluent cultures of SKMES were incubated with varying concentrations of dexamethasone for 3 days and then assayed.
Mean $\pm$ s d (n=3)


PA
%
100
75
50
25
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
g/ml
SK-MES
MDX

when incubated with dexamethasone and this effect was dose dependant (Table 4). However, not all the cell lines sensitive to dexamethasone had the same responsiveness. The cell lines A549 and L-DAN were responsive to dexamethasone to about the same extent as was SKMES, their PA levels being lowered by 98% and 95% respectively. However, the PA activity of WIL cells was only reduced by 75% in the presence of 26μM dexamethasone. H23 had a very low PA activity but this could be lowered by a significant amount. At a concentration of 26μM there was a 69% reduction of PA activity.

Of all the cell lines examined only one H125 did not have its PA activity lowered by dexamethasone (Figure 13). At a concentration of 26μM there was an increase in PA activity but this was not significant.

2.2 Possible Correlation between PA Activity and State of Differentiation

There have been previous reports of an inverse correlation between the state of differentiation of neoplastic tissue and PA levels; the higher PA levels accompanied the more differentiated neoplasms, although the results (Table 5) from this study are from a very small sample. For example H125 a well differentiated adenocarcinoma has the highest PA activity of all the lines examined while the undifferentiated H23 has the lowest PA of all 3 the adenocarcinoma cell lines examined. Thus there seems to be a positive correlation between degree of differentiation and PA activity. Unfortunately there are too few lines of each of the tissue types to allow similar investigations to be made.

2.3 Amount of Activity of the t-PA Type

One cell line WIL was investigated for the presence of t-PA. Using fibrinogen as a stimulator and acidification of the reaction mixture to break down enzyme inhibitor monolayers were investigated for tPA, none was found.

TABLE 4 P A ACTIVITY OF CELL LINES AND EFFECT OF DEXAMETHASONE

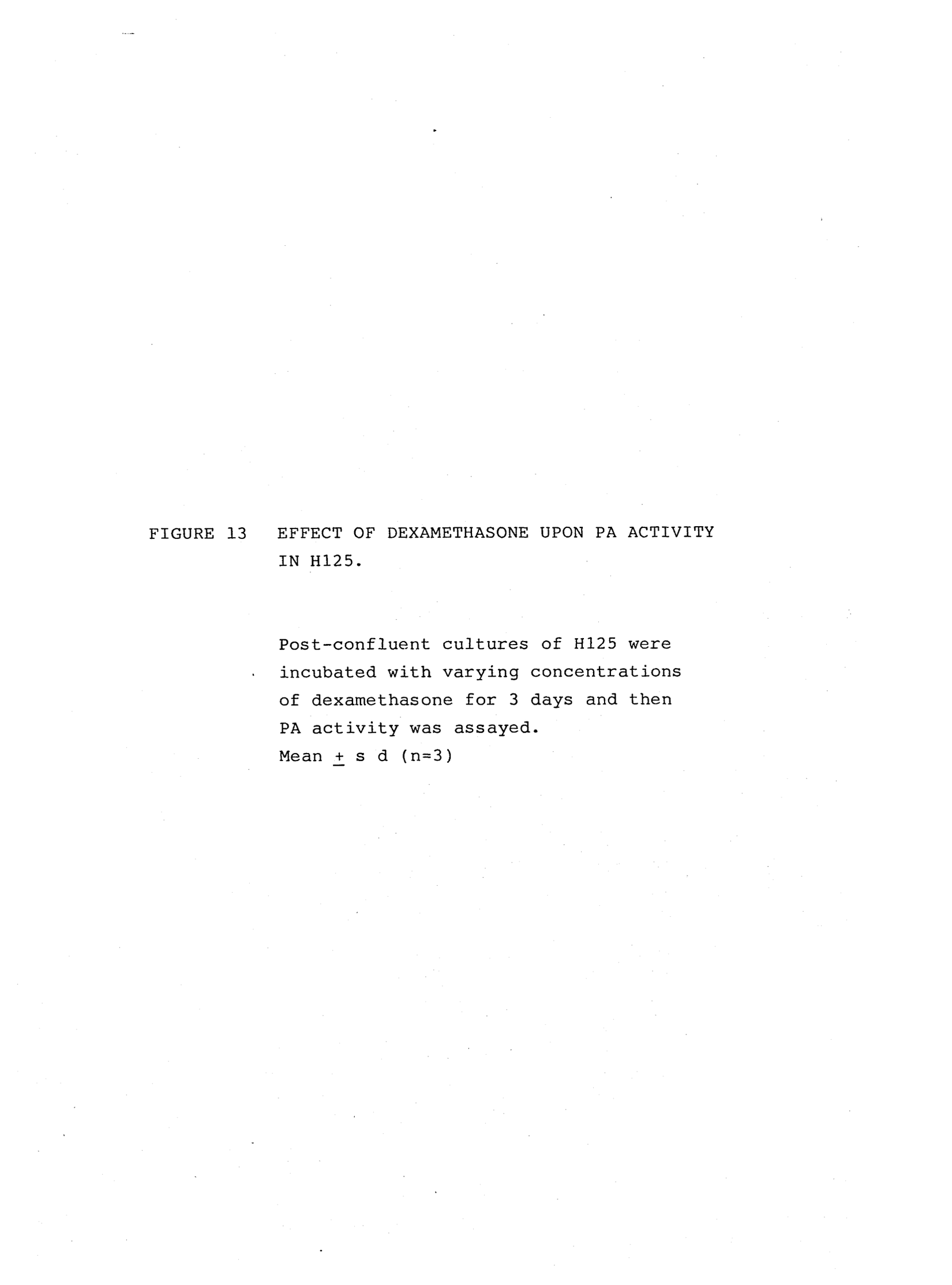
	Activity	% Reduction by Dexamethasone		
	P A Activity			
	P U 10^{-6} Cells	26μM	0.26μM	2.6μM
A549	1.60	98*	95*	63*
L-DAN	1.00	95*	95*	70*
SKMES	1.18	95*	85*	69*
H125	23.8	-48	-10	-10
H23	0.015	69*	81*	33*
WIL	9.32	78*	83*	76*

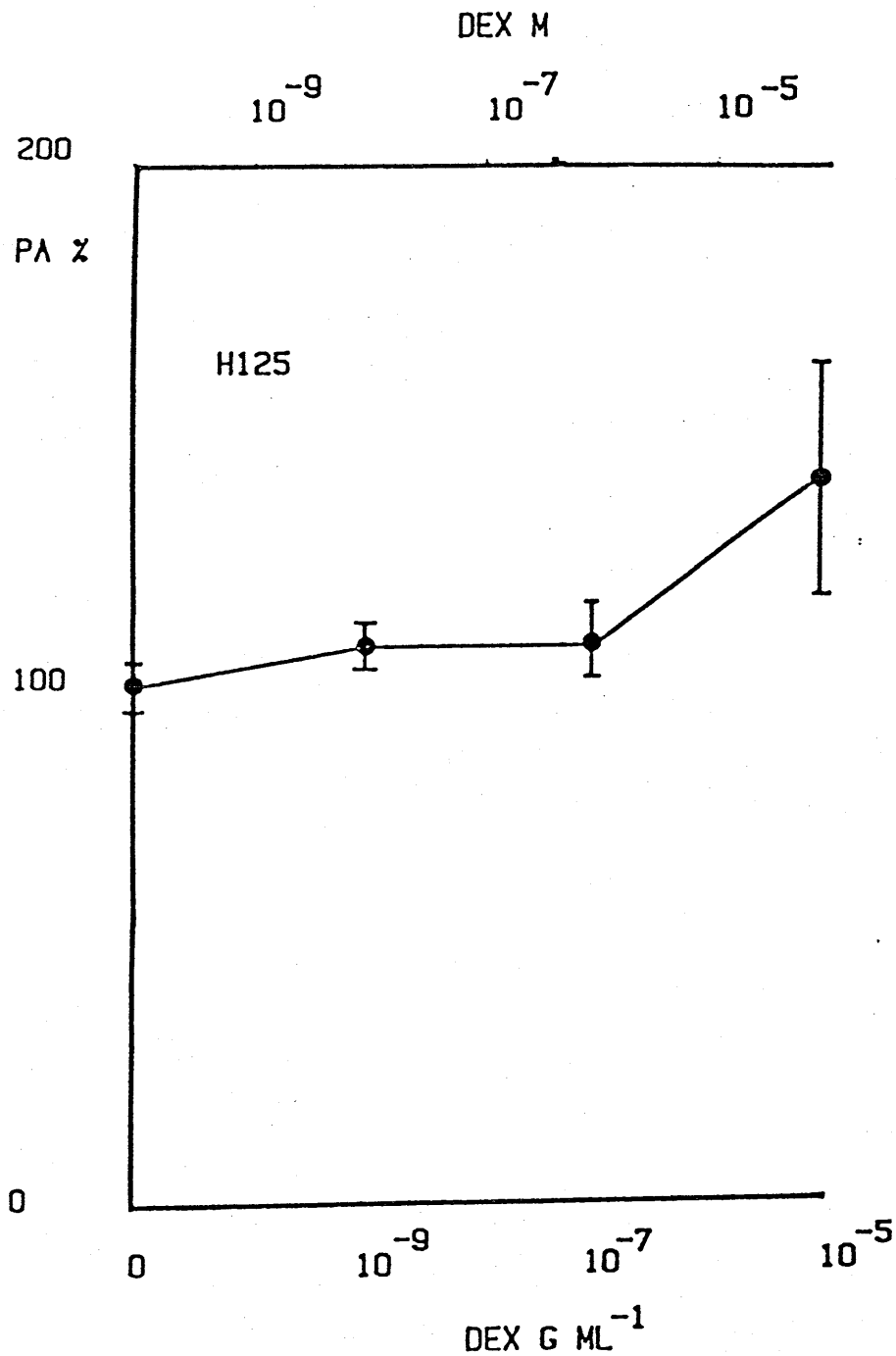
* Significant at 5% Level
(-n) increase in P A

TABLE 5 RELATIONSHIP OF P A ACTIVITY WITH DIFFERENTIATION OF ADENOCARCINOMA CELL LINES

Cell Line	P A Activity P U 10^{-6} Cells	State of Differentiation
H125	23.8	Well differentiated
WIL	9.32	Moderately differentiated
H23	0.015	Poorly differentiated

FIGURE 13 EFFECT OF DEXAMETHASONE UPON PA ACTIVITY IN H125.




DEX M
10 -9
10 -7
10 -5
200
PA %
H125
100
0
0
10 -9
10 -7
10 -5
DEX G ML -1

It may be that there is tPA activity present in WIL but the assay was unable to detect any activity.

DISCUSSION

Dexamethasone was found to reduce significantly the PA activity of 5 out of 6 cell lines investigated. The reduction was found to be time dependent with the majority of the reduction induced by dexamethasone seen in the first 25 hours of addition of drug. Although maximal inhibition was achieved with non-physiological concentrations of glucocorticoid (26μM) there was still a significant reduction with physiological concentrations of glucocorticoid (2.6nM). This indicates that dexamethasone is able, in some cell lines, to reduce significantly the levels of PA, an enzyme often associated with the malignant phenotype. Interestingly the percentage inhibition seen with dexamethasone is higher than usually reported (59, 61) for glucocorticoids, indicating the better efficacy of this reagent with respect to other steroids. This could be due to a number of reasons, e.g., increased affinity for receptor and little metabolism of the drug resulting in repeated binding to the receptor and low inactivation. Dexamethasone is also more stable than other glucocorticoids. One cell line, H125, did not have significantly altered PA levels in the presence of dexamethasone. At higher concentration of dexamethasone (26μM) an increase of about 50% over controls was seen but this was not significant. In the presence of dexamethasone H125 had increased growth rate and terminal density. This is in line with the work of Mira-y-Lopez et al who found a correlation between hydrocortisone induced inhibition of PA activity and reduction of growth *in vivo* with murine mammary tumours (64). However this correlation exists in human non-small cell lung cancer only up to a point. The cell line H23's growth rate is unaltered in the presence of dexamethasone while its PA is significantly reduced (Table 4). But at a general level there does seem to be a relationship between alteration of PA and proliferation by dexamethasone.

3. ANGIOGENESIS

INTRODUCTION

3.1 The Process of Neovascularisation and Its Investigation

For a solid tumour to grow beyond a few millimetres in diameter requires the establishment of an adequate blood supply (65). This process of the ingrowth of new blood vessels is known as angiogenesis and almost certainly tumour induced. Following the establishment of a blood supply a formerly dormant tumour will not only begin to grow but will also have the potential to be disseminated to other sites via the circulatory system (66). In 1971 (67) it was demonstrated that angiogenesis may be induced by a soluble factor or factors produced by the tumour.

A number of experimental systems exist for investigating angiogenesis. The most commonly used are :-

1. A transparent chamber, inserted into the dermis of the rabbit ear, hamster cheek pouch or dorsal skin of mouse (68, 69, 70).
2. The chorioallantoic membrane (CAM) of 9 to 10 day old chick embryos (71).
3. The rabbit cornea (72).

All of the aforementioned assays allow direct observations of angiogenesis. With these assays complete formation of blood vessels is achieved resembling the in vivo response. Although it is possible to measure the rate of growth and count the number of new capillary sprouts by these methods they cannot be considered truly quantitative. Another problem is the possibility of obtaining a false positive response; that is angiogenesis not caused by the test sample but by the carrier (e.g. Millipore filters, slow release polymers) or by mechanical injury via an inflammatory reaction.

Although the overall angiogenic response is complex the reaction of the endothelial cells in angiogenesis can be examined in appropriate experimental systems in vitro. Basically the process of neovascular growth with respect to the endothelial component can be summarised as follows:-

Pre-existing endothelial cells become more permeable and engorged.

Endothelial cells become blebbed, cell junctions loosened and there is a general increase in the number of cytoplasmic organelles. Due to basement membrane disruption endothelial cell processes penetrate into the perivascular tissue. The endothelial cells then migrate in the perivascular tissue stroma towards the source of the stimulus. Division of endothelial cells now occurs. The labelling index of resting endothelial cells is usually about 0.1% but during angiogenic stimulation or embryonic development the labelling index can rise to about 40% measured in animals.

Finally, maturation of capillary sprouts occurs with fibroblastic cells migrating towards the new vessels, a basement membrane is set down and this results in a reduced overall permeability (73). Endothelial cells from various sources have been isolated and cultured e.g. human umbilical veins (74), bovine heart and aorta (75) and bovine adrena cortex (76).

Cultured endothelial cells form at confluence a "cobblestone" monolayer of closely opposed polygonal cells. The quiescent cells within such monolayers are not stimulated to synthesise DNA by the addition of fresh serum or other mitogens; however disruption of the monolayer stimulates proliferation and migration (77).

Endothelial cell division has been monitored by measuring total cell number (75), incorporation of ^{3}H thymidine into the nucleus (78), total amount of DNA present (79) and colony number (80).

It should be noted that although a great number of factors have been shown to stimulate endothelial cell proliferation in vitro, only some of these stimulating factors are angiogenic when examined in vivo. Conversely, other factors are angiogenic when examined in vivo have been reported to have either no effect or inhibit endothelial cell growth in vitro (81, 82), but some of these factors have been found to be stimulatory when examined under different assay conditions (83). Zetter has used capillary endothelial cells in a migration assay to measure angiogenesis (84).

3.2 Tumour Induced Neovascularisation

The proliferation of endothelial cells has been reported to be stimulated by tumour cell conditioned medium (79), co-culture with sarcoma 180 cells (80), Walker 256 carcinoma cell homogenates (75) and semi-purified Walker 256 carcinoma extracts (83).

Since angiogenesis is so important in the establishment and growth of solid tumours it may be a possible target for chemotherapy of neoplasms. An agent that could inhibit angiogenesis may reduce growth and possibly cause regression of tumours without affecting normal tissue.

3.3 Modulation of Neovascularisation

This, as an avenue for chemotherapy, has, and is still being investigated, and results have given some hope for the future. Taylor and Folkman (85) have demonstrated that in vivo angiogenesis can be promoted by heparin and inhibited by protamine, a heparin antagonist. Previously it had been shown that mast cells accumulated at a tumour site before the ingrowth of new capillary sprouts (86) and that these cells release heparin (87). The presence of heparin caused migration of capillary endothelial cells in vitro (88) and also enhanced tumour angiogenesis on the CAM (89).

3.3 cont'd
Protamine inhibited the ability of mast cells or heparin to cause angiogenesis (87) and inhibited tumour angiogenesis in the chick embryo (85).

Using the CAM as an assay, background inflammation can commonly create false positive results and to get around this problem corticosteroid is added to suppress inflammation. When added with heparin (90) it was found that cortisol abolished angiogenesis. During this study it was further shown that the two agents together could inhibit tumour growth and there was evidence that this approach may cause the regression of some established tumours. Heparin and cortisol had no cytostatic effect on the examined tumour cells in vivo or in vitro. Further the inhibitory effect of the drugs was shown to be on growing and not mature blood vessels. However not all tumours investigated responded to heparin/cortisol.

Medoxyprogesterone, dexamethasone and corticosterone have all been found to inhibit neovascularisation and growth of the V-2 carcinoma implanted in the rabbit cornea (91). This was accompanied with a concomitant drop in collagenolytic activity.

METHODS

The bovine pulmonary artery endothelial cell line, CPAE, was used to measure the presence of mitogenic factors by monitoring the effect of tumour cell conditioned medium on proliferation of the endothelial cells. The cells were inoculated at a density of 5×10^4 cm^{-2} in 6 well plates of diameter 35mm. CPAE's were grown in 15% serum and were at confluence for at least 4 days before being used in experiments. Conditioned medium was obtained in the following way. Tumour cells were allowed to reach confluence in 75 cm^{-2} flasks. Cells were then incubated with either medium with 5% serum for control conditioned medium or medium with 5% serum and 26μM dexamethasone (3 days) for conditioned medium from dexamethasone-treated cells.

After this incubation period the medium was aspirated and the monolayers washed twice with medium. At this point fresh medium (5% serum) was added to the cells and incubated for 6 hours. The function of this incubation was to remove dexamethasone that is bound to the receptor and that may through the presence of the drug interfere with the assay. Fresh medium (25mls) containing 5% serum was added for 18 hours. The medium was collected and 4mls added to each well containing CPAEs. After 3 days the cells were trypsinized and counted electronically. Parallel cultures were set up containing 5% serum not conditioned.

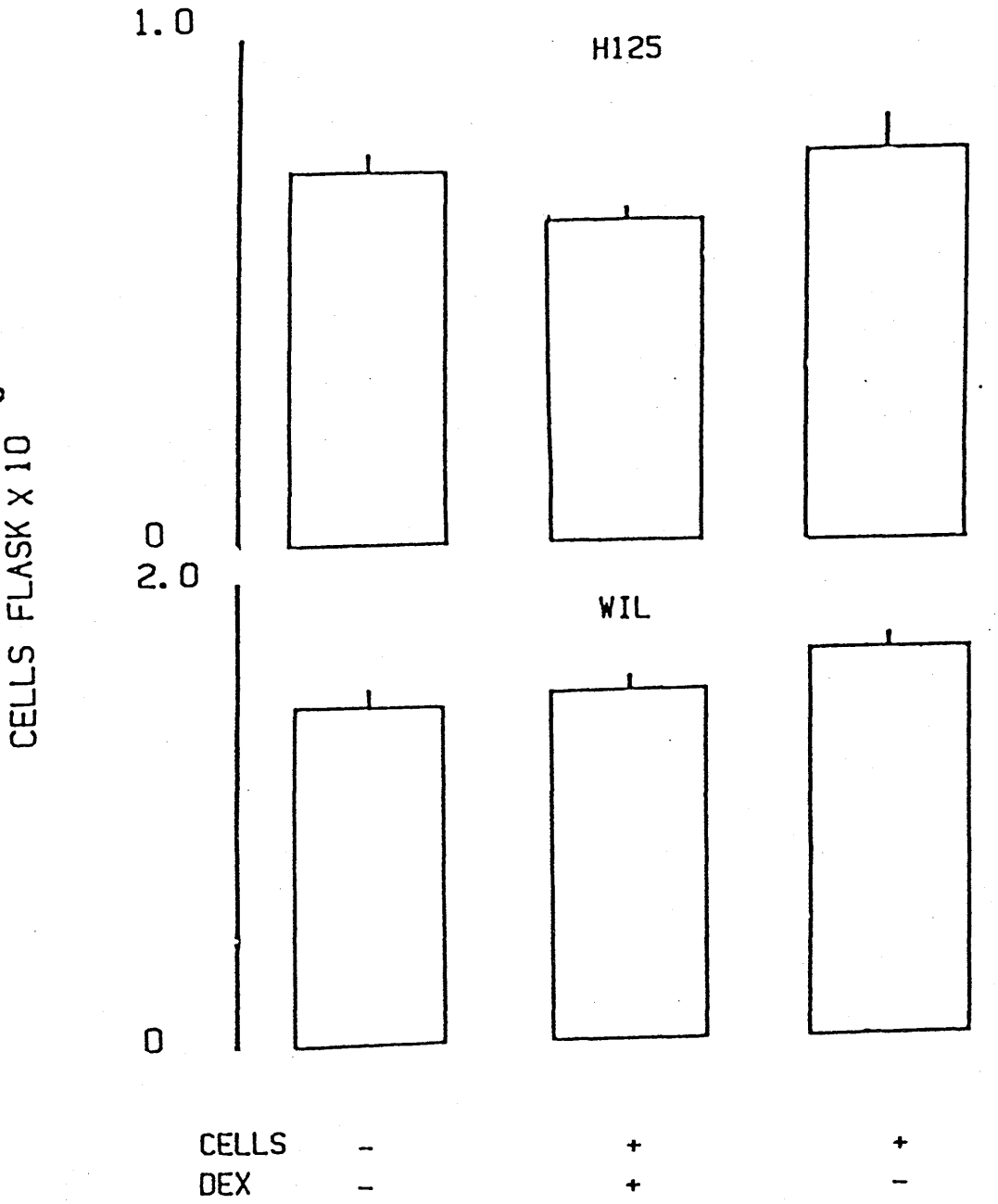
RESULTS

The results for WIL and H125 are shown in Figure 14. As can be seen conditioned medium from WIL cells stimulates the growth of CPAEs significantly with respect to cells incubated with medium not conditioned. This indicates the probable presence of one or more TAFs in the medium collected from WIL cells in culture. Proliferation of cells when incubated with medium from dexamethasone treated cells was significantly reduced compared to medium collected from untreated cells, indicating a possible reduction in secretion of mitogenic factors by WIL in the presence of dexamethasone. However, it is possible that the reduced in proliferation could be due to the presence of dexamethasone that has "leaked off" receptors into the medium and directly reduces the growth rate of CPAE. WIL grows well as a xenograft in nude mice (see Animal Experiments) and thus must be able to induce tumour anigogenesis.

Conditioned medium from the cell line H125 does not significantly increase the proliferation of CPAEs compared to the control medium indicating a lack of mitogenic factors detectable by this system. The line H125 must secrete mitogenic factors since the cell line grows well in nude mice as a solid tumour. Furthermore, there is a significant drop in cell number in cultures incubated with conditioned medium from H125 cells treated with

FIGURE 14 ASSAY OF SECRETED ANGIOGENIC FACTORS BY 2 LUNG CELL LINES.

Angiogenesis was measured by monitoring endothelial mitogenesis as stated in methods.
Mean $\pm$ s d (n=6)


1.0
H125
0
2.0
WIL
0
CELLS FLASK X 10 -6
CELLS - + +
DEX - + -

dexamethasone compared to unconditioned medium in cultures of CPAEs. This can be explained by the presence of dexamethasone in the medium that has been carried over following treatment with drug.

The reduction in proliferation seen with conditioned medium from WIL cells treated with dexamethasone may then be due to the presence of the drug and not totally attributable to a reduction in TAF secretion.

In conclusion this in vitro assay has two flaws. Firstly, the system does not detect all mitogenic factors. The two cell lines WIL and H125 must produce mitogenic factor since they both grow well in nude mice but only the former stimulates endothelial mitogenesis in vitro. Secondly, dexamethasone interferes with the assay and so an assay is needed that is unaffected by dexamethasone directly.

DISCUSSION

Tumour induced angiogenesis is indicated by mitogenesis of pulmonary aortic endothelial cells (CPAE) by conditioned medium from the cell lines A549 and WIL. The conditioned medium from these cells increased the rate of proliferation of cultures of CPAEs. Conditioned medium from cells pre-treated with dexamethasone caused a significantly reduced proliferation with respect to conditioned medium from untreated cells. However the results from experiments carried out with the cell line H125 indicate problems with these studies. Firstly there is evidence of dexamethasone carry-over; that is following treatment of the cells with dexamethasone there is incomplete removal of the drug before collecting conditioned medium. Secondly there does not seem to be any secretion of mitogenic factors secreted by as detected by this model. However the cell line H125 when injected into nude mice grows well as a tumour and thus must secrete angiogenic factors. It is possible however that in vitro these factors are not secreted.

It seems then that another experimental model may be needed to investigate angiogenesis. As stated earlier an assay exists that utilizes capillary endothelial cells (84).

This assay has the added advantage that it is these capillary endothelial cells which are the site of action of the tumour angiogenic factors. Also there is the possibility that migration of these endothelial cells is unaltered by glucocorticoid though this is unlikely. The key to this problem may be to get rid of the dexamethasone. This can be done in a number of ways. Firstly a more readily metabolized analogue of dexamethasone thus reducing the amount of glucocorticoid. Another approach is purification of the mitogenic factor or physical removal of it from the conditioned medium in the latter case by charcoal stripping for example. The theoretical amount of dexamethasone that could be carried over can be easily calculated. The amount of dexamethasone bound per cell is known from receptor assays and if we assume 100% carry-over the calculated dexamethasone concentration is 50nM which would almost certainly affect proliferation.

4. CARBOHYDRATES OF THE TUMOUR CELL SURFACE

INTRODUCTION

Cancer is often described as "a social disease at the cellular level" and if this is so then many of the symptoms may show on the cell surface (91). It now appears that there is a correlation between carbohydrate changes and malignancy. The predominant glycoconjugates are glycoproteins containing 80% of all cell surface located carbohydrate. These carbohydrate units have an asymmetrical distribution on the outer cell surface. The monosaccharides predominantly occurring in membrane glycoconjugates are the neutral sugars; mannose, galactose and fucose, the aminosugars; glucosamine and galactosamine and their acetylated derivatives N-acetylglucosamine and N-acetylgalactosamine including the negatively charged sialic acids (92). The oligosaccharides on the cell surface are placed into two groups depending on their amino-acid viz the N- and O-glycosidic types. The N-glycosidic can be further divided into the so called complex and simple type sugars (Figure 15). Both types contain a common core region and several branches of different types in complex type or mannose in simple type sugars. These sugar components on the cell surface are reported to be involved in specific cellular recognition (93). Processes associated with tumour growth and spread; loss of cell-cell adhesion, cell-cell interactions and the ability of cells to migrate, may be directly attributable to changes in the cell surface carbohydrates.

4.1 Surface Carbohydrates of Transformed Cells

The general observation to be discussed in this review is an alteration in the molecular weight of fucose containing glycopeptides derived from the proteolytic digestion of the cell surface (94). When analysed by gel filtration an overall increase in molecular weight was seen in tumour cells compared to their controls.

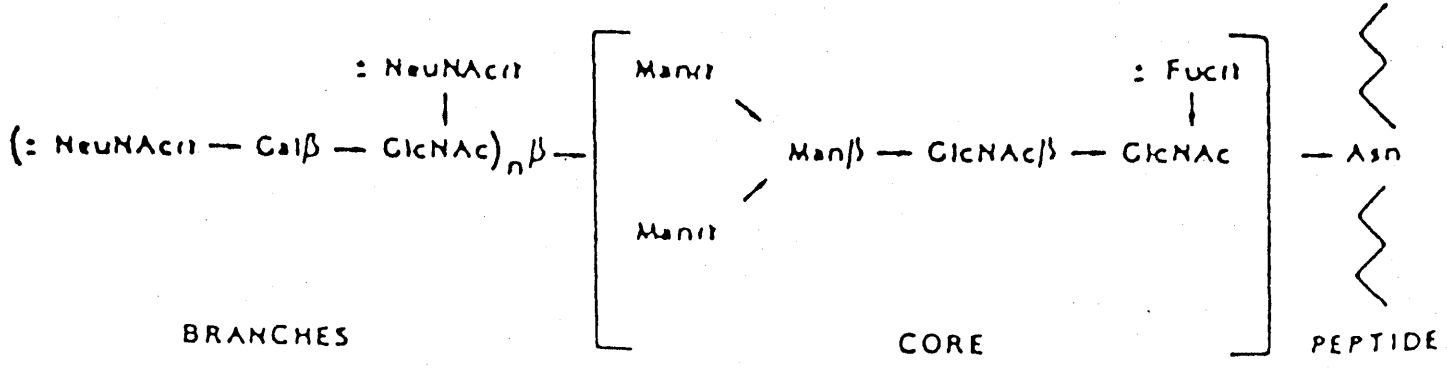


Fig15 COMPLEX CARBOHYDRATE

Accompanying this there is a decrease in binding to the lectin Concanavalin A (Con A). Following treatment with neuraminidase, an enzyme specific for sialic acid residues, the differences between control and tumour glycopeptides were abolished in most cases.

Overall this can be interpreted as an increase in branching at the mannose units of complex oligosaccharides. This increased branching reduces or abolishes mannose specific binding to Con A and augments sialylation sites. In summary there is often the appearance of more large glycopeptides upon tumourigenic transformation.

4.2 Glycosyltransferases and Glycosidases

After the transfer of a high mannose precursor from a lipid intermediate to the nascent protein, protein carbohydrates are synthesized during a complex interplay of several glycosidases and glycosyltransferases (95). Cancer related changes must somewhere be generated along this route. As the increase in apparent molecular weight is mainly detected by an increase in the level of sialylation many attempts have been made to correlate the changes in tumour cells with changes in the activity or specificity of glycosyltransferases, notably of sialyl transferases. Warren et al (96) were the first to report on the increased amount of specific sialyltransferase in transformed as well as dividing cells. Elevated levels of sialyltransferases have subsequently been found in a range of transformed cells (97). However SV 40 transformed 3T3 cells had a decreased sialyltransferase activity (98). Interestingly SV 40 transformed cells are often negative for the more highly sialylated membrane glycoproteins. Overall it can be said that there is an increase in sialyltransferase activity in surface molecules of tumour cells.

4.3 Changes in Individual Membrane Molecules

Although mainly studied in isolates of trypsin sensitive surface molecules, the glycosylation change of transformed cells is a general phenomenon. Glycopeptide alterations associated with transformation of Balb/c cells have been observed in several individual proteins (99). Further, the glycopeptides prepared from fibronectin of transformed cells contain more branches per core and a higher degree of sialylation without, however, affecting the adhesive properties (100). On the other hand no differences have been found in the glycopeptides derived from the transplantation alloantigens of normal and malignant cells (101).

4.4 Effects of Growth and Differentiation

Neoplastic cells often resemble undifferentiated, rapidly growing or embryonic cells in many phenotypic properties. Confluent non-growing, normal, cells contain more complex type surface glycopeptides and fewer oligo-mannosyl type glycopeptides than during active proliferation (102,103). In addition to this difference in amount, the size and distribution of complex type glycopeptides can also be a function of proliferation state. A good example are hamster cells. Growing they have an enlarged quantity of the larger surface glycopeptides compared to the quiescent cultures (104).

Mouse (105) and human (106) teratocarcinoma cells contain a class of very large fucosyl glycopeptides with terminal galactose residues. These very large glycopeptides of embryonal carcinoma cells disappear following induction of differentiation, while the sialylated glycopeptides of the more common size range (107, 108) differ from adult tissue in the same manner as observed when comparing other pairs of normal and malignant cells. Differentiation of mammalian cells can be accompanied by structural changes in fucosylated surface carbohydrates (109).

An interesting case to note is that the (^{3}H)-glucosamine labelled glycopeptide profile of gliomas can be made similar to normal glia by treatment of the tumour cells with dexamethasone which is a putative inducer of differentiation in this system. Treatment of trypsinates with neuraminidase abolishes this difference between tumour and normal cells implying that sialic acid is responsible (110).

4.5 Genetic Studies

It is known that cancer is caused at the molecular level by changes in the cellular DNA so changes in the surface carbohydrate sialylation may be related to changes in genomic rearrangement or gene expression. Transformation by oncogenic viruses usually appears as a single-step process by an *en bloc* transfer of the relevant information (111). Several studies with temperature sensitive mutants of transforming viruses has revealed that expression of the viral genome is required for the emergence of tumour associated carbohydrate changes (112). The entire viral genome need not however always be used in the investigation of transformation. De Leij et al (113) studied the carbohydrate changes following the transfection of rat kidney cells with restriction enzyme fragments of adenovirus 12 DNA. Transfection with a small left hand fragment resulted in immortalized and morphologically transformed but not tumourigenic cell lines whereas the entire E1 region was needed for the manifestation of a tumourigenic phenotype expressing characteristic changes in surface glycopeptides. Transfection of mouse 3T3 cells with DNA isolated from neuroblastoma cells resulted in tumourigenic clones expressing more highly branched glycopeptides (114). Also Collard and co-workers (115) transfected 3T3 cells with DNA from T24 bladder carcinoma and from HL-60 leukemia cells. Again a more highly branched structure was found and the DNA responsible for these changes was shown to be the *ras* oncogene, the continuous presence of which was required to maintain tumourigenic potential.

This evidence suggests that tumour specific carbohydrate changes are caused by activation of the ras gene either directly or indirectly. This possibility is attractive for a number of reasons. Firstly, ras genes are analogous to the E1B region of adenovirus 12 in the malignant transformation of rat kidney cells. Moreover activation of ras oncogenes is increasingly observed in a wide range of tumours of different histogenic origins.

4.6 Biological Consequences of Altered Carbohydrate Changes

The dissemination of tumour cells into distant organs is a complex multi-step process involving release from the primary tumour, invasion into blood vessels or lymphatics, transport and survival of blood-borne tumour cells, extravasation from the capillaries and infiltration of the target tissue (116). Surface molecules are almost definitely involved in these processes. For instance Finne et al (117) reported that in poorly metastasising clones from mouse melanoma the amount of sialic acid in complex type carbohydrates was decreased with a concomitant increase in the amount of fucose residues while revertants of this phenotype were highly metastatic. On the other hand Steck et al were unable to detect alterations in surface glycoproteins of high and low metastatic cell lines of a rat mammary carcinoma (118). The answer to these conflicting reports may come from studies investigating single aspects of metastasis. One such study has been to examine the role of sialic acid in attachment to substrate glycoproteins by metastatic and non-metastatic murine tumours (119). The amount of neuraminidase accessible sialic acid was reduced on the surface of the non-metastatic clone compared to the highly metastic clone. Furthermore, this reduction in sialic acid was accompanied with a reduction in ability to attach to fibronectin and type IV collagen but not laminin, compared to the metastatic clone.

Neuraminidase treatment of the metastatic cells enhanced their attachment to type IV collagen but not to laminin. Neuraminidase had no effect on the ability of the non-metastatic cell to attach to any of the substrates.

In conclusion alterations in the carbohydrate may be responsible for directly causing the appearance of malignancy associated properties which are essential for the progression of tumours.

METHODS

4.1 Affinity Chromatography

Affinity chromatography is a powerful biochemical tool that utilises the inherent specificity of biological systems. A ligand is covalently attached usually by a linker molecule to an inert support, most often Sepharose. In this case the lectin Con A which has a specificity for mannose residues was used.

In this study cells were treated with dexamethasone (26μM) for 3 days and then labelled with ^{3}H-L-6 fucose (27 Ci mmol^{-1}) for 16 hours in the presence of the drug. The medium was then collected, the monolayers washed with PBS and 10 ug ml^{-1} of pronase in PBS added to the cells. After 30 minutes the cells were removed and centrifuged. Fresh pronase was added daily to the pronase removed components for 3 days. Samples were then centrifuged in a Beckman Microfuge for 10 minutes. The supernatant was decanted and used in studies in this form or frozen at -70 ^{o}C till required.

About 2000cpm of glycopeptide was applied to a column of Con A Sepharose (0.9 x 15 cm) using 0.01M Tris pH 7.5 with 0.1M NaCl as the buffer. The specific eluent was 0.2M α-methylmannoside and 1% SDS was used to remove any radioactivity. Fractions of 2 mls were collected for scintillation counting.

Further characterisation of the glycopeptides was achieved by sequential digestion of the carbohydrate chains. Labelled glycopeptides were treated as by Muramatsu et al (120) but with the following alterations. Neuraminidase (C.perfrinigens) was added to about 100 µl of sample, after the pH of the sample was reduced to 6.1 giving a final concentration of 0.05 U ml^{-1}. After 2 hours at 37°C the samples were heated at 100°C for 5 minutes to stop any further activity. The reaction mixture was allowed to cool and 0.28 U ml^{-1} of β-galactosidase (Jack bean) and 0.77 U ml^{-1} of β-N-acetylglucosaminidase (Jack bean) were added and the incubation allowed to continue at 37°C for 40 hours. A small amount of toluene was added to stop bacterial contamination.

4.2 Gel Exclusion Chromatography

High performance liquid chromatography was used to investigate the molecular weights of the glycopeptides. TSK SW columns were chosen. TSK G2000 SW has a working range of 70 to 5 kilodaltons while TSK G3000 SW operates between 150 and 15 kilodaltons. The columns were calibrated with protein molecular weight standards. The columns (0.75 x 30cm) were used with PBS as the buffer. The flow rate was 0.2 ml min^{-1} and 0.2ml fractions were collected. A constant flow rate was maintained, and when detecting compounds spectrophotometrically an Altex flow cell coupled to a Hitachi spectrophotometer was incorporated into the system.

4.3 Lectin Mediated Agglutination

Cells were grown with or without dexamethasone for 3 days before harvesting for assay. This was done using EDTA (1mM) since trypsinization would remove cell surface glycoproteins. Cells were washed with EDTA for 30 seconds and then fresh EDTA was added for 15 minutes or till the cells detached.

The cells were then washed with PBS twice and adjusted to 10^{-6} cells ml^{-1}. Greater than 95% of the cells were judged to be viable by trypan blue exclusion. Lectin (0.1ml) was added to give final concentrations between 25 to 0.39 $\mu g ml^{-1}$. Agglutination was observed and ranked from ++++ to 0 by the author and an independant observor, ++++ being maximal agglutination and 0 the level of agglutination observed in controls.

RESULTS

4.1 Affinity Chromatography

Affinity chromatography gave complex results. The cell line WIL's glycopeptides had a decreased binding capacity for Con A Sepharose when treated with dexamethasone from 49% to 21% while the binding of glycopeptides from dexamethasone treated A549 was increased from 32% to 37%. In the latter case the percentage increase is quite small and thus may not be significant. These changes could be due to an increase in sialylation in the case of WIL and a decrease in the case of A549. The percentage of glycopeptides from H125 bound to the affinity column was not altered when the cells were treated with dexamethasone. These results are summarised in Table 6.

Sequential treatment with the enzymes neuraminidase, β-galactosidase, and β-N-acetylglucosaminidase caused an increase in binding to the Con A Sepharose thus indicating the fucose labelled carbohydrate was of the complex type in all the cell lines investigated.

4.2 Gel Exclusion Chromatography

4.2.1 Proof for Pure Carbohydrate Content of Fucose Labelled Species

It had to be insured that the pronase digestion caused the carbohydrate chain to be cleaved from the protein portion.

TABLE 6 BINDING OF FUCOSYLATED GLYCOPEPTIDES TO IMMOBILISED CON-A SEPHAROSE (% BOUND)

	WIL	A549	H125
CON	49	32	17
DEX	21	37	19

% bound is the percentage of radio activity released from the column following elution with α methyl mannoside (0.2M) and 1% SDS.

To examine this a sample of the glycopeptide was added to chloroform:methanol (2:1; v/v) vortexed and the phases allowed to separate. Any carbohydrate attached to protein will be present in the chloroform phase. To 1ml of PBS 50 µl of sample was added and this mixed with chloroform. The two phases chloroform and methanol/PBS were added to scintillation fluid and counted. Typical results are shown in Table 7. As can be seen there are no counts above background in the organic phase.

4.2.2 Gel Exclusion Chromatography Studies

Differences between the carbohydrates of normal and transformed cells can be monitored by gel exclusion chromatography (see introduction). As previously stated there is increased sialylation in transformed cells with respect to normal and this is manifested by an increase in molecular weight that this difference is abolished on treatment with neuraminidase.

Figure 16 shows the profiles of labelled glycopeptides of A549 generated from cells grown with or without dexamethasone. From the profiles it is obvious that there was no difference between the molecular weights of control and treated cell glycopeptides. Similar results were seen with WIL glycopeptides.

There is no evidence here to support dexamethasone treated cells having altered sugar components attached to cell surface components. It is unlikely that any changes in the carbohydrate were missed due to a lack of column resolution, since resolution is already seen on the column and the working range of the column can handle the probable molecular weight range of the glycopeptides. Furthermore the possibility that it is due to incomplete protein removal is dismissed after the results from the previous section.

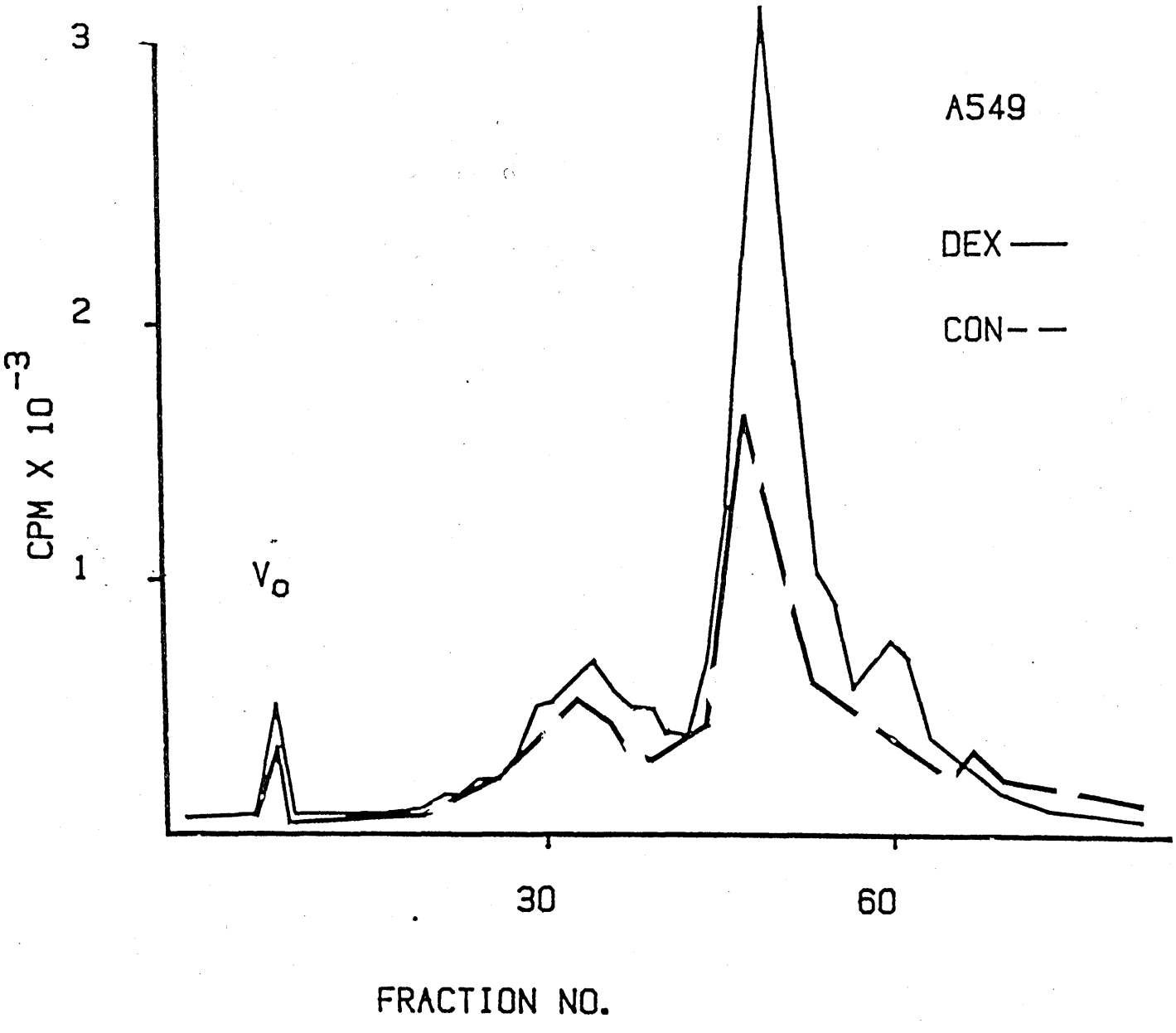
TABLE 7 AMOUNT OF (^{3}H) FUCOSE LABELLED GLYCOPEPTIDE IN EACH PHASE

		(^{3}H) CPM PER PHASE	
		CHLOROFORM	PBS/METHANOL
A549	CON	37	1071
	DEX	31	3187
WIL	CON	26	1116
	DEX	20	3331

A sample of (50µl) of labelled glycopeptides was added to 1ml of PBS. To this chloroform: methanol (2/1:v/v) was added and the mixture vortexed. After being allowed to separate each phase was counted for radioactivity.

FIGURE 16 EFFECT OF DEXAMETHASONE UPON ELUTION PROFILES OF A549 CELL SURFACE PRONASE DIGESTS.

Aliquots of pronase digests were applied to a TSK-G2000SW column. The flow rate was 0.5 ml min^{-1} and 0.5 ml fractions were collected and counted in a scintillation counter.


3
2
1
CPM X 10^{-3}
V_o
A549
DEX ——
CON- -
30
60
FRACTION NO.

4.3 Agglutination of Cells by Plant Lectins

An easy and quick method of examining cell surface carbohydrates is to monitor lectin induced cell agglutination. The results of the cell agglutination experiments are shown in Table 8. Agglutination by Con A was inhibited by α-methyl-mannoside and that by the wheat germ lectin WGA by N-D-glucosamine.

As can be seen dexamethasone had the greatest effect on WIL decreasing agglutination by WGA and Con A markedly. A549 was affected in a converse manner, dexamethasone increasing Con A agglutination albeit only slightly. Agglutination by WGA was unaffected by dexamethasone treatment. The cell line H125 did not have changed agglutination characteristics when grown with the drug.

4.4 Effect of Dexamethasone Upon ^{3}H Fucose Incorporation into Pronase Sensitive Fraction

Incubation of dexamethasone with A549 and WIL caused an increase in the ^{3}H fucose incorporation into the pronase sensitive fraction. However with H125 a significant decrease was observed. The results for A549 and H125 are shown in Table 9.

DISCUSSION

Dexamethasone was found to have different effects on the carbohydrates of the three cell lines WIL, A549 and H125. The results for the whole section are summarised in Table 10. In the case of A549 the percentage of label that bound to Con A Sepharose was increased indicating a probable reduction in sialylation. Conversely with WIL there seems to be an increase in sialylation and with H125 the percentage of label bound to the column was not altered.

TABLE 8 AGGLUTINATION OF H125, A549 AND WIL CELL LINES BY WHEAT GERM AND CONCANAVALIN A LECTINS

	Lectin Concentration ($\mu g\ ml^{-1}$)	W.C.A.		Con A	
		Dex	Con	Dex	Con
H125	25.0	+	+	++	++
	12.5	+	+	++	++
	6.25	-	-	+	+
	3.13	-	-	-	-
	1.56	-	-	-	-
	0.78	-	-	-	-
	0.39	-	-	-	-
	0	-	-	-	-
A549	25.0	++++	++++	++++	+++
	12.5	++	++	++++	++
	6.25	-	-	+++	++
	3.13	-	-	+	+
	1.56	-	-	+	-
	0.78	-	-	-	-
	0.39	-	-	-	-
	0	-	-	-	-
WIL	25.0	++	+++	+	+++
	12.5	+	++	+	+++
	6.25	-	+	-	+++
	3.13	-	-	-	+
	1.56	-	-	-	-
	0.78	-	-	-	-
	0.39	-	-	-	-
	0	-	-	-	-

TABLE 9 EFFECT OF DEXAMETHASONE UPON (^{3}H) FUCOSE INCORPORATION INTO PRONASE SENSITIVE FRACTION

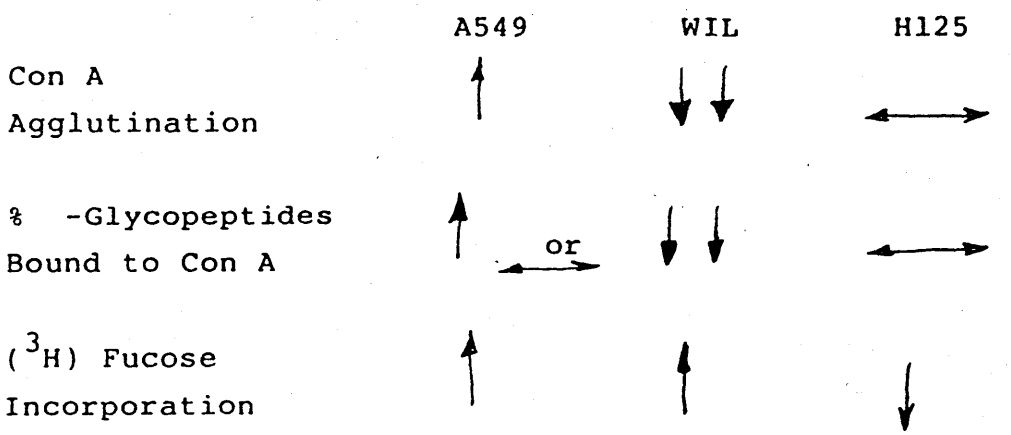
		CPM X 10^{-6} CELLS	
H125	DEX	8532 $\pm$	1988 *
	CON	17377 $\pm$	5887
A549	DEX	66366 $\pm$	13665 *
	CON	19504 $\pm$	2960

Results are mean $\pm$ s.d (n = 4)

Cells were labelled with (^{3}H) fucose 5uCi ml^{-1} for 16 hours. The pronase sensitive fraction was prepared as in methods.

* Significant at the 5% level

TABLE 10 SUMMARY OF EFFECTS MEDIATED BY DEXAMETHASONE RELATIVE TO CONTROLS ON CELL SURFACE



The data obtained from the agglutination studies agree with that from affinity chromatography experiments. However no alterations in the size of the glycopeptides were seen on gel exclusion chromatography. As stated earlier it is unlikely that this is due to a lack of column resolution since three definite peaks are seen for example with A549 glycopeptides. A reasonable answer may be that the glycopeptides may be so large that the differences in molecular weight between control and dexamethasone glycopeptides is negligible. The effect of dexamethasone on the amount of fucose labelled material in the pronase sensitive fraction is increased in the case of WIL and A549 but decreased in the case of H125. This agrees with other data in which the effect of the glucocorticoid was different between H125 and the other cell lines. These results are at odds with those obtained using lung tumour biopsy specimens and normal adjacent tissue in which it was found that in general the tumour had an increase in the amount of sialic acid, galactose, glucosamine and mannose (121). The increase in malignancy associated carbohydrate changes in the case of WIL, which is the opposite of that seen in other parts of this thesis, may be explained by the fact that the cell lines normal function is secretory and the changes seen are related to this function. Overall the results from investigations into tumour cell surface protein bound carbohydrates are in agreement with other findings in this thesis.

INTRODUCTION

5.1 Glycosaminoglycans

Glycosaminoglycans, GAGs, formerly known as mucopolysaccharides, are long, unbranched polysaccharide chains composed of repeating disaccharide units. They are known as GAGs because on of the two repeating units is always an amino-sugar. GAGs are highly negatively charged due to the presence of sulphate or carboxyl groups or both on many of the sugar residues (Figure 17). Seven groups of GAGs are known. They are chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate, heparan sulphate and keratan sulphate. The last member of the family is hyaluronic acid which is not sulphated (Table 11). Hyaluronic acid exists as a single chain of several thousand sugar residues in a regularly repeating sequence of disaccharide units (Figure 18). However hyaluronic acid is not typical of GAGs. Firstly, the others tend to contain a number of different disaccharide units arranged in a more complex manner. Secondly, the others have very much shorter chains, consisting of fewer than 300 sugar residues. Third, all of the other GAGs are covalently attached to protein to form proteoglycans. In lung mucus, GAGs, are involved in pulmonary defence. The viscous substance traps and dissolves pollutants and particles entrapped in the air passages. Ciliated epithelial cells move the entrapped species to the larynx where it is removed by swallowing

5.2 Modulation of Glycosaminocan Synthesis

A number of studies have investigated the factors which control lung GAG synthesis. Wu, Nolan and Hunter indicated the importance of both vitamin A and a collagen substratum for the expression of the fully differentiated properties of hamster tracheal epithelial cells (122). Electron micrographs found functional cilia and biochemical studies indicated that mucin was synthesised and secreted.

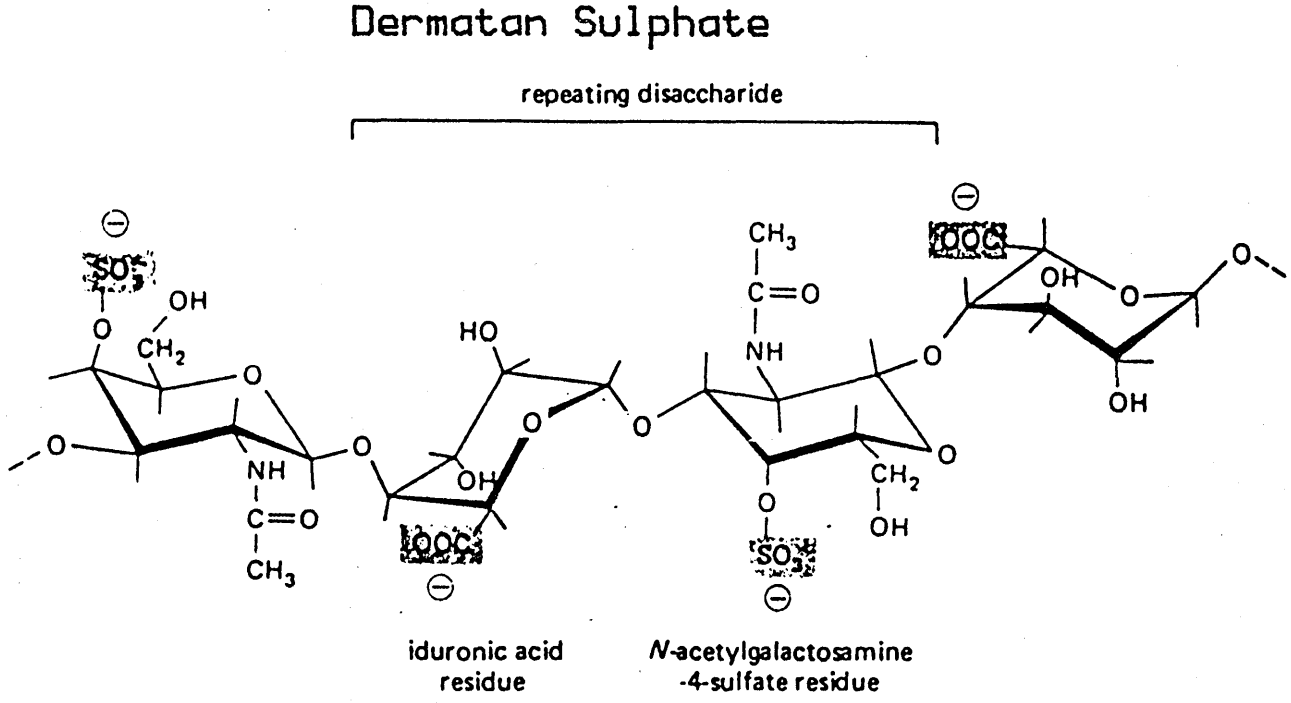


Fig 17 General Strucure of GAGs

TABLE 11 STRUCTURE OF GAGs

Glycosaminoglycan	Molecular Weight	Repeating Diaaccharide(A-B) Monosaccharide A	Monosaccharide B	Sulphates per Disaccharide Unit	Linked to Protein	Other Sugar Components	Tissue Distribution
Hyaluronic acid	4000 to 8×10^6	D-glucuronic acid	N-acetyl-D-glucosamine	0	-	0	various connective tissues, skin, vitreous body, cartilage, synovial fluid
Chondroitin 4-sulfate	5000-50,000	D-glucuronic acid	N-acetyl-D-galactosamine	0.2-1.0	+	D-galactose D-xylose	cartilage, cornea, bone, skin arteries
Chondroitin 6-sulfate	5000-50,000	D-glucuronic acid	N-acetyl-D-galactosamine	0.2-2.3	+	D-galactose D-xylose	cornea, bone, skin arteries
Dermatan sulfate	15,000-40,000	D-glucuronic acid or L-iduronic acid	N-acetyl-D-galactosamine	1.0-2.0	+	D-galactose D-xylose	skin, blood vessels, heart, heart valves
Heparan sulfate	5,000-12,000	D-glucuronic acid or L-iduronic acid	N-acetyl-D-glucosamine	0.2-3.0	+	D-galactose D-xylose	lung, arteries, cell surfaces
Heparin	6000-25,000	D-glucuronic acid or L-iduronic acid	N-acetyl-D-glucosamine	2.0-3.0	+	D-galactose D-xylose	lung, liver skin, mast cells
Keratan sulfate	4000-19,000	D-galactose	N-acetyl-D-glucosamine	0.9-1.8	+	D-galactos-amine D-mannose L-fucose, Sialic acid	cartilage, cornea, inter-vertebral disc

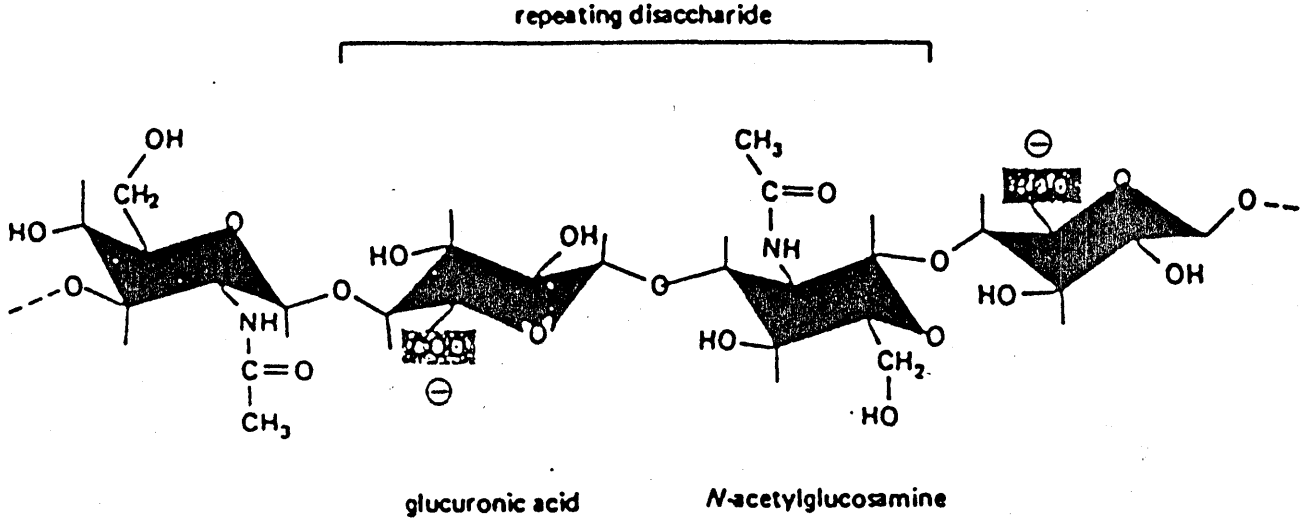


Fig 18 HYALURONIC ACID.

Hyaluronic acid was synthesised in significant amounts accounting for 15% of high molecular weight label. However the majority of radioactivity was associated with mucin glycoprotein i.e. not GAGs. Interestingly the production of mucin was related to kinetic parameters; confluent cultures produced more mucin per cell than did log phase cultures indicating a possible relationship between proliferation and differentiation. A similar study with rabbit tracheal epithelial cells found that 85% of secreted high molecular weight glucosamine labelled material was hyaluronic acid when cells were grown on collagen compared to exclusively hyaluronic acid with cells grown on plastic (123).

Only one report of synthesis of GAG by pure cultures of normal human bronchial epithelial cells has been published. When grown in serum free medium retinoids were found to stimulate the secretion of hyaluronic acid as well as altering the pattern of keratin synthesis (124). Thus it seems that normal human bronchial epithelial cells secrete purely hyaluronic acid and no mucin at least in this system. Retinoids stimulate the growth of these cells and so it seems that hyaluronic acid secretion is related to proliferation also although in a converse fashion to the previously mentioned hamster system (122). Jetten and Smits using rabbit tracheal epithelial cells found that the cells which could undergo squamous differentiation could be induced to differentiate into a mucin secreting cell by retinoic acid (125). In this system at least there seems to exist a precursor cell whose pathway of differentiation can be controlled.

METHODS

Cells are inoculated in either 25cm^2 flasks or 24 well plates and dexamethasone (26 μM) was added for 3 days during the log phase of growth. Parallel cultures with no dexamethasone were set up as controls.

Cells were labelled with 10 μCi ml^{-1} of 6 - ^{3}H (n) - glucosamine (29Ci $mmol^{-1}$) for 24 hours. For 25cm^2 flasks 2ml and for 24 well plates 0.3ml of medium was added. At the end of labelling period the medium was collected and the monolayers washed with 1ml (25cm^2 flasks) or 0.1ml (24 well plates) of medium the samples pooled and lyophilized. Prior to application to a TSK G3000 SW column the samples were resuspended in PBS with 2% mercaptoethanol (v/v). The column was run at a flow rate of 0.5mlmin^{-1} using PBS with 5mM dithiothreitol (DTT) as the eluent. Radioactivity in the void volume (Vo) was pooled and lyophilized. Experiments were carried out to identify the class or classes of GAGs present. This was done in three ways; enzymatically, chemically and by ion-exchange chromatography. To identify hyaluronic acid the enzyme hyaluronidase (bovine testes) was added to the samples at 10Uml^{-1} in PBS adjusted to pH6.0 with 0.1M citric acid and incubated at 37°C for 16 hours. After digestion, samples were applied to the TSK G3000 column and elution profiles gathered. Samples were also treated with chondroitinase AC 0.3Uml^{-1} for 5 hours at 37°C in 50mM acetate buffer (pH 5.5) and elution profiles gathered. To test for the presence of heparin aliquots were incubated for 2 hours at 37°C with 0.75M $NaNO_2$ and 2.5M HCl. The incubation was terminated with the addiion of 0.1M ammonium sulphamate (126). Again elution profiles were gathered. Prior to ion-exchange chromatography samples were desalted on Sephadex G-10. The desalted samples were applied to a TSK DEAE 5PW column (0.75 x 7.9 cm) equilibrated with 50mM acetate buffer (pH 5.5) containing 0.15M NaCl. Attached species were removed either stepwise or with a linear gradient of NaCl (0.1-0.7M). The column was run at 0.5 mlmin^{-1} and 2ml fractions were collected. The concentration of NaCl in each fraction was determined by conductivity using a standard curve.

RESULTS

5.1 WIL

5.1.1 Types of GAGs Secreted

High molecular weight ^{3}H-glucosamine labelled molecules ($> 5 \times 10^{5}$ daltons) were secreted into the medium by WIL cultures in considerable amounts. High molecular weight material is that found in Vo fractions. There appears to be reproducible differences in elution profiles between dexamethasone treated cultures and controls with respect to the void volumes (Figures 19 and 20). Conditioned medium from dexamethasone treated cells appeared to have two peaks when elution profiles were examined. Furthermore there were considerable alterations in the labelled species upon the column, between Vo and Vt, but these changes were not further investigated. Pooled Vo fractions were treated with hyaluronidase which is quite specific for hyaluronic acid and this caused the whole label to appear in fractions at Vt with both control and dexamethasone treated samples. A small fraction of the sample stays within the Vo fractions but this accounts for less than 0.1% of all radioactivity applied to the column (Figure 21). This radioactivity is digested hyaluronic acid. This indicates that the bulk of the high molecular weight label is hyaluronic acid. However further work is needed to ensure that this is the case. Contaminating enzymes in the hyaluronidase preparation may degrade non-hyaluronic acid material. Further hyaluronidase is not totally specific for hyaluronic acid also digesting chondroitin sulphates. To gain further evidence for the presence of hyaluronic acid samples were treated with chondroitinase AC which degrades hyaluronic acid but not to the same extent as the chondroitins. As can be seen degradation of samples does occur but not to the same extent as is seen with hyaluronidase (Figure 22). Samples treated with nitrous acids did not have altered elution profiles (results not shown).

FIGURE 19 ELUTION PROFILE OF CONDITIONED MEDIUM FROM CONTROL CULTURES OF WIL

Plateau phase WIL cells were incubated with 2mls of 10 μCi ml^{-1} of (^{3}H) glucosamine for 24 hours and the conditioned medium collected.

The medium was lyophilized and then resuspended in PBS containing 2% mercaptoethanol. Aliquots were applied to a TSK G3000SW column and elution carried out with PBS containing 5% DTT and 0.02% Na azide at 0.5ml min^{-1}. Fractions of 0.5ml were collected.

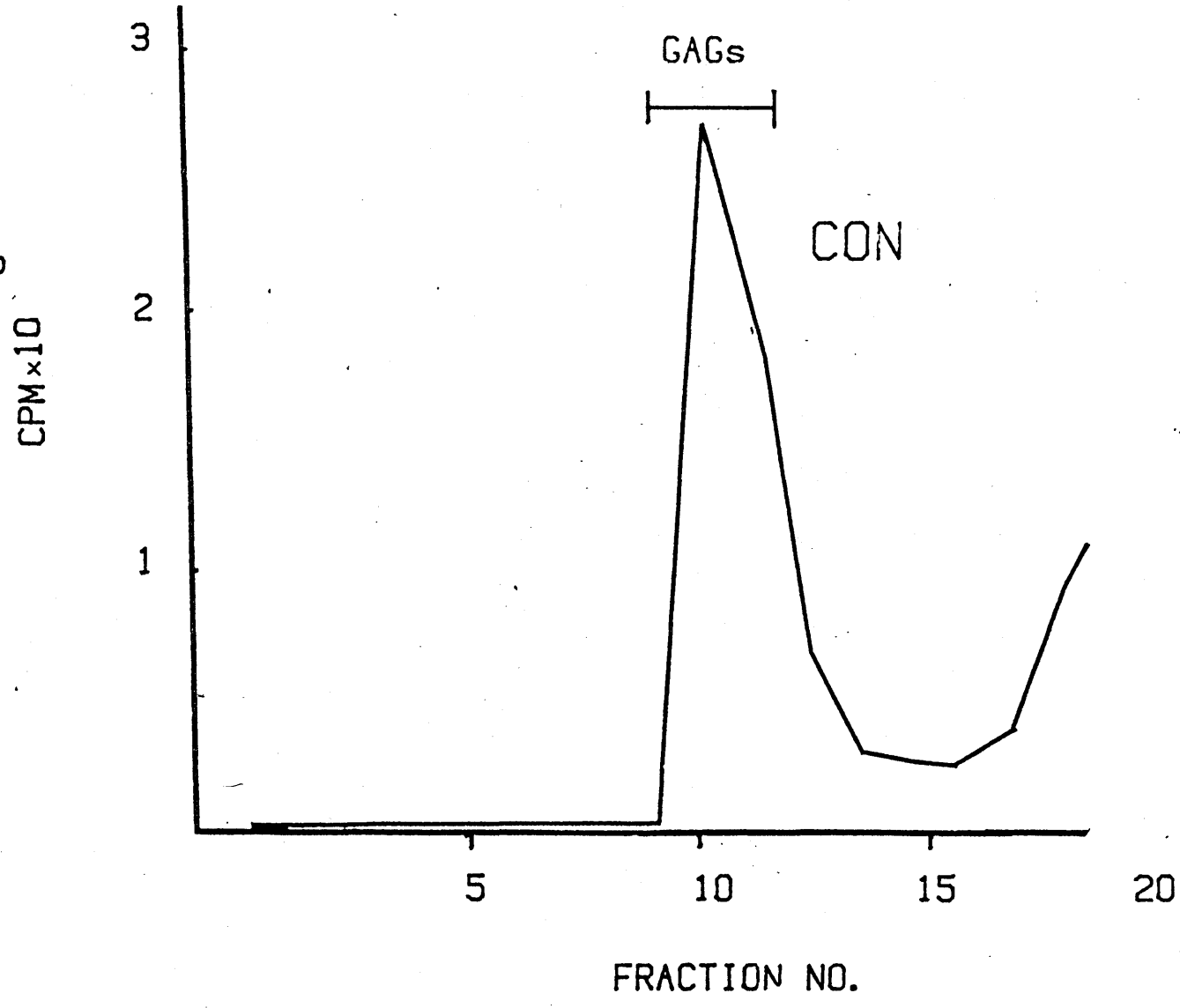

GAGs
CON
CPM×10 -3
3
2
1
5
10
15
20
FRACTION NO.

FIGURE 20 ELUTION PROFILE OF CONDITIONED MEDIUM FROM DEXAMETHASONE TREATED CULTURES OF WIL

Plateau phase WIL cells were pre-treated with dexamethasone (26 μM) for 3 days prior to incubation with 2mls of 10 μCi ml of (^{3}H) glucosamine for 24 hours and then medium collected. The medium was lyophilized and then resuspended in PBS containing 2% mercapto ethanol. Aliquots were applied to a TSK G3000SW column and elution carried out with PBS containing 5% DTT and 0.02% Na azide at 0.05 ml min^{-1}. Fractions of 0.5ml were collected.

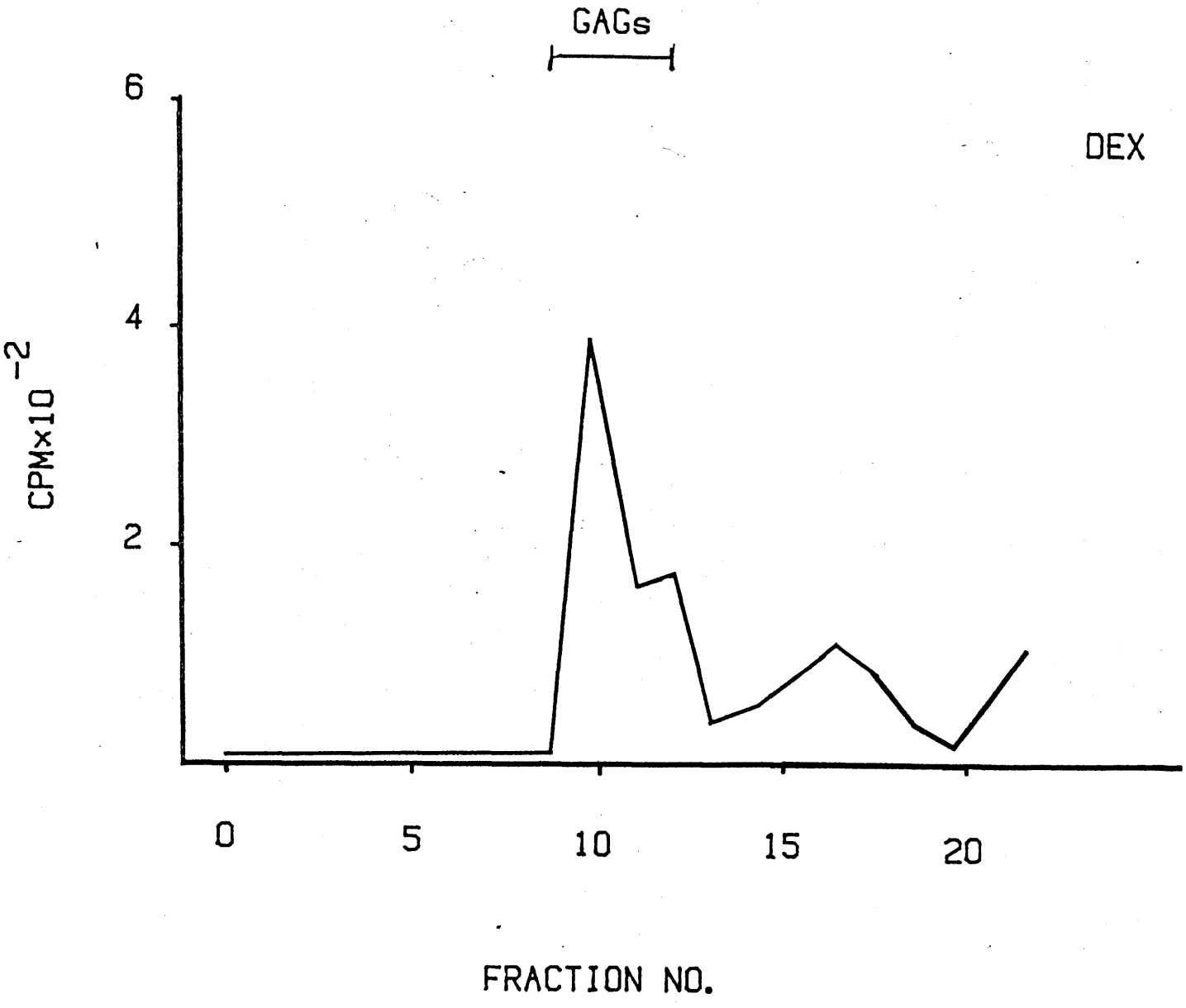

GAGs
DEX
6
4
2
CPM×10 -2
0
5
10
15
20
FRACTION NO.

FIGURE 21 HYALURONIDASE TREATMENT OF V_O RADIOACTIVE MATERIAL.

Aliguots of V_O material were treated with 10 U ml^{-1} of hyaluronidase for 16 hours at 37°C. Samples were then applied to a TSK-G3000SW column and elution profiles gathered. The flow rate was 0.5ml min^{-1} and 0.5ml fractions gathered.

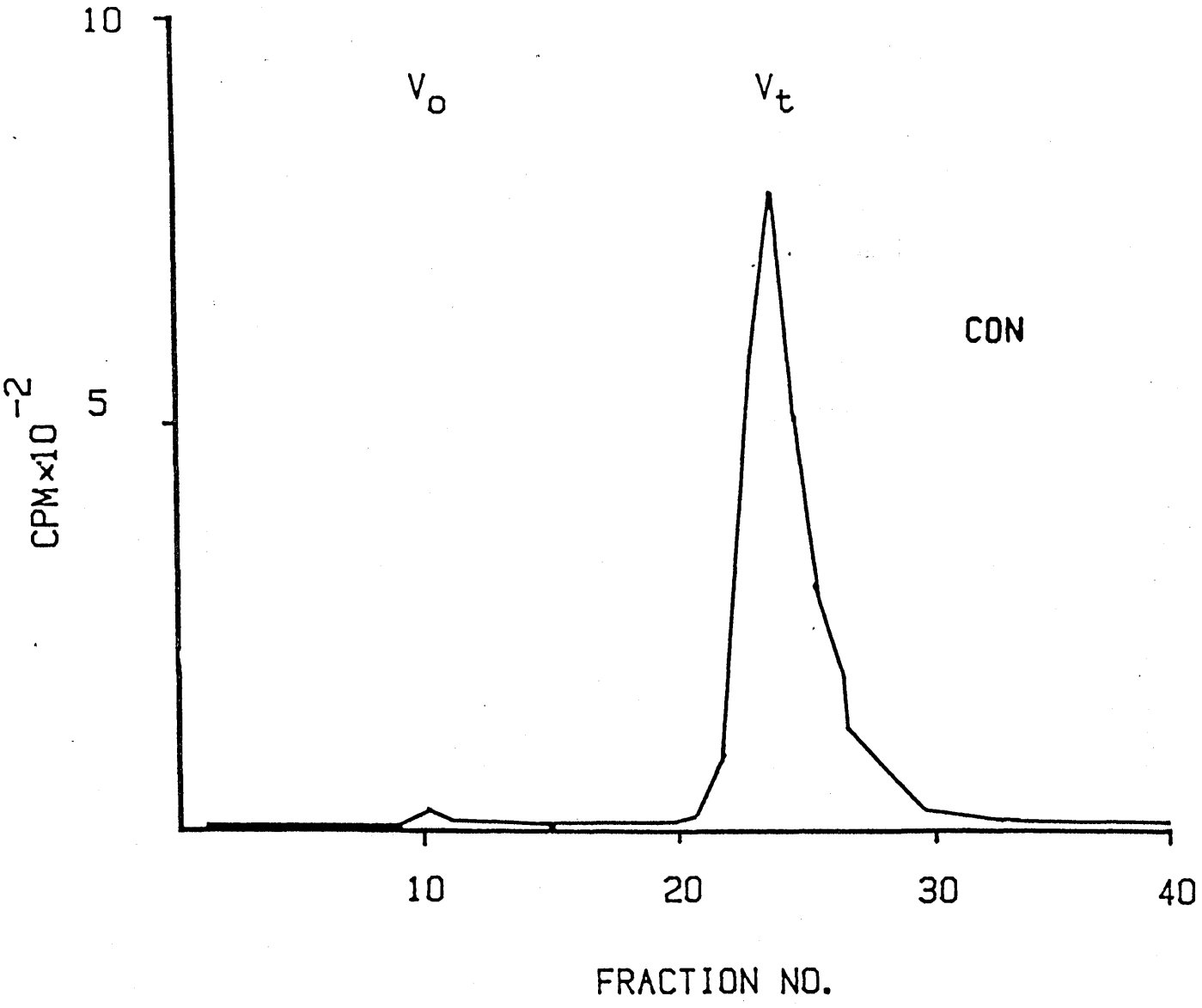
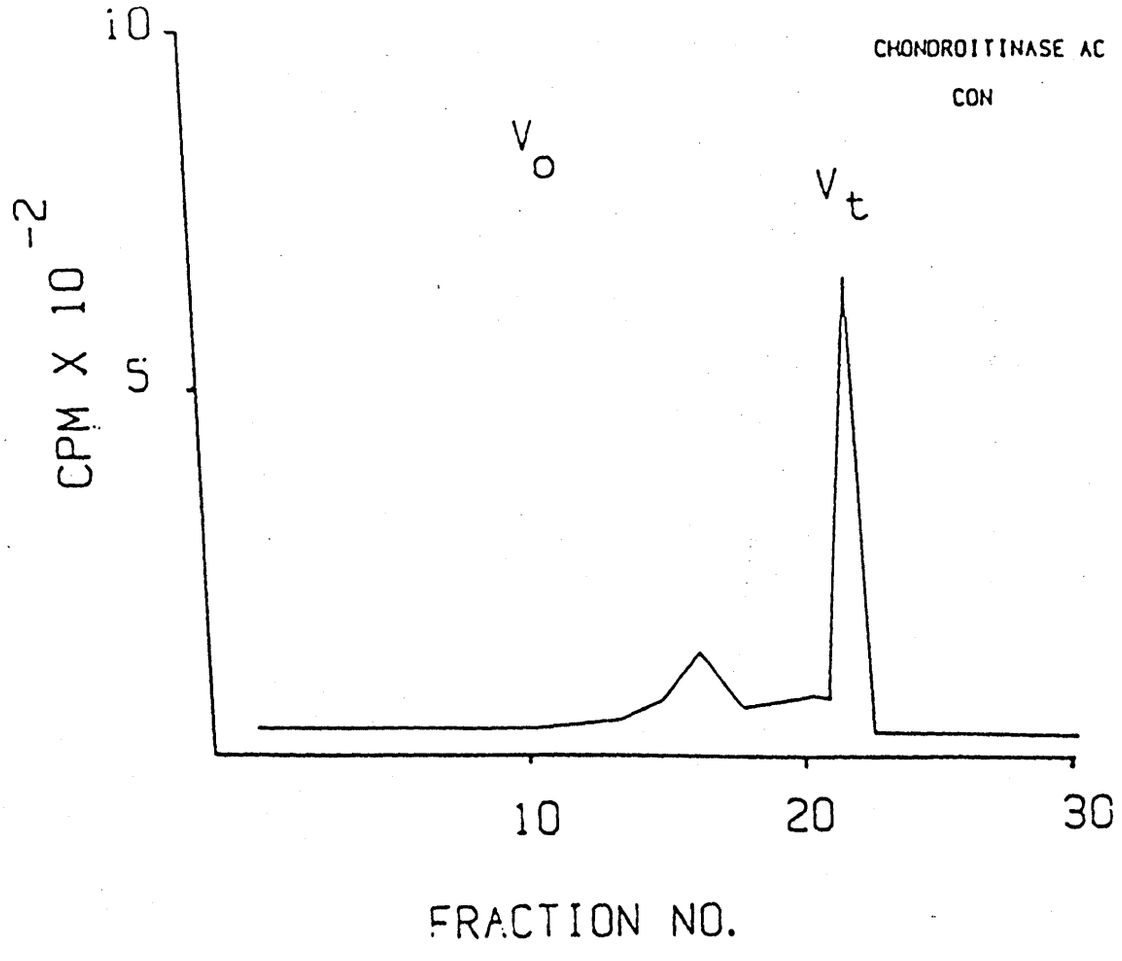

10
5
V_o
V_t
CON
CPM×10^{-2}
10
20
30
40
FRACTION NO.

FIGURE 22 CHONDROITINASE AC TREATMENT OF Vo RADIOACTIVE MATERIAL.

Aliquots of Vo material were treated with 0.3 U ml^{-1} of chondroitinase AC for 5 hours at 37°C. Samples were then applied to a TSK G3000SW column and elution profiles gathered. The flow rate was 0.5ml min^{-1} and 0.5ml fractions gathered.


CHONDROITINASE AC
CON
V_o
V_t
10
5
CPM X 10^{-2}
10
20
30
FRACTION NO.

Hyaluronic acid has a characteristic elution profile when using DEAE ion-exchange chromatography due to the negative charge of the GAGs and so bind to the positive charge of the column packing material. All counts attached to the column when equilibrated with 0.15M NaCl. When eluted with NaCl a single peak coming off at a NaCl concentration of 0.25M was seen suggesting that hyaluronic acid is the only species present (Figure 23). Hyaluronic acid is known to elute at this salt concentration (124).

It is clear that hyaluronic acid is secreted by the adenocarcinoma WIL in vitro. No differences were seen between control cultures or those incubated with dexamethasone with respect to class of GAG released into the medium.

5.1.2 Modulation of Hayluronic Acid Secretion in WIL by Dexamethasone

To ascertain if dexamethasone modulated the secretion of hyaluronic acid, cells were grown in 24 well plates and material isolated as in methods. The amount of radio-activity that stayed in within the Vo fractions was taken to be hyaluronic acid and the results expressed as Vo cpm 10^{-6} cells. When grown with dexamethasone (26 µM) WIL cells had significantly reduced hyaluronic acid secretion (Table 12). The control levels found for this cell line are very similar to those obtained for hamster tracheal epithelial cells (122).

FIGURE 23 ION EXCHANGE CHROMATOGRAPHY OF V_o MATERIAL.

Desalted V_o material was applied to a DEAE column equilibrated with acetate buffer PH 5.5 (50 mM) containing O.1m NaCL. Attached species were removed by elution with a linear gradient of NaCl. The flow rate was O.5ml min^{-1} and O.5ml fractions collected.

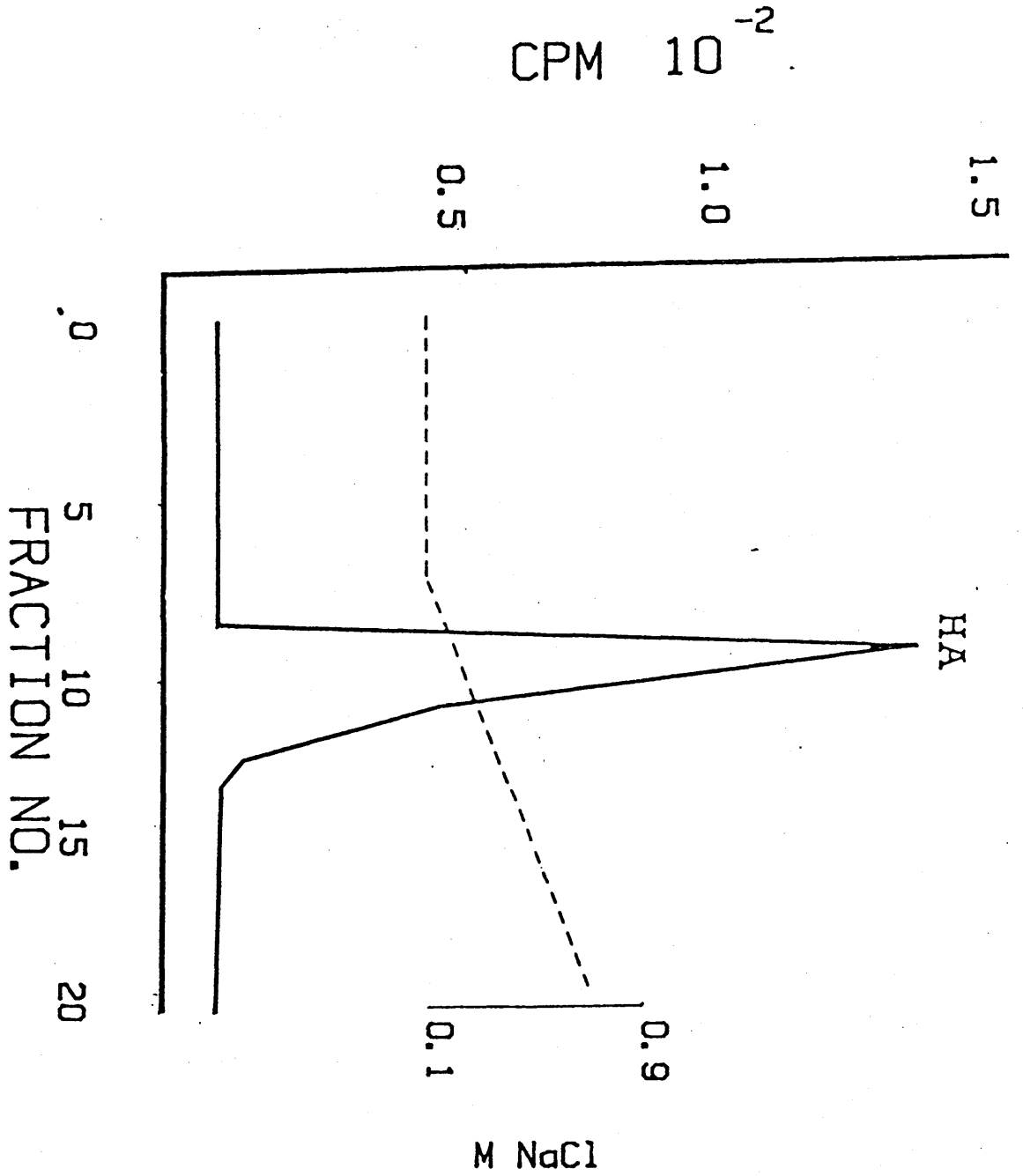

CPM 10 -2
0.5
1.0
1.5
0
5
10
15
20
FRACTION NO.
HA
0.1
0.9
M NaCl

TABLE 12 HYALURONIC ACID SECRETION BY WIL AND H125 AND ITS MODULATION BY DEXAMETHASONE (26μM)

CPM $X10^{-6}$ CELLS

	CON	DEX
WIL	9161 ± 100	6797 ± 387 *
H125	8992 ± 1087	14465 ± 3157 *

* Significant at the 5% level.

mean ± standard deviation (n = 3).

Hyaluronic acid is counts in Vo fractions on TSK G3000SW.

5.2 H125

5.2.1 Types of GAGs Secreted

As with WIL cells, H125 secreted high molecular weight compounds into the medium. No differences were seen in the profiles of material from dexamethasone treated or control cells. Utilizing the same approach as in section 5.1.1, it was found that the only class of GAG secreted was hyaluronic acid (results not shown).

5.2.2 Modulation of Hyaluronic Secretion in H125 by Dexamethasone

Dexamethasone significantly increased the amount of hyaluronic acid by H125 (Table 12).

DISCUSSION

The human lung adenocarcinoma cell lines WIL and H125 secrete hyaluronic acid *in vitro*. This was ascertained by a variety of biochemical techniques. Although the marker of differentiation in these secretory cells is mucin neither WIL or H125 synthesised either protein bound GAGs or mucin glycoprotein. This may be explained by the fact tht the addition of a substratum is needed for full expression of the differentiated phenotype as was seen in previous reports (121, 122, 123). It is possible that the secretion of hyaluronic acid is indicative of an undifferentiated phenotype and if this is true then the action of dexamethasone upon WIL and H125 would agree with other results in this thesis. Dexamethasone reduces malignancy associates parameters in WIL while increasing these in H125. If hyaluronic acid is a marker of de-differentiation then the results observed would agree with the hypothesis that dexamethasone induces differentiation in WIL but malignancy in H125.

As stated earlier dexamethasone reduces hyaluronic acid secretion in WIL while in H125 it is increased. No mucin synthesis was seen because of the lack of a substratum either added chemically or by the presence of mesenchyme. Thus it may be that although dexamethasone is essential, in this system, for differentiation it is not sufficient.

Another interesting result from these experiments is that the control of hyaluronic acid in these two cell lines is intrinsically linked to the level of proliferation. In the presence of dexamethasone the growth rate of WIL is decreased while that of H125 is increased.

6. PULMONARY SURFACTANT

INTRODUCTION

Pulmonary Surfactant

The synthesis of pulmonary surfactant is essential for the normal functioning of the lungs and its production and presence is characteristic of mature lung. The material reduces surface tension within alveoli and thereby prevents alveolar collapse and transduction. Surfactant composition has been studied and the components are well documented. Chemical analyses indicate that it is a complex mixture of phospholipids and proteins. The component in largest supply is dipalmitoylphosphatidyl choline (DPPC). Together with small amounts of other anenoic phosphatidycholines it comprises from 41 to 58% of total surfactant lipid (128, 129). It is likely this DPPC is responsible for the surface tension lowering properties of surfactant. Monoenic phosphatidylcholines account for approximately 11 to 29% of total surfactant lipid and of the remaining phospholipids phosphatidylethanolamine (PE) leads at 5 to 10%. Phosphatidylglycerol (PG) may be present in amounts of up to 10% and when full saturated may even contribute to surface tension lowering ability. Neutral lipids comprise 5 to 13% of total surfactant lipid.

Another minor (less than 10%) but important component of surfactant is non serum protein. The surface associated glycoproteins (SAG) are lung specific proteins of molecular weight 30 to 40 kilo daltons (Kd) (130). These subunits are cross-linked by means of di-sulfide bonds to form a high molecular weight aggregate of greater than 400 Kd (131). Using antibodies against SAG s the relationship of phospholipid production with the protein component of surfactant were investigated (132). The results indicated that synthesis and secretion of these glycoproteins involved the same cytoplasmic organelles for synthesis, packing, storage and exocytosis of surfactant phospholipids. However, it did not appear that the phospholipids and

SAG were stored together within the cells responsible for producing surfactant type II pneumocytes. A typical alveolar type II cell will, if examined with electron microscopy (E.M.), have distinct organelles called lamellar bodies which seem to act as stores of surfactant (133). The lamellar bodies are secreted into the sub-phase of the alveoli where they are arranged to form surfactant.

Although the chemical and physical composition of surfactant is well understood, the mechanisms controlling production are still under investigation. Much information has come from fetal tissue due to the fact that premature children often have problems in breathing due to a lack of pulmonary surfactant. Avery led a large number of investigators in discovering that DPCC production and low surface tension of extracts were promoted by glucocorticoids (134). The exact mechanism by which the glucocorticoids exert their action has been the subject of much investigation and although much is known more work is needed.

Using the human pulmonary alveolar carcinoma derived cell line A549 it was found that it synthesized and secreted saturated phosphatidylcholine (135). Cortisol significantly stimulated (^{3}H) choline incorporation into the phospholipid by 26%. The steroid also increased the amount of saturated phosphatidylcholine that could be released by acetylcholine, carbachol and isoprotorenol (isoprenaline), but had no effect on secretion itself. However, the same author found that glucocorticoids incubated with fetal human alveolar type II pneumocytes showed only minimal response on surfactant production unless other lung cells (endothelial and mesenchymal) were present (136, 137, 138).

Serum free medium collected after incubation of fetal human lung fibroblasts with cortisol contained a factor that caused accelerated lung maturation when injected into fetal rats (139). This fibroblast-pneumocyte factor

(FPF) had a molecular weight of between 5 and 7Kd, was heat stable, dialyzable and a polypeptide. Furthermore, FPF increased (^{3}H) choline incorporation into disaturated phosphatidylcholine by 56%. More recently it has been shown that FPF also increase formation of phosphatidylglycerol from labelled glycerol and palmitate. As well as observing an increase in (^{3}H) choline incorporation into disaturated phosphatidylcholine a concomitant and equal increase is seen of the radioactive tracer into phosphatidylcholine (140). Incubation with onoclonal antibodies raised against FPF cortisol stimulated synthesis of saturated phosphatidylcholine in fetal rat lung organotypic cultures. Furthermore, injection of the antibody into embryonic chicks delayed lung maturation (141).

Although there is a role for epithelia-mesenchymal interactions in surfactant production and its stimulation by glucocorticoid there is also evidence of a direct action of glucocorticoids upon type II pneumocytes. Maniscaleo and Shapiro (142) found that dexamethasone increased the number of B adrenergic receptors by twofold in fetal rat lung explants and the authors suggest, but have no evidence, that it is in type II cells on which this increase occurs. Using Wl-38 human fetal lung fibroblasts and its SV 40 transformed counterparts VA_2, along with VA_4, SV 40 transformed Wl-26. Hydrocortisone was observed to cause an increase in β receptor concentration in a dose dependant manner (143). Since β agonists cause release of saturated phosphatidylcholine and cortisol augments this effect on the cell line A549, it is possible that there is a physiological role for glucocorticoids directly on type II cells.

Although glucocorticoids are obviously important in controlling synthesis of surfactant there are other agents which can affect its production and are probably of physiological importance. The "second messenger"

cyclic adenosine monophosphate (cAMP) along with phosphodiesterase inhibitors was found to increase (methyl-^{14}C choline) incorporation into both saturated and unsaturated phosphatidylcholine in fetal rabbit type II alveolar cells (144). Nites and Matarski (145) have demonstrated that cAMP analogues increased total phosphatidylcholine and disaturated phosphatidylcholine in A549 cells.

Myo-inositol potentiated the dexamethasone and thyroxine induced increase in saturated phosphatidylcholine in fetal rabbit lung (146) and insulin reduced glucose incorporation into phosphatidylcholine in fetal rat lungs (147).

Methods

The measurement of synthesis of surfactant was carried out utilizing the technique of Smith (135). Cells were plated at a concentration of $5x10^{-4}$ cm $^{-2}$ and after growing to confluence the medium was replaced with serum free medium. The cells were incubated with serum free medium containing 0.26 µm dexamethasone for 48 hours in the case of treated cells. Parallel cultures as controls were also set up. The cells were then labelled with 0.1µCi ml^{-1} of ^{3}H methyl choline chloride (80 Ci $mmol^{-1}$) for 24 hours.

6.1 Cellular Surfactant Synthesis

To examine phosphatidylcholine biosynthesis cells in 25cm $^{-2}$ flasks were incubated with label as stated. The medium was removed and replaced with 5ml of methanol. The cells were scraped from the flask and 5ml of 100mM KCl with 10ml of chloroform added to the suspension. At this stage 0.2mg of dipalmitoylphosphatidylcholine and 1.0mg of phosphatidylcholine added as "cold" carriers. To correct for recovery ^{14}C dipalmitoylphosphatidylcholine

(approximately 2000 cpm) was added. The extracts were shaken, allowed to separate and the organic phase was removed and evaporated to dryness in a vortex evaporator (Bu chler).

Disaturated phosphatidylcholine was isolated by column chromatography on neutral alumina (148). Evaporated chloroform extracts were treated with 3.1mg of osmium tetroxide in 0.5ml of carbon tetrachloride for 15 minutes. Samples were evaporated to dryness and redissolved in 0.5ml of chloroform-methanol 20:1 (v/v) and applied to isolation columns. These consisted of 1g of neutral alumina on a plug of glass wool in a 9 inch Pasteur pipette. The neutral lipids, which did not bind to the column, were eluted with 20ml of chloroform-methanol 20:1 (v/v). The disaturated diphosphatidylcholine is then eluted with 10ml chloroform-methanol-7M ammonia 70:30:2 (v/v). The eluted disaturated phosphatidylcholine in eluent was evaporated, scintillant added and counted in a double channel counter (Packard).

6.2 Secreted Surfactant

Measurement of secreted surfactant was similar to that of cellular surfactant synthesis. After the labelling period the medium was decanted and the cultures washed 3 times with 5ml of PBS. Cells were incubated with 3ml of serum free medium containing 10^{-6} isoproteronol for 15 minutes. Dexamethasone acts directly on alveolar cells to increase β adrenergic receptor number and β agonists stimulate surfactant release (135, 143). The media was collected, spun down to remove any contaminating cells and then 3ml of methanol added. The mixture was vortexed and 6ml of chloroform added and the sample revortexed for 30 seconds. The sample was spun down at 2000 rpm (4 °C) for 10 minutes, the aqueous phase removed and the chloroform layer evaporated to dryness as before. All the remaining purification steps were as for cellular investigation studies (6.2).

RESULTS

Effect of Dexamethasone on Surfactant Synthesis

Dexamethasone was found to increase the effect of isoproteronol on disaturated phosphatidylcholine release in A549 cells. A significant increase of about 2 fold was seen. However, with the adenocarcinoma WIL there was a decrease.

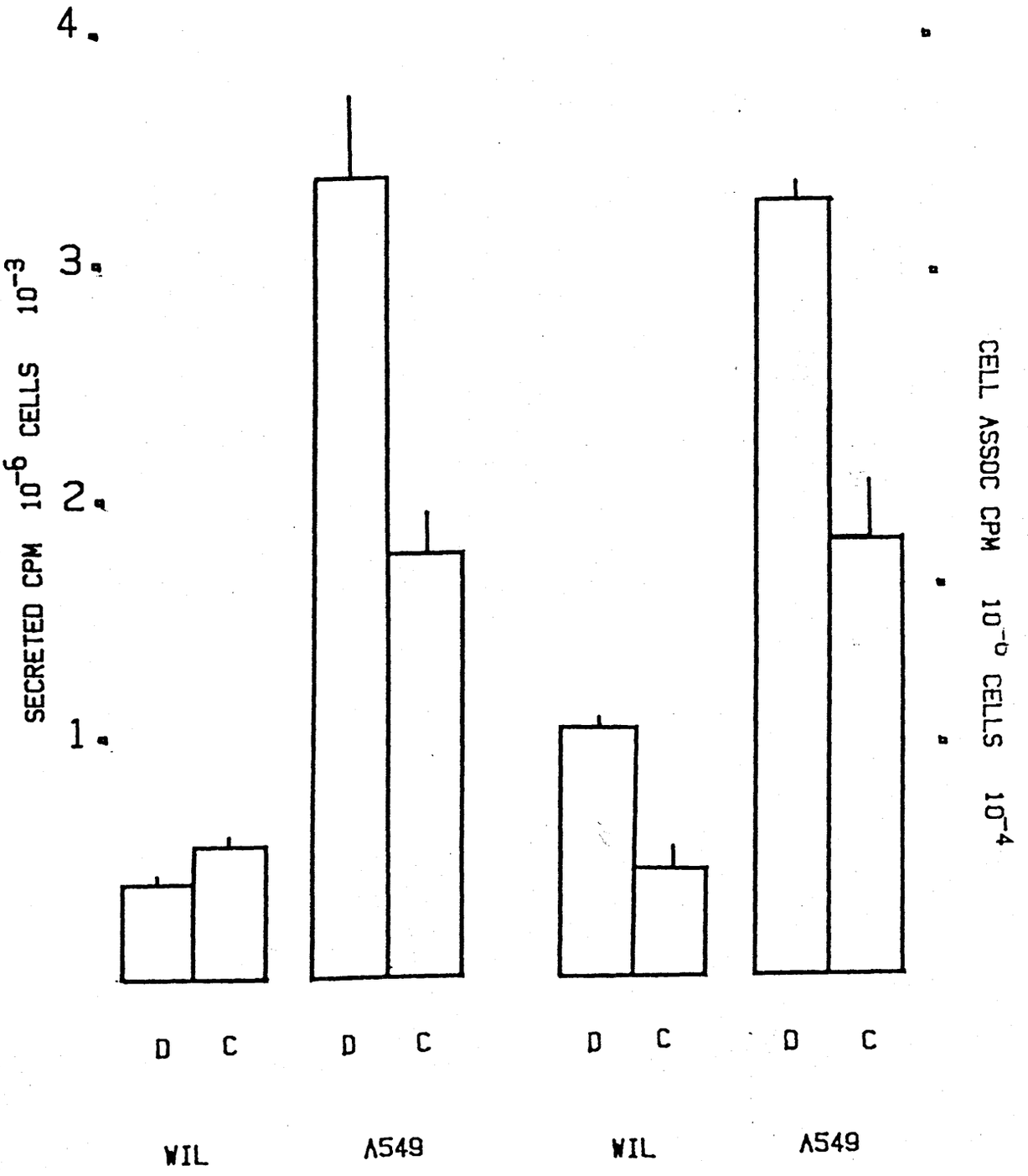
Dexamethasone was found to significantly increase disaturated phosphatidylcholine in monolayers of both cell lines. The increase was about twofold in each case (Figure 24).

DISCUSSION

Dexamethasone increased cellular synthesis and secreted surfactant in A549 cells. These results are similar to those previously reported. It appears then that dexamethasone can increase differentiation in this alveolar carcinoma cell line. Interestingly, it was reported that the amount of surfactant synthesised was inversely related to the rate of growth ie the state of proliferation (135). However, the fact that the cell line WIL synthesises and secretes surfactant is unusual since it is thought to be an adenocarcinoma. The results can be rationalized. Firstly, the increase in disaturated phosphatidylcholine in the cell membrane may be a general membrane effect since these compounds are a common component of membranes and thus dexamethasone modulation of its presence could be indicative of a change in the membrane. This would not however explain the presence of secreted phospholipid. It is possible that WIL is a pluripotent (at least bipotent) precursor or stem cell that can have the properties of a surfactant and mucus secreting cell. This is not the first time that this has been proposed (149).

FIGURE 24 EFFECT OF DEXAMETHASONE UPON PULMONARY SURFACTANT SYNTHESIS AND SECRETION.

Pulmonary surfactant was measured as in methods.
Mean $\pm$ s d (n=3)


4.
3.
2.
1.
SECRETED CPM 10^{-6} CELLS 10^{-3}
CELL ASSOC CPM 10^{-6} CELLS 10^{-4}
D C D C D C D C
WIL A549 WIL A549

The fact that dexamethasone does not increase secretion of disaturated phosphatidylcholine may be due to the fact that there is little surfactant secreted initially and the levels are near those that the assay can measure.

7. ANIMAL EXPERIMENTS

INTRODUCTION

The ability to change the phenotype of carcinoma cells *in vitro* has been well documented but if differentiation inducing agents are to be of any use in chemotherapy then they must act *in vivo*. An agent may be very promising *in vitro* but when examined *in vivo* exhibit little activity. This can be due to a number of reasons; e.g. metabolism, distribution, penetration, or the dose needed is not pharmacologically attainable. The *in vivo* response in relation to that *in vitro* is interesting also as a scientific exercise.

Animal tumours grow readily in rats, mice and other animals but if human tumours are to be grown *in vivo* the problem of tumour rejection has to be overcome. Human tumours cannot be grown in animals unless the animal's immune system is removed or greatly suppressed.

Animal (studies) are now available for the investigation of chemotherapy of human tumours. In 1969 the growth of human tumours (xenografts) was first reported (150). The serially transplanted tumours very often had pathology similar to the initial tumour although xenografts rarely exhibit metastases (151). Chromosomal studies indicated only human genetic material in the transplanted tumours (152). In many cases the xenograft tumour response to chemotherapy correlates with patient response. This was the case of bronchialcarcinoma xenografts (153). Xenografts have also been used in investigating the activity of interferon a differentiating agent (154).

Methods

7.1 Mice

MF-1 (nu/nu) were used as immuno-incompetent animals. The mice lack T-cells. These mice were kept under sterile conditions and had sterile food and water ad libitum. Animals were used between 6 and 9 weeks of age.

AKR mice were used for the growth of the murine ROS tumour.

7.2 Tumour

All tumours were passaged and grown as follows:-

Dissected tumours were cut up into pieces of 3 x 3 mm and placed sub-cutaneously (s.c.) in the animals. The tumours were used for experiments about 2 weeks post-passage.

The tumours WIL and H125 were obtained from cells grown *in vitro* and injected into mice. The ROS tumour was derived as reported previously (155).

Tumour diameters were converted to approximate tumour volume by the formula :-

$$\text{Tumour Volume} = \frac{(\text{mean tumour diameters})^3 \times \pi}{6}$$

Tumours were excised, treated and controls, placed in formol saline and processed for histology.

To examine for the effect of dexamethasone upon tumorigenicity confluent cultures of WIL cells were incubated with or without dexamethasone (26 μM) for 3 days, the medium aspirated and the monolayers washed twice with PBS/EDTA and then trypsinised. The trypsinised cells were then washed with PBS and suspended in PBS at a concentration of 5×10^{-6} cells ml^{-1}.

Cells were inoculated into nude mice (1×10^6 cells in 0.2 ml) subcutaneously and at various times the mice were examined for palpable tumours.

Drug was injected intra-peritoneatally (I.P.).

RESULTS

7.1 Effect of Dexamethasone Upon Tumour Growth

7.1.1 Effect of Dexamethasone Upon ROS Tumour Growth

Mice carrying the Ridgeway Osteogenic Sarcoma (ROS) tumour were injected with either dexamethasone or PBS. Two regimes were used with their corresponding controls : day 0 was the date of the first injection.

1a. Treated on day 0 with 0.005 ml of PBS g^{-1}
1b. Treated on day 0 with 100µg of dexamethasone g^{-1}

2a. Treated on days 0-4 with 0.005 ml of PBS g^{-1}
2b. Treated on days 0-4 with 20µg of dexamethasone g^{-1}

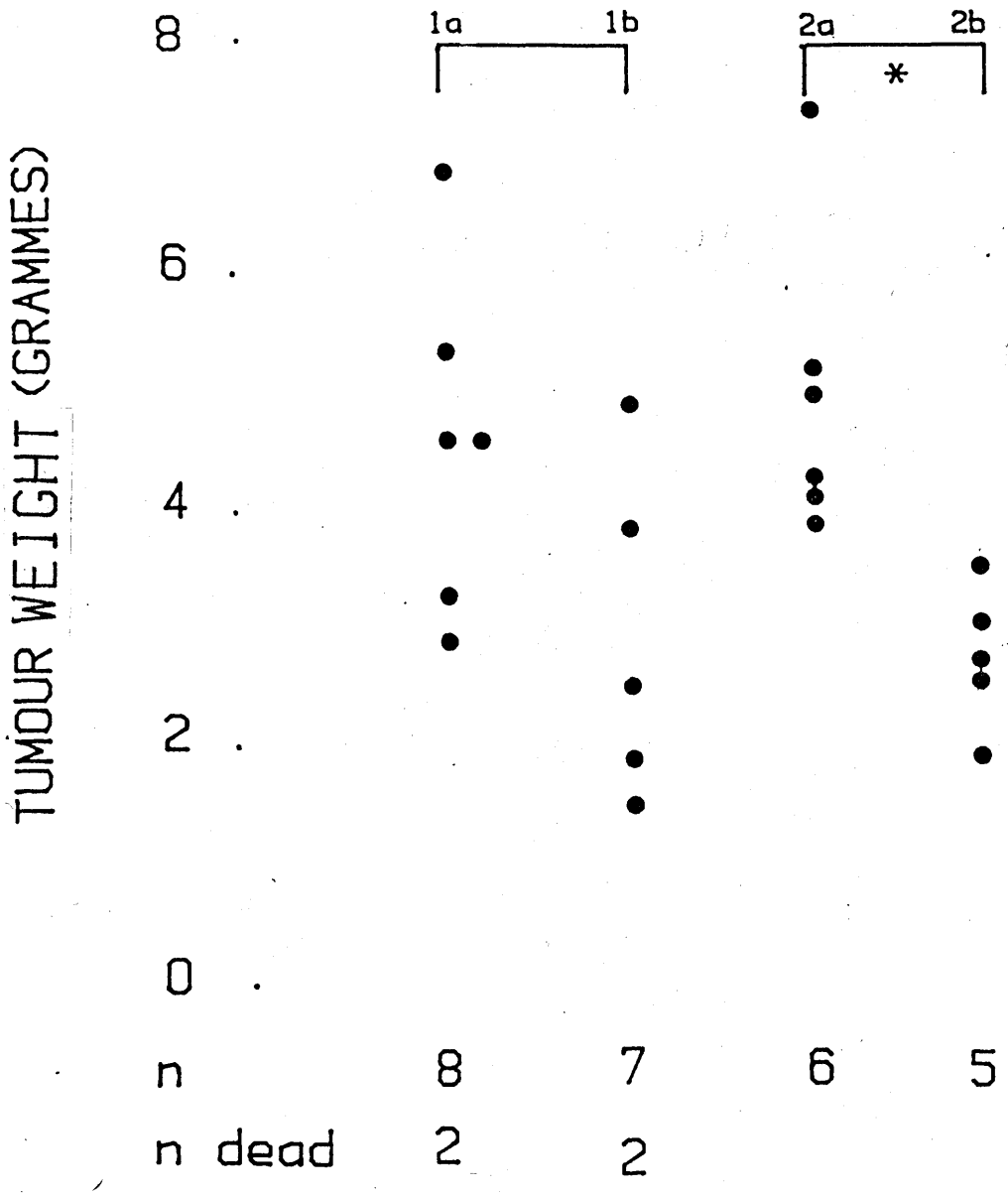
Results are shown in Figure 25. Dexamethasone given as 5 doses I.P. caused a significant reduction in the size of the tumour compard to the controls. Controls over a 10 day period had a 4-fold increase in tumour size to 5g while the dexamethasone treated tumours to 2.9g. From this experiment it is clear that the glucocorticoid caused a significant decrease in the growth rate of ROS tumour in AKR mice.

7.2.1 Effect of Dexamethasone Upon WIL Xenograft Growth

Nude mice were implanted with WIL xenograft. After approximately two weeks most tumours were of a size that could be used for experiments. Dexamethasone (20 µg g^{-1}) was injected I.P. 10 times over 12 days. Tumour size was measured by callipers as stated in methods.

FIGURE 25 EFFECT OF DEXAMETHASONE UPON ROS TUMOUR GROWTH.

Dexamethasone (20μg g-1) was administered I.P. into AKR mice bearing ROS tumour. After 10 days tumours were excised and weighed.



ROS

1a; 1 PBS

1b; 1 DEX

2a; 5 PBS

2b; 5 DEX

* at the 5% level.

After 12 days tumours were either excised and sent for histology or injected with PBS to investigate reversibility of growth inhibition or dexamethasone was given to see if tumour regression could be achieved.

Response ~~to tumour with or without~~ dexamethasone over the first 12 days is shown in Figure 26. Inhibition of tumour growth was seen after 2 days of treatment. At the end of the regime mean control tumour volume was over 4-fold the volume of the tumours at the initiation of the experiment. Dexamethasone treated tumours had very similar volumes of those at the initiation of the experiment i.e. dexamethasone totally inhibited the growth of WIL xenograft. Furthermore, from 9 days onwards the mean tumour volume fell and was still falling at day 12. Thus at this point there is evidence of tumour regression. Experiments were carried out to see if tumour regression could be attained by maintaining the regime beyond 12 days. However, it was found that there was no loss in tumour size when the regime was extended (results not shown).

The effect of dexamethasone upon WIL xenograft was found to be reversible. After a regime of ten injections over twelve days the tumours were measured at regular intervals in the absence of further treatment with dexamethasone. All tumours previously treated with dexamethasone resumed growth with doubling times very similar to untreated control tumours. Table 13 shows the doubling times of tumours treated and controls. The doubling times represent the time needed for a doubling of tumour volume once the tumours started regrowing. No significant difference was found.

7.2.2 Effect of Dexamethasone on Tumorigenecity of WIL

Mice were inoculated with 5×10^5 WIL cells with or without dexamethasone at 26μM for 3 days. The results are shown in Figure 27.

TABLE 13 DOUBLING TIME OF WIL XENOGRAFTS AFTER DEX WITHDRAWAL

	1st Doubling	2nd Doubling	Control (No DX)
Mean	17.4	7.0	8.6
S.E.	± 3.6	± 1.65	± 0.5
n	5	5	5

FIGURE 26 EFFECT OF DEXAMETHASONE UPON THE GROWTH OF WIL IN VIVO.

Xenograft bearing nude mice were given dexamethasone (20 g g^{-1}) on 10 days out of 12. Tumour volume was measured by callipers.
Mean ± s d (n=6)

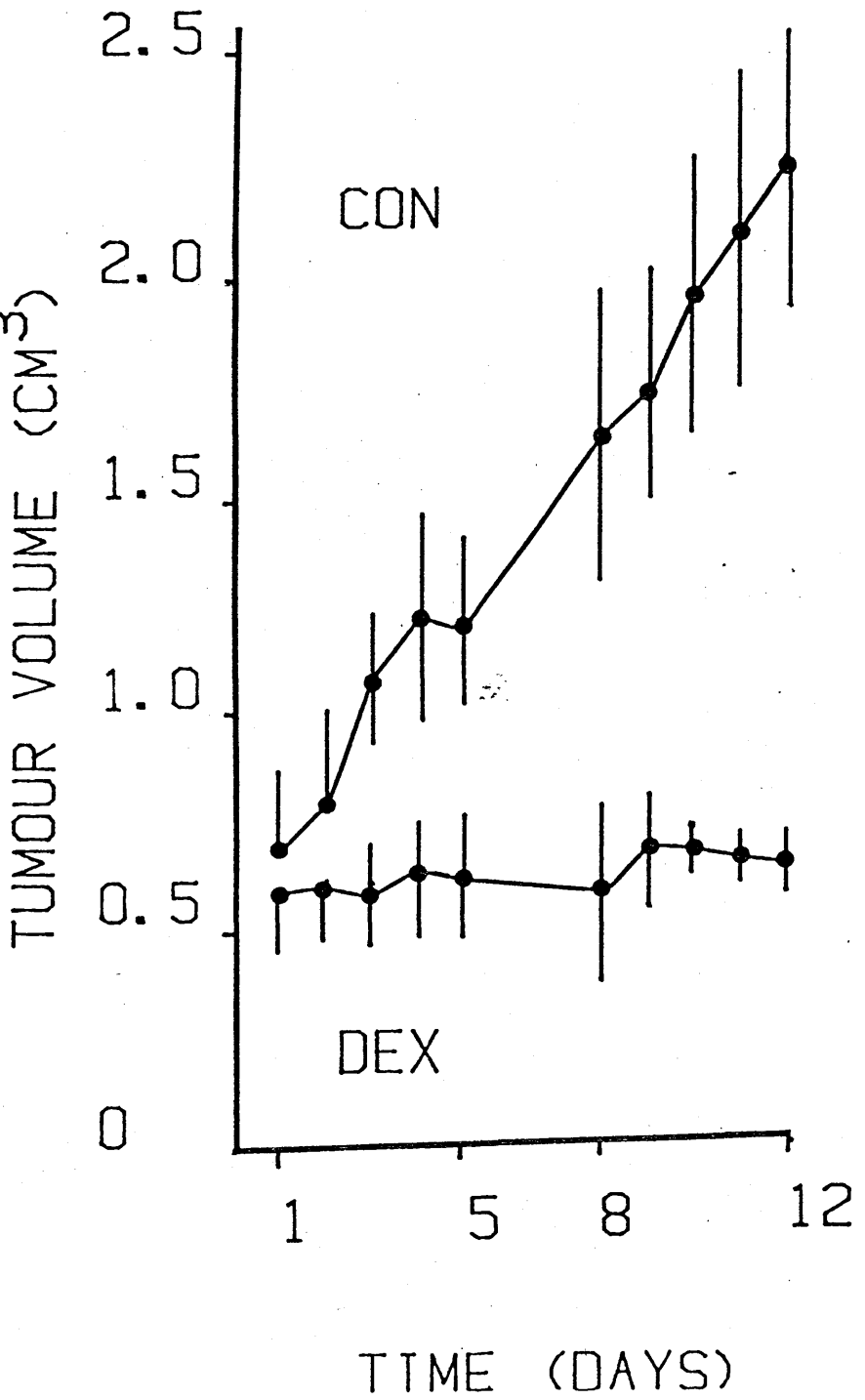
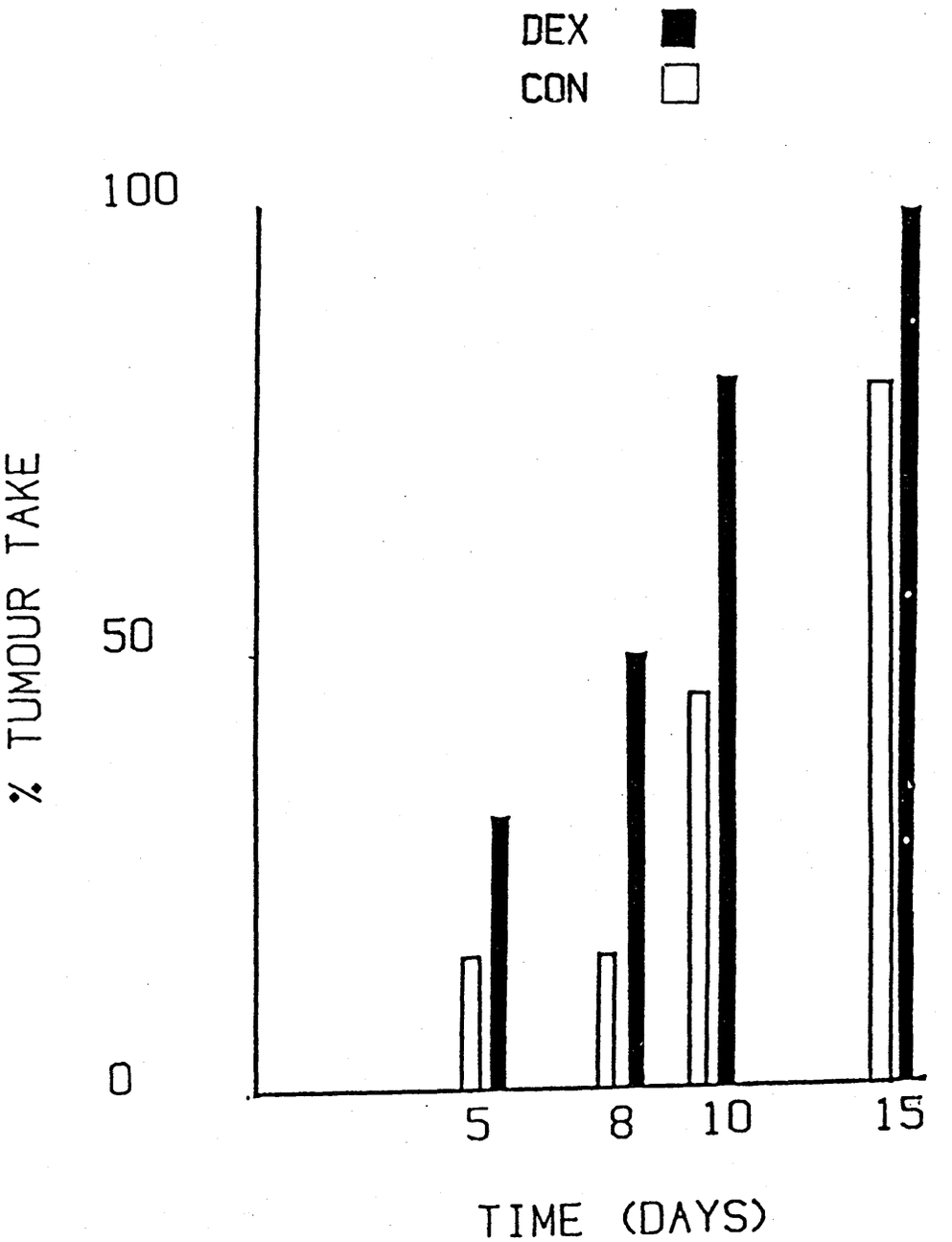

2.5
2.0
1.5
1.0
0.5
0
TUMOUR VOLUME (CM3)
CON
DEX
1
5
8
12
TIME (DAYS)

FIGURE 27 EFFECT OF DEXAMETHASONE ON TUMORIGENICITY OF WIL.

Confluent cultures of WIL were incubated with or without dexamethasone (26 μM) for 3 days. Cells (10^6) were then injected into mice (S.C.). Mice were inspected daily for palpable tumours. Six mice are in each group.


DEX
CON
100
50
0
% TUMOUR TAKE
5
8
10
15
TIME (DAYS)

Dexamethasone increased the tumorigenecity of WIL cells in nude mice with respect to control cells. At 8 days post-inoculation 50% of mice bearing dexamethasone treated cells have palpable tumours compared to 15% with control cells. At 15 days 100% of mice innoculated with dexamethasone treated WIL cells compared to 80% with untreated WIL cells. It appears that initially dexamethasone treated WIL cells are more tumorigenic than untreated cells.

7.4 Effect of Dexamethasone Upon H125 Tumour Growth

Nude mice bering H125 tumour were treated with dexamethasone or PBS in exactly the same way as WIL xenograft (Section 7.2.1). In this case dexamethasone was found to have no effect on the growth of H125 when compared to controls (Figure 28).

7.5 Histology of Treated Xenografts

Tumours were excised, fixed in formalin and mounted for staining. Control and dexamethasone treated tumours stained with H and E are shown in Figures 29a and 29b. As can be seen there is much more central necrosis in the dexamethasone treated tumours. In Figure 29a there is a much deeper band of viable cells compared to the dexamethasone treated tumour 29b.

At higher magnification mitotic cells (M) can be seen in the centre of the dexamethasone treated WIL tumour. However, there is also much evidence of vacuolation of cells. On the whole there are fewer mitotic cells in dexamethasone treated tumours 30.

DISCUSSION

The growth of the ROS tumour was significantly reduced by dexamethasone although at higher doses toxicity was observed.

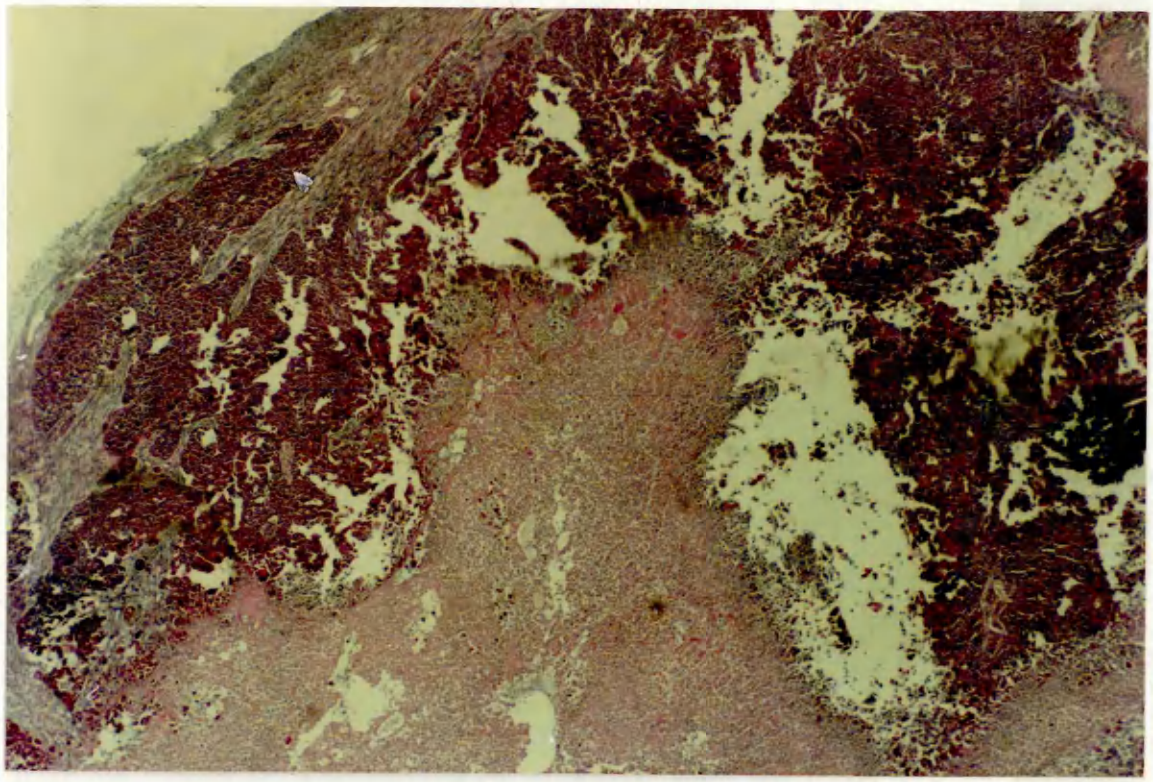


Fig 29a

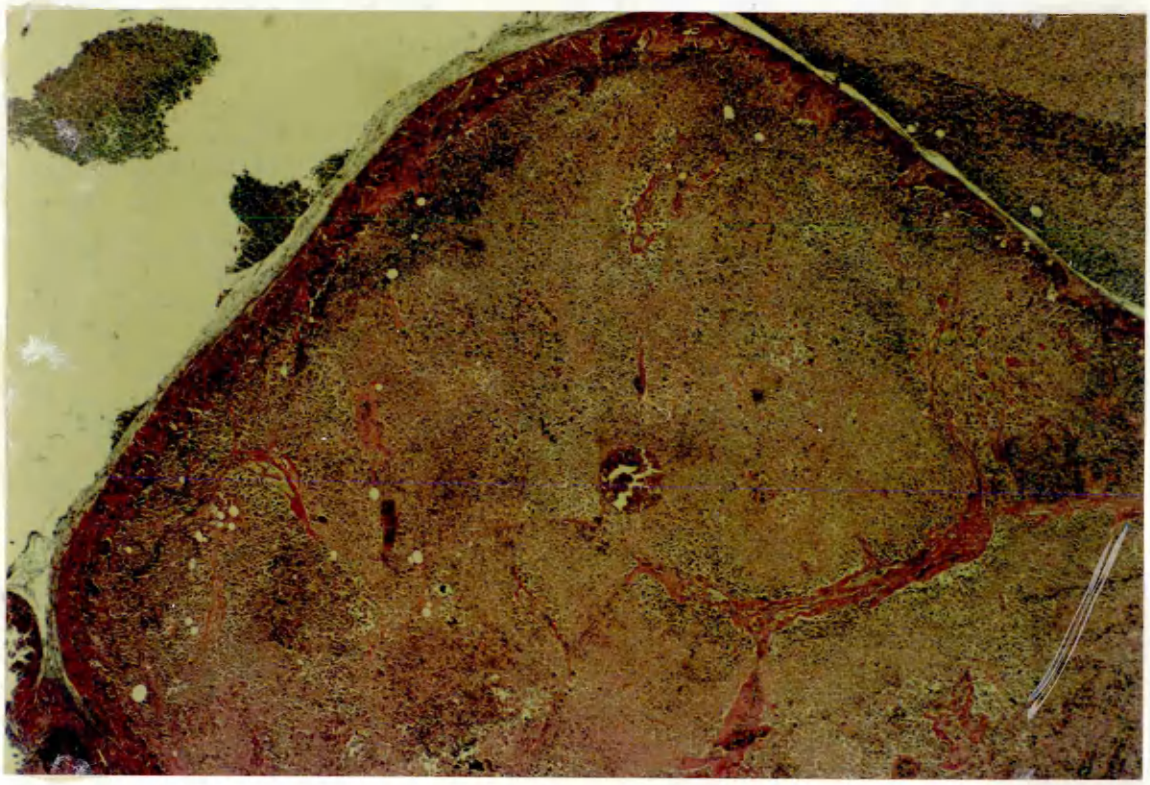


Fig 29b

x4 (H&E)

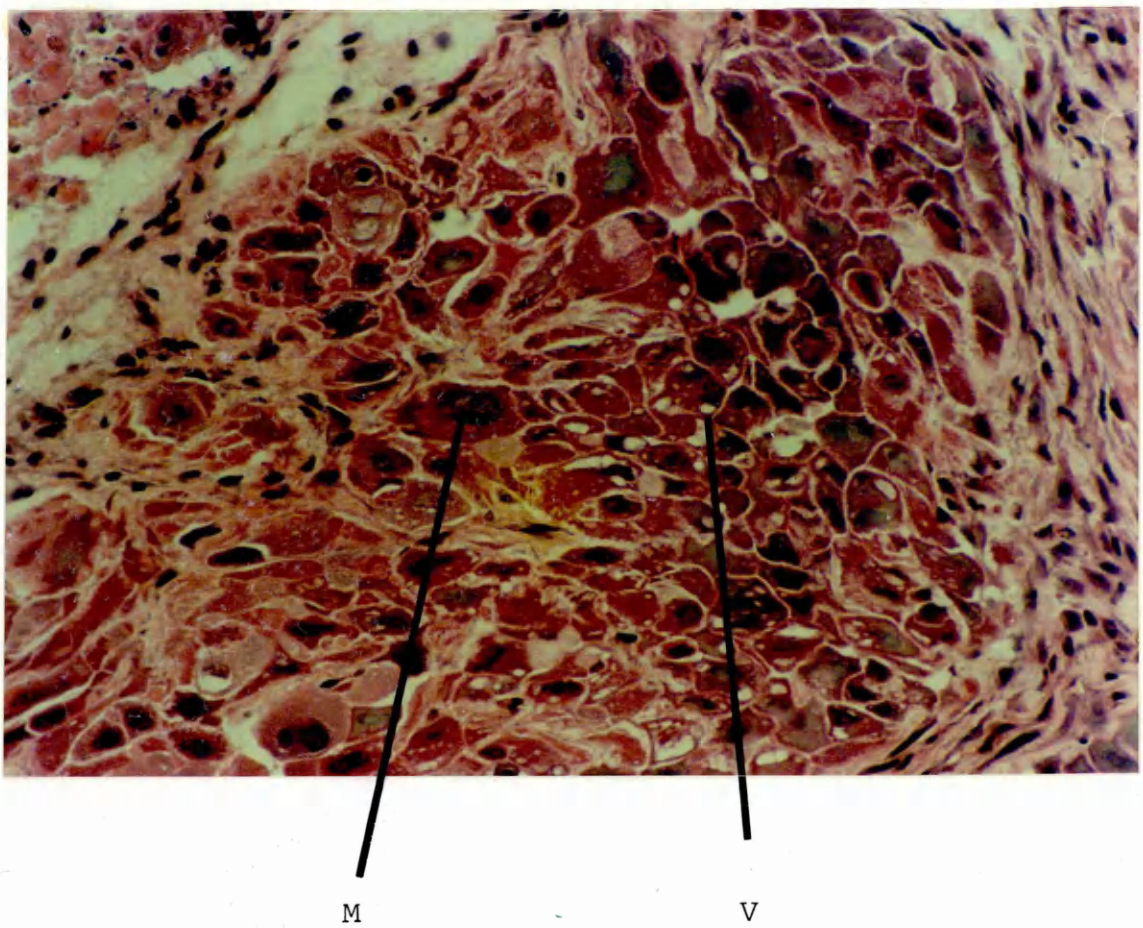
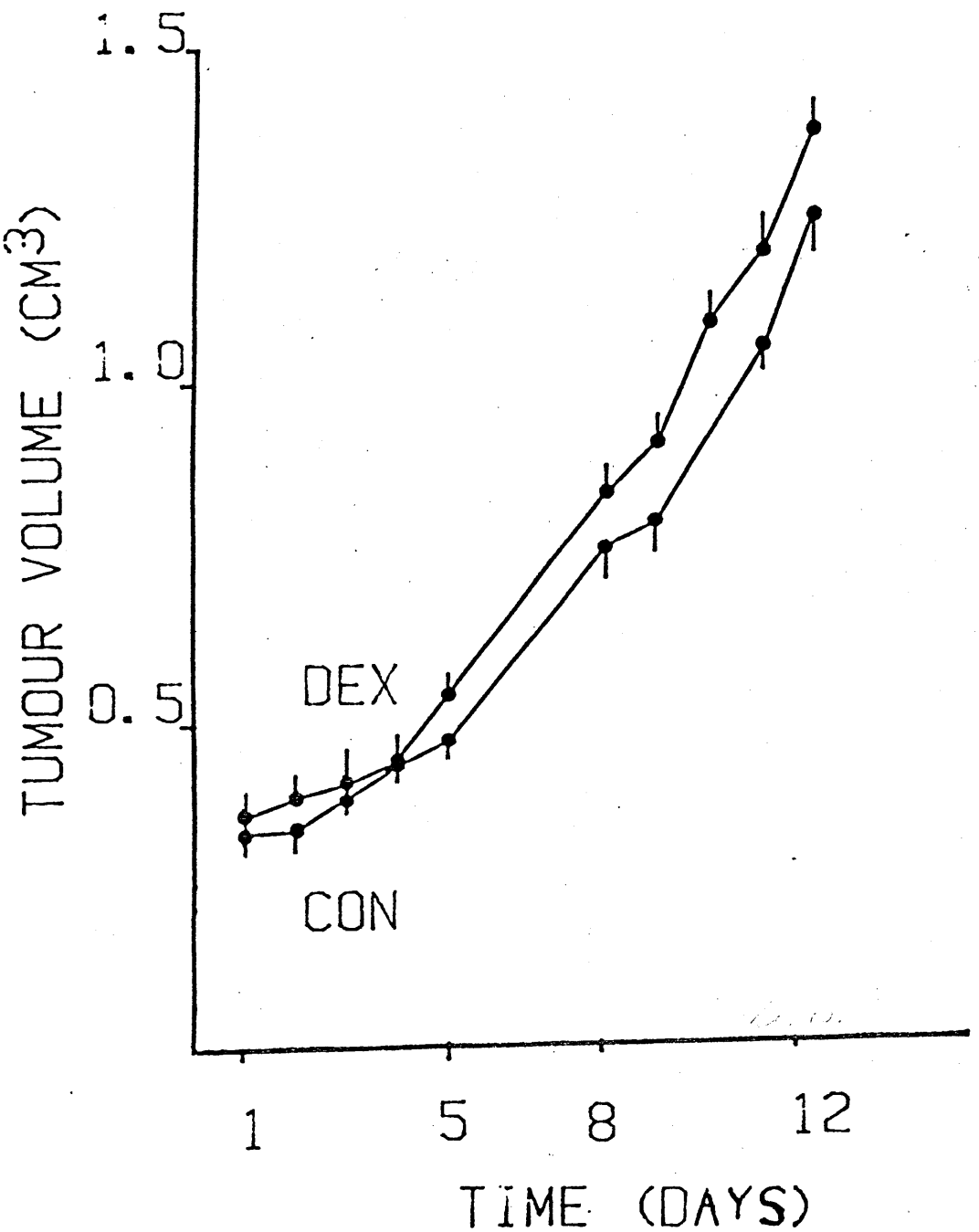


Fig 30

x40 (H&E)

FIGURE 28 EFFECT OF DEXAMETHASONE UPON THE GROWTH OF H125 IN VIVO.

Xenograft bearing nude mice were given dexamethasone (20$\mu g\ g^{-1}$) on 10 days out of 12. Tumour volume was measured by callipers.
Mean $\pm$ s d (n=6)


1.5
1.0
0.5
TUMOUR VOLUME (CM3)
DEX
CON
1
5
8
12
TIME (DAYS)

Studies with the lung xenografts show that the *in vivo* response to dexamethasone correlated with that *in vitro* in the case of the lung xenografts. *In vitro* the cell line WIL had lowered growth rate (at normal passage densities), terminal cell density, clonogenicity in agar, PA levels, in the presence of dexamethasone. Clonogenicity as a monolayer was increased by dexamethasone. The growth of the xenograft derived from the cell line was totally arrested by dexamethasone.

The growth of the tumour H125 in nude mice was unaltered by dexamethasone. *In vitro* the cell line had an increased growth rate (at regular passage densities) terminal cell density and PA activity in the presence of dexamethasone. Clonogenicity in agar was reduced but monolayer cloning was unaffected by the glucocorticoid. It appears then that the response (or lack of it) to dexamethasone *in vitro* correlates with that *in vivo*.

These results are in agreement and expand upon the study using a murine mammary carcinoma where reduction in *in vivo* PA content of tumours was directly related to reduction in *in vivo* growth rate (62). In our study with the cell line WIL, *in vitro* PA reduction correlated with growth rate reduction *in vitro* and inhibition of xenograft growth. The cell line H125 had a raised PA content and growth rate *in vitro* while the growth rate *in vivo* was unaffected by dexamethasone. The mode of action of dexamethasone's inhibition of growth of WIL *in vivo* appears to be due to vascular collapse. Histology of the excised WIL tumours showed that in treated tumours there was only a thin layer of viable cells on the outside of the xenograft with central necrosis. With control tumours a deeper band of viable cells is seen with much less central necrosis. If the growth inhibition was due to a direct cytotoxic action then the outer layers would not be viable as they are the first cells to "see" the drug.

The vascular collapse was not due to a direct effect on the growing endothelial cells in the vasculature since the growth of H125 is unaffected by dexamethasone. Thus in the case of WIL dexamethasone seems to abolish growth by inhibiting angiogenesis. More conclusive evidence could come from investigating angiogenic factors by the cell lines WIL and H125 or by directly examining the vasculature of the tumours in vivo as done by Braunschwieger (156).

Dexamethasone was found to increase the tumour take of WIL cells in nude mice, albeit transiently. This result is not in line with the observation that dexamethasone reduces malignancy associated parameters. Also a reduction of tumorigenecity would be expected to accompany the reduction in growth rate. As a chemotherapeutic agent an increase in tumorigenecity would not be beneficial. There have been reports however that more differentiated cells are more tumorigenic (157). This anomaly can be explained by the fact that the increase in tumorigenecity is due to an increase in adhesiveness in the WIL cells thus aiding tumour take. If this is the case then what is being observed is actually an increase in a characteristic that may reduce tumour spread.

There are two possible reasons for the transient nature of dexamethasone's increase in tumorigenecity in WIL cells. Firstly, the action of dexamethasone in vitro is reversible and so, after a certain time, any difference between treated and control cells will disappear. Secondly, the increase in tumour take may be due to the presence of dexamethasone that has dissociated from the receptor in the WIL cells and in some way make the host tissue more amenable to tumour take. The dexamethasone induced growth inhibition of WIL tumour was reversible as was found with murine mammary carcinoma (62). This indicates either a resistant fraction of the tumour to dexamethasone or, since growth inhibition appears to be due to inhibition of angiogenesis, there may exist two or more types of vasculature.

SUMMARY

The effect of dexamethasone on human non-small cell lung carcinoma *in vitro* and *in vivo* was investigated. Dexamethasone caused a significant reduction in growth rate in most of the cell lines at normal passage densities with the exception of H125 which had an increased growth rate in the presence of dexamethasone. In both cases the effect was dose dependent. Clonogenicity as a monolayer was increased by dexamethasone in all cell lines investigated except H125. Cloning efficiency in agar of all the cell lines examined was reduced by dexamethasone in a dose dependent manner. Significant levels of glucocorticoid receptor were found in all cell lines investigated. PA activity was lowered by dexamethasone in all cell lines except H125 where it was raised though not significantly.

Some alterations in cell surface carbohydrates were found to correlate with alterations in cell proliferation. The cell lines WIL and H125 were examined for mucin/GAG secretion but no mucin secretion was found. Both cell lines secreted hyaluronic acid only. Dexamethasone increased hyaluronic acid secretion by H125 and decreased it in WIL. Pulmonary surfactant synthesis and secretion was increased by dexamethasone in the alveolar carcinoma A549.

Dexamethasone reduced the growth of the murine ROS tumour *in vivo*. The growth of WIL tumour was inhibited totally by dexamethasone in a reversible manner although pre-treatment of WIL cells *in vitro* with dexamethasone increased the frequency of successful tumour takes.

Apparently dexamethasone could increase differentiation and reduce malignancy associated properties a manner which suggested they were inter-related. The expression of the

differentiated phenotype is also correlated with a reduction in cell proliferation. Furthermore the *in vivo* response reflected the *in vitro* response.

8.1 The Effect of Dexamethasone Upon Malignancy Associated Properties

The effect of dexamethasone upon certain malignancy associated properties was investigated. PA activity was reduced in 5 out of 6 cell lines examined. The other cell line H125 had increased PA activity. In the assay used it is not possible to distinguish tPA from uPA but in one cell line examined no tPA was found.

Angiogenesis may also be reduced *in vitro* by dexamethasone. Conditioned medium from dexamethasone treated WIL and A549 cells caused less stimulation of endothelial cells than control conditioned medium. However conditioned medium from the cell line H125 did not stimulate mitogenesis of endothelial cells. This indicates a possible deficiency in the system used. H125 is angiogenic *in vivo* but *in vitro* no mitogenic activity was detected. Thus the system may not detect certain tumour derived growth factors. Evidence also exists of dexamethasone "carry over" which may interfere with the assay.

The effect of dexamethasone upon tumour cell surface glycopeptides was examined. As stated earlier an increase in sialylation and/or carbohydrate size accompanied an increase in malignancy associated properties. In this study it was found that this did not totally hold true. With WIL which had a reduced PA activity with dexamethasone there was evidence of reduced sialylation in the presence of the drug and with A549 which also had a reduced PA activity with dexamethasone there was evidence of an increase, albeit low, in sialylation. There was no evidence of any alteration in H125 glycopeptides; this cell line had an increased PA activity in the presence of dexamethasone.

Thus there is a slight correlation between dexamethasone modulation of PA activity and alteration in cell surface glycopeptides. Differences may arise due to the secretory function of the cells.

Clonogenicity in agar often accompanies malignant transformation. A reduction in clonogenicity in agar would therefore imply a reduction in malignancy. In the presence of dexamethasone it was found that the clonogenicity of the cell lines H125, WIL and A549 were reduced in a dose dependent manner. Most interesting was the fact that H125 had reduced clonogenicity while PA activity, angiogenic capacity and sialylation were either increased or unaffected by dexamethasone. This may be explained by the fact that dexamethasone reduces growth rate at clonal cell densities. This in turn may reduce colony size in agar.

In conclusion dexamethasone reduces malignancy associated parameters of human non-small cell lung cancer in vitro. One cell line, H125, appears to have increased malignancy associated parameters in vitro but this is an exception.

8.2 Effect of Dexamethasone on Differentiation

For some tissue types markers of differentiation are readily available e.g. melanin in melanocytes/melanoma. However for many tissues especially those of epithelial origin these markers are either not readily available or difficult to assay and lung cells fall into this category. The alveolar carcinoma A549 has a marker in pulmonary surfactant synthesis/secretion and in agreement with previous reports it was found that dexamethasone caused a significant increase in both synthesis and secretion of pulmonary surfactant. As stated earlier secretion of pulmonary surfactant is a better marker of differentiation of alveolar function since pulmonary surfactant components are found in membranes.

Markers of differentiation for mucin secreting cells are more difficult to use since mucin/GAGs are highly heterogeneous and their measurement is difficult. Using complete and time consuming methods the presence and quantity of these compounds can be ascertained. In both of the cell lines investigated, H125 and WIL, no mucin secretion was found and the only GAG secreted was hyaluronic acid. It may be that for full differentiation a basement membrane is needed. As stated earlier (chapter 5) mucin secretion and a decrease in hyaluronic acid secretion is indicative of differentiation.

When WIL was incubated with dexamethasone there was a significant reduction in hyaluronic acid secretion. An increase in hyaluronic acid secretion was seen with H125 when incubated with dexamethasone. Thus dexamethasone appears to induce differentiation to an extent in WIL and de-differentiation in H125. Hyaluronic acid secretion seems to be related to proliferation; as the growth rate of WIL decreases hyaluronic acid secretion is reduced, while H125 has an increased growth rate and hyaluronic secretion in the presence of dexamethasone.

The experiments need to be repeated with a substratum present for the attainment of optimal induction of differentiation. Furthermore it may be worthwhile examining the effect of dexamethasone on trans-glutaminase activity and cross-linked envelope formation, two accepted markers of differentiation of the squamous phenotype.

8.3 Inter-relationship between Malignancy and Differentiation

It has been proposed that malignancy and differentiation are inversely related; an increase in differentiation would be accompanied by a decrease in malignancy. It appears that this now also applies for human non-small cell lung cancer.

The cell line WIL had reduced malignancy associated parameters i.e. PA activity, angiogenic capacity, agar cloning and increased differentiation albeit partial i.e. hyaluronic acid secretion. The opposite effect applies for H125 when incubated with dexamethasone - dexamethasone causes an increase in malignancy associated parameters and a decrease in differentiation.

Furthermore with A549 dexamethasone induces differentiation (surfactant synthesis and secretion) and reduces malignancy (PA, agar cloning angiogenic capacity etc).

Thus in the case of human non-small cell lung cancer there appears to be an inverse correlation between differentiation and malignancy.

8.4 Relationship between State of Differentiation and Cell Proliferation

Dexamethasone can modulate proliferation. In 4 out of 6 cell lines dexamethasone reduced proliferation while H125 had an increased growth rate in the presence of the drug. These experiments were carried out at regular passage densities. This modulation of proliferation is accompanied by changes in phenotype

In the case of WIL the reduced growth rate in the presence of dexamethasone is accompanied by a decrease in hyaluronic acid secretion, PA activity, angiogenic capacity and clonogenicity in agar. Thus as differentiation is increased there is a decrease in proliferation. Although the effect of dexamethasone is reversed in H125 the relationship still holds. An increase in proliferation is accompanied by a decrease in differentiation.

Thus cytostasis correlates with differentiation while proliferation correlates with expression of malignancy.

8.5 In Vivo Results

Dexamethasone significantly reduced the growth of ROS tumour in mice and abolished growth of WIL as an xenograft in nude mice. The growth of H125 xenograft was unaffected by dexamethasone. This result implies that the inhibition of tumour growth *in vivo* is cellular and not systemic. The reason for the reduction in growth rate of WIL and lack of inhibition on H125 may be ascertained. Histology of dexamethasone treated WIL xenografts and *in vito* angiogenesis experiments using the cell line imply a reduction in angiogenesis. It is possible that the inhibition of tumour growth may relate to a reduction in PA activity as reported by Mira-y-Lopez et al (62). Similarly the lack of inhibition of tumour growth in H125 may relate to the lack of inhibition of PA in the cell line. The cytostatic effect of dexamethasone could also explain the reduced tumour growth in WIL.

The inhibition of growth of WIL as an xenograft is not due to a reduction in tumorigenicity. Pre-treated WIL cells *in vitro* when injected subcutaneously into nude mice showed a transient increase in tumour take relative controls. This reversibility may be due to the reversibility of the actions of dexamethasone.

8.6 Future Experiments and Prospects

An interesting avenue would be to carry out many experiments with a natural substratum present to see if a greater increase in differentiation is obtained.

The combination of dexamethasone with conventional cytotoxic drugs in xenograft experiments may improve tumour growth inhibition and suggest a role for the steroid in the chemotherapy of human non-small cell lung cancer.

SOURCE OF MATERIALS

AMERSHAM INTERNATIONAL

(^{3}H) methyl choline
L-(6-^{3}H) fucose
D. -(C-^{3}H (N)) - glucosamine hydrochloride
(1, 2, 4, (n) - ^{3}H) triamcinolone acetate

B.D.H.

Acetic acid
Ammonia
Ammonium Sulphamate
Carbon Tetrachloride
Chloroform
Ditriothreitol
Ethanol
EDTA
EGTA
Giemsa
Glycerol
HEPES
Magnesium Chloride
Mercapto Ethanol
Sodium Chloride
Sodium Nitrite
Tris Hydrochloride

Bio Rad

Neutral Alumina
Protein Assay Kit

Boehringer

Pronase

Flow

Hams F-10 (10x)
Hanks balanced salt solution (HBSS)
Plasminogen
Serum
S-2251
S-2238
S-2288

T-PA assay Kit

Gibco

Agar (2%)
Bicarbonate
DMEM
Glutamine
Serum
Trypsin

Leo Laboratories

Urokinase

Merck Sharp and Dohme

Dexamethasone

Northumbria Biologicals Limited

Serum

Pharmacia

Sepharose - conconavalin A

Scotlab

TSK columns

Sigma

Chondroitinase AC
Concanavilin A
Hyaluronidase
α - methyl mannoside
N - acetyl glucosamine
Osmium Tetroxide
Poly - D - lysine
Sodium Dodecyl Sulphate (S.D.S.)
Wheat Germ Agglutinin

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