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Glucocorticoid Receptors and Growth Inhibitory Effects of Dexamethasone in Human Lung Cancer Cell Lines

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Expression of glucocorticoid receptors (GR) and growth effects of dexamethasone and the antiglucocorticoid RU-486 were investigated in six cell lines originating from small cell lung cancer (SCLC) and 13 cell lines from non-small cell lung cancer (NSCLC; four adenocarcinoma, four squamous cell carcinoma, four large cell carcinoma and one mesothelioma). All cell lines contained specific and saturable binding sites for the synthetic glucocorticoid dexamethasone, as determined by whole cell assays and by cytosolic receptor assays. The presence of GRs in the carcinoma cells was confirmed by immunocytochemistry. In NSCLC cell lines, GRs were present in large amounts (37–638 fmol/mg cytosolic protein). In SCLC cell lines, GRs were also detectable but in considerably lower concentrations. Growth inhibitory effects of dexamethasone were seen in the cultures of two squamous cell carcinoma lines (EPLC-32M1 and NCI-H157), one adenocarcinoma line (A-549), one large cell carcinoma cell line (LCLC-97TM1) and the cell line of a mesothelioma (MSTO-211H). All cell lines responsive to dexamethasone had high GR concentrations (≥ 164 fmol/mg cytosolic protein). The antiglucocorticoid RU-486 was virtually inactive when administered alone but was able to block the growth-inhibitory effect of dexamethasone. The results indicate that glucocorticoids may inhibit the progression of individual non-small cell lung carcinoma.

Key words: lung cancer, cell lines, glucocorticoids, receptors, growth inhibition
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INTRODUCTION

GLUCOCORTICOIDS PLAY a well-established role in the treatment of leukaemia and lymphoma, acting through cytosolic and nuclear receptor proteins in malignant cells [1, 2], thereby inducing cytolysis [3]. Growth-regulatory effects of glucocorticoids in cell systems derived from solid tumours have also been observed by using xenografts [4] or cell lines *in vitro* [5, 6].

The glucocorticoid receptor (GR) is widely distributed in human tissues, but receptor activation and regulation are tissue specific. Stimulation as well as inhibition of malignant transformation by glucocorticoids has been reported [7]. Glucocorticoids may lead to a down-regulation of proto-oncogenes. In the human leukaemia cell line CEM-C7, triamcinolone leads to a decrease of *C-MYC* mRNA level and *C-Ki-RAS* mRNA level resulting in growth arrest.

As far as lung cancer is concerned, the possible influence of glucocorticoids has not been thoroughly evaluated [7]. A biological role for steroid hormones has been suggested by epidemiological investigations on small cell lung cancer (SCLC) [8] and by the observed stimulation of SCLC cell growth *in vitro* by dihydrotestosterone [9]. GRs have been observed in a number of cytosol preparations from non-small cell lung cancer (NSCLC) biopsies, together with smaller amounts of androgen, oestrogen and gestagen receptors [10, 11]. GRs have also been described in the permanent lung cancer cell lines A-549, originating from an alveolar carcinoma [12] and COR L103, established from a SCLC [13].

For A-549 cells, which may be regarded as a model of type 2 alveolar cells [12], glucocorticoids have marked inhibitory effects on cell growth [12, 14, 15]. In addition, the growth of a human lung adenocarcinoma xenograft [11], and the proliferation of the *V-HA-RAS* transformed normal human bronchiolar epithelial cell line, TBE-1 [15], are reduced by glucocorticoids. As has been pointed out [7], however, the published information about glucocorticoid effects on lung cancer cells is scarce.

In the present study, we demonstrate the common appearance of GRs in permanent cell lines derived from small, squamous and large cell carcinoma and from adenocarcinoma of the lung. Moreover, growth inhibition by dexamethasone was observed in

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five of the 13 NSCLC cell lines investigated; this effect could be blocked by the simultaneous addition of the antiglucocorticoid RU-486.

MATERIALS AND METHODS

Cell lines

The SCLC cell lines used in this study were DMS-79 [16], NCI-H60, NCI-H69, NCI-H82, NCI-H146 and NCI-H510 [17]; all grown in suspension cultures. In contrast, all NSCLC cell lines were grown in surface-adherent cell layers. A-549 [18], NCI-H23, NCI-H322 and NCI-H596 [19] were the cell lines originating from adenocarcinomas. The cell lines EPLC-32M1 [20], EPLC-272H [21], U-1752 [22] and NCI-H157 [17] were derived from squamous cell carcinomas, while the lines LCLC-97TM1, LCLC-103H [20], U-1810 [23] and NCI-H661 [24] were established from large cell carcinomas. MSTO-211H cells [20] were derived from a mesothelioma.

Culture media and hormones

The growth medium for the long-term culture of the cell lines was RPMI 1640 (Gibco, Grand Island, New York, U.S.A.) supplemented with 10% fetal bovine serum (FCS) (Boehringer, Mannheim, Germany). For the receptor determinations by whole cell assays and for the proliferation assays, a steroid-depleted medium (DC-medium) was used [25]; this was based on phenol red-free RPMI 1640 with 10% FCS which had been treated with dextran-coated charcoal [26].

Hormones were dissolved to a concentration of 10 mM in ethanol, and further diluted with phosphate-buffered NaCl solution (PBS) containing 2.5 g/l bovine serum albumin (Code No. A7030, Sigma; München, Germany) (PBS–BSA); test and control media were then adjusted to contain equal concentrations of PBS–BSA and ethanol.

Glucocorticoid receptor determinations

For the detection of GRs, three different methods were used, based on ligand binding and immunochemistry. GR values were calculated by the method of Scatchard [27].

Cytosolic receptor measurements of NSCLC cell lines. The cells were harvested from the culture bottles near confluence by treatment with 0.02% EDTA in PBS and washed twice with 0.9% NaCl. The cells were then mixed with 2 volumes of 0.9% NaCl, lysed by ultrasonic treatment and centrifuged at 104 000 *g*. The GR content of the cytosols (2–6 mg protein/ml) was determined by the dextran–charcoal method [28, 29]. [6,7-^{3}H(N)]dexamethasone (specific activity 44.7 Ci/mmol, Du Pont de Nemours, Dreiech, Germany) at final concentrations ranging from 0.5 to 16 nM was used as ligand; non-specific binding was determined by parallel incubations with 10 μM unlabelled dexamethasone (Sigma). Protein concentrations were determined with a commercial test kit (Bio-RAD Laboratories, München, Germany).

Whole cell receptor assays: NSCLC cell lines. Cells were plated in multiwell dishes (four wells, 2 cm^2 per well, Nunc, Roskilde, Denmark) at a density of 50 × 10^3 cells per well in growth medium. After a 1-day attachment phase, the cells were grown for 3 more days in DC-medium. Thereafter, the cell numbers were determined, and the wells destined for the binding studies were supplied with unused DC-medium (200 μl per well). Tritiated dexamethasone was added in 25 μl aliquots of PBS–BSA to achieve final concentrations varying from 0.1 to 25 nM in quadruplicate cultures. Non-specific binding was determined by adding 25 μl volumes of PBS–BSA with or without unlabelled dexamethasone (final concentration of 10 μM). The wells were incubated at 37°C for 2 h. Then the cellular monolayers were washed twice with cold DC-medium and lysed with 200 μl 1 M NaOH per well at room temperature overnight. After the addition of 200 μl 1 M HCl and 100 μl 1 M HEPES-buffer per well for neutralisation, the radioactivity of the solutions was determined in a β-counter [25].

Whole cell receptor assays: SCLC cell lines. Cells grown for 3 days in DC-medium were washed, and portions of 2 × 10^6 cells in 200 μl of DC-medium were pipetted into multiwell dishes (Nunc). Incubation with the ligands was performed as described for the NSCLC cell lines. Thereafter, 50 μl volumes of a 0.05% trypan blue solution were added for facilitating the following manoeuvre. The cell suspensions were laid on 2 ml cushions of cold PBS–BSA/20% glycerol in 5 ml tubes. The tubes were then centrifuged at 1000 *g* for 10 min, and the liquid contents discarded. The radioactivity of the resultant cell pellets was determined as described above.

Immunocytochemical receptor analyses. Cytospin preparations of the cell lines were fixed in Zamboni's fixative for 10 min. The monoclonal IgG antibody anti-GR 788–795, which detects an amino acid sequence of the C-terminal steroid binding region of the human GR, was used as the primary antibody [30]. Immunoreaction was performed with the avidin–biotin complex method (Vectastain ABC Kit, Camon Laborservice, Wiesbaden, Germany). To control for non-specific binding, the primary antibody was presaturated with an excess of the peptide GR 788-795.

Proliferation assays

Cell suspensions of 4 × 10^3 cells in 0.5 ml medium were pipetted into multiwell dishes (Nunc). While SCLC cells were plated directly in fresh DC-medium, NSCLC cells were plated in growth medium and changed to DC-medium after a 1-day attachment phase. Dexamethasone or RU-486 (kindly supplied by Schering A.G., Berlin, Germany) were added alone or in combination. For each hormone concentration and for the controls, at least quadruplicate cell cultures were employed [5, 31]. After an experimental incubation period of 6 days, the cell numbers in the wells were determined with a haemacytometer. SCLC cells were counted in aliquots derived directly from the incubation suspensions. NSCLC cells were first detached from the culture wells by treatment with defined volumes of EDTA–PBS.

RESULTS

Glucocorticoid receptor determinations

Glucocorticoid binding sites were found in all lung cancer cell lines tested. Whole cell assays and, as a confirmatory method, cytosolic receptor assays were used for the determination of receptor concentrations in NSCLC cell lines. For SCLC lines, which had much lower receptor concentrations, only the highly sensitive whole cell assays were employed. Saturation curves and Scatchard plots for both types of assays are exemplified by the NSCLC cell line, EPLC-32M1 (Figures 1 and 2).

As determined in the whole cell assays, NSCLC lines contained GR concentrations ranging from 31 × 10^3 to 314 × 10^3 sites per cell (Table 1). In particular, the cells originating from squamous cell carcinomas exhibited high receptor numbers

Table 1. Glucocorticoid receptor concentrations in non-small cell lung cancer cell lines as determined by cytosolic receptor assays and by whole cell assays

	Cytosolic assay		Whole cell assay	
Histology Cell line	GR (fmol/mg)	K_d (nM)	GR (sites/cell) $\times 10^3$	K_d (nM)
Adenocarcinoma				
NCI-H23	82	1.2	31	3.5
NCI-H322	43	0.5	42	10.0
A-549	638	0.6	314	9.3
NCI-H596	140	1.0	186	8.2
Squamous cell carcinoma				
EPLC-32M1	316	2.4	141	8.2
NCI-H157	200	0.2	211	7.7
EPLC-272H	282	0.5	147	8.7
U-1752	315	0.9	157	7.6
Large cell carcinoma				
LCLC-97TM1	164	0.6	139	7.7
LCLC-103H	63	0.8	66	7.3
NCI-H661	80	0.6	86	2.6
U-1810	37	6.1	47	12.0
Mesothelioma				
MSTO-211H	177	0.8	63	5.4

Table 2. Glucocorticoid receptors in small cell lung cancer cell lines as determined by whole cell assays

Cell line	GR (sites/cell) $\times 10^3$	K_d (nM)
NCI-H60	1.9	1.3
NCI-H69	2.0	3.2
DMS-79	24.0	14.0
NCI-H82	1.1	3.1
NCI-H146	5.4	5.4
NCI-H510	+*	

* Detected, not quantified.

($\geq$ 141 $\times$ 10^3 sites per cell). Among the other NSCLC types, the adenocarcinoma cell lines A-549, NCI-H596 and the large cell line LCLC-97TM1 also had high GR concentrations ($\geq$ 139 $\times$ 10^3 sites per cell). The dissociation constants K_d in the whole cell assays ranged from 2.6 to 12 nM.

With separate batches of the NSCLC cell lines, GR concentrations were analysed by cytosolic receptor assays. By this method, receptor concentrations ranging from 37 to 638 fmol per mg cytosolic protein were found in the NSCLC cell lines, with dissociation constants ranging from 0.5 to 6.1 nM. As in the whole cell assays, A-549 cells exhibited the highest concentration of GR (638 fmol/mg). Among the other NSCLC cell lines, the squamous cell carcinoma lines contained marked amounts of GR as measured by the cytosolic assay (200–316 fmol/mg). The GR concentrations determined by the two methods were well correlated. With the exception of MSTO-211H cells, high GR concentrations measured by the cytosolic assay ($\geq$ 140 fmol/mg cytosolic protein) corresponded to high GR values measured by the whole cell assay ($\geq$ 139 $\times$ 10^3 sites per cell).

SCLC lines had a comparatively low GR content; the whole cell measurements yielded concentrations from 1.1 $\times$ 10^3 to 24 $\times$ 10^3 sites per cell with dissociation constants ranging from 1.3 to 14 nM (Table 2). DMS-79 was the only SCLC cell line in which the glucocorticoid binding sites (24 $\times$ 10^3 sites per cell) approximated that of the NSCLC cell lines with low receptor content.

The presence of GR in SCLC and NSCLC cell cultures was also detected using immunocytochemistry. Using the monoclonal antibody anti-GR 788–795, specific staining was observed with all cell cultures examined. Generally, while a distinct reaction was detected in the nuclei of the cells, the cytoplasm exhibited only a faint reaction pattern. This is illustrated for the cell line EPLC-32M1 (Figure 3). A heterogeneity in staining intensity was typically observed.

Proliferation assays

In the proliferation experiments, five NSCLC cell lines (EPLC-32M1, LCLC-97TM1, NCI-H157, MSTO-211H and A-549) exhibited a dose-dependent growth inhibition due to dexamethasone, tested at 10 nM and 1 μM concentrations. In an assay with EPLC-32M1 cells, higher concentrations of the glucocorticoid (10 μM) were no more effective than 1 μM dexamethasone (data not shown). RU-486 had negligible effects

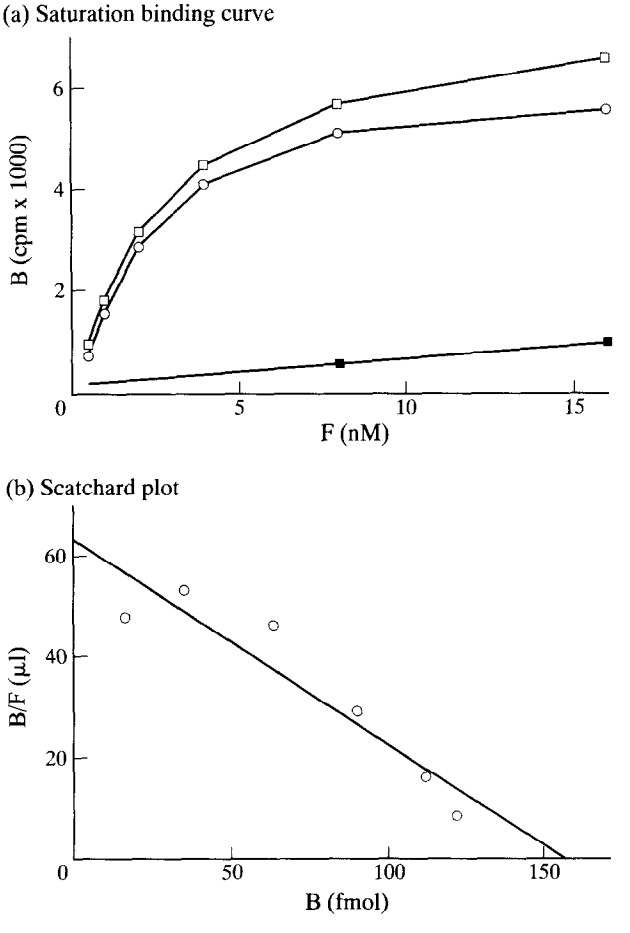

Figure 1. Cytosolic assay for glucocorticoid receptors in EPLC-32M1 cells. Open squares: total binding, solid squares: non-specific binding, open circles: specific binding.

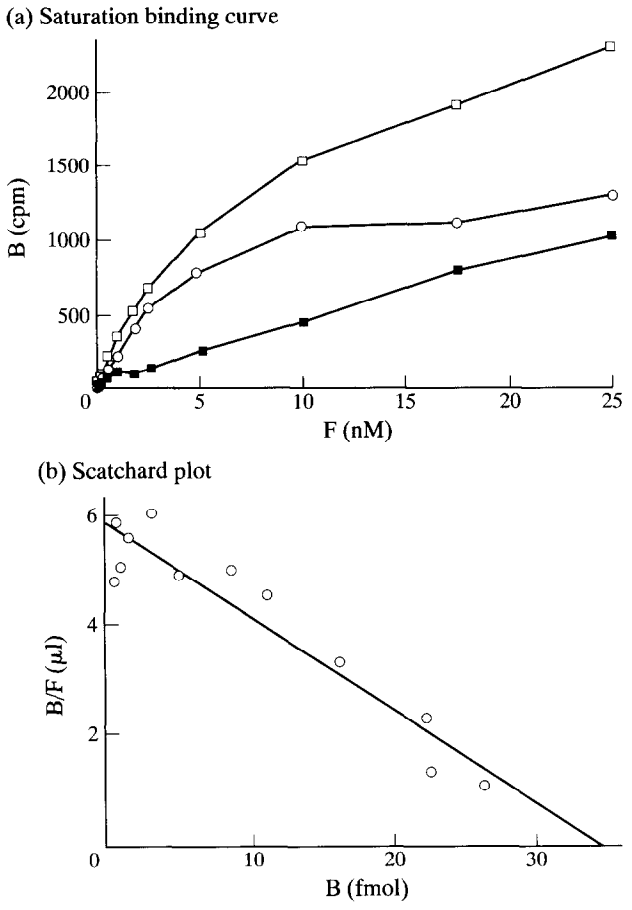

Figure 2. Whole cell assay for glucocorticoid receptors in EPLC-32M1 cells. Open squares: total binding, solid squares: non-specific binding, open circles: specific binding.

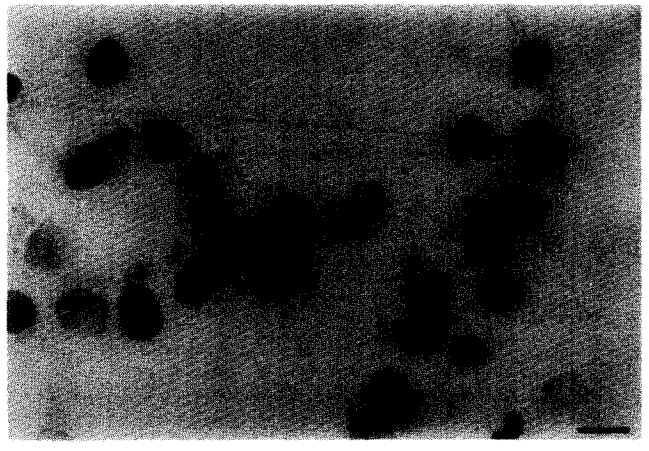

Figure 3. Demonstration of glucocortoid receptors in EPLC-32M1 cells by immunocytochemistry with the monoclonal antibody anti-GR 788-795. Bar corresponds to 13 µm.

on the growth of the NSCLC cell lines, when administered alone at either 10 nM or 1 µM concentrations. The antiglucocorticoid at 1 µM concentration was able to block the growth inhibition induced by dexamethasone in the glucocorticoid-sensitive cell lines. The effects of the synthetic hormones on the cell growth

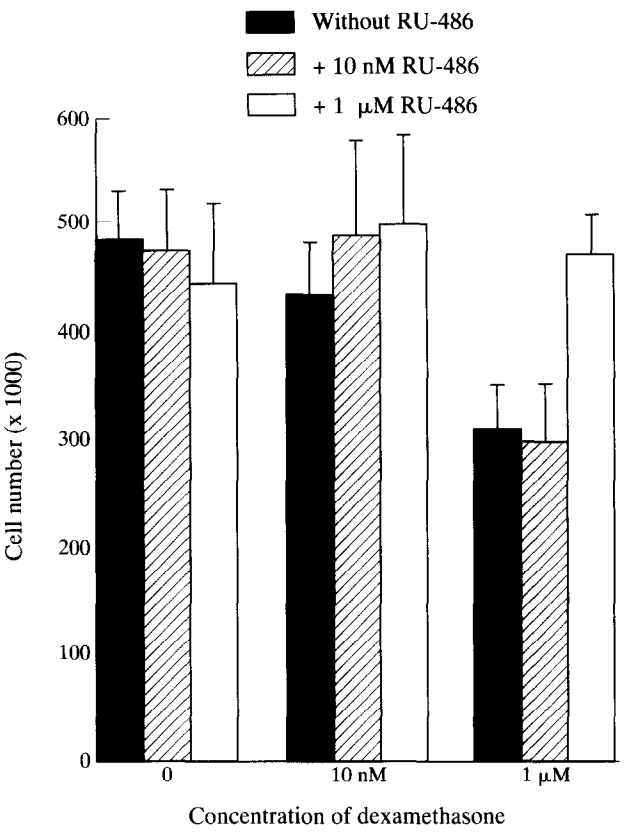

Figure 4. Inhibition of the proliferation of EPLC-32M1 cells by dexamethasone and reversal of the glucocorticoid induced growth inhibition by RU-486. The cell numbers were determined at the end of a 6-day incubation period; standard deviations are indicated. An effective growth inhibition is observed at 1 µM dexamethasone in the incubation medium; this effect is completely reversed by RU-486 at concentration 1 µM. The lower concentrations (10 nM) of the synthetic hormones were ineffective in this experiment.

are illustrated by an assay using the squamous carcinoma cell line EPLC-32M1 (Figure 4).

The growth of the most sensitive cell line, A-549, was inhibited by 1 µM dexamethasone to 6% compared to the corresponding control cultures. This cell line was also strongly inhibited by 10 nM dexamethasone to 26% of the control growth. EPLC-32M1, LCLC-97TM1, NCI-H157 and MSTO-211H cells required the higher dexamethasone concentration (1 µM) for effective growth reduction. The inhibition of cell proliferation by 1 µM dexamethasone is compared in Table 3 for the five

Table 3. Growth inhibition of non-small cell lung cancer cell lines by dexamethasone

Cell line	Cell counts after a 6-day incubation in the presence of 1 µM dexamethasone (% of counts in hormone-free controls)
EPLC-32M1	61 ± 4*
LCLC-97TM1	73 ± 1*
NCI-H157	59
MSTO-211H	46 ± 8†
A-549	6

*,†, means ± S.D. of two (*) or four (†) independent experiments. For each experiment, the results were highly significant ($P < 0.05$) as determined by the unpaired, two-tailed Student's t-test.

glucocorticoid-sensitive cell lines. The growth inhibition was highly significant for all cell lines demonstrated. With regard to the other NSCLC cell lines and all SCLC cell lines used in this study, no distinct effects of dexamethasone on the cell growth were observed.

DISCUSSION

In this investigation we were able to demonstrate the presence of GR in six SCLC and 13 NSCLC cell lines. Whole cell assays and cytosolic receptor assays were used to quantify the glucocorticoid binding sites, and immunocytochemistry confirmed the presence of GRs in the cell lines. Moreover, we found that the glucocorticoid dexamethasone could inhibit the growth of five of the NSCLC cell lines; reversal of growth inhibition by the antiglucocorticoid RU-486 indicated that the antiproliferative effects were mediated by GRs.

NSCLC cell lines exhibited a great diversity in GR content. Except for the alveolar carcinoma cell line A-549, squamous carcinoma cell lines had the highest receptor concentrations. Among the cell lines originating from other types of pulmonary cancer, NCI-H596 cells (adenocarcinoma), LCLC-97TM1 cells (large cell carcinoma) and MSTO-211H cells (mesothelioma) had high GR concentrations ($\geq$ 140 fmol/mg cytosolic protein). The high glucocorticoid binding capacity of A-549 cells as determined in this study is similar to a value reported previously (570 fmol/mg) [12].

The amounts of GR present in the NSCLC cell lines with low glucocorticoid binding capacity are in the range of values determined in primary lung carcinoma: in biopsies of squamous cell carcinoma and of adenocarcinoma of the lung, mean GR contents of 12.8 and 123 fmol/mg, respectively, were found [32]. In another study [10], mean GR concentrations of 50.3 for squamous, 66.3 for large cell carcinoma and 87.8 fmol/mg for adenocarcinomas of the lung were determined.

Related to the protein content, the GR concentrations of the cell lines derived from squamous cell carcinoma and four of the other cell lines (LCLC-97TM1, MSTO-211H, A-549 and NCI-H596) were considerably higher than the mean biopsy values quoted above. This may be explained by the frequent contamination of tumour biopsies by adjacent vessels, normal lung epithelium and other non-tumorous tissues. In agreement with this hypothesis, other authors [11] reported high GR concentrations in nude mouse xenografts of primary human lung tumours that had been passaged several times (13.3–437.4 for adenocarcinomas, 33.2–510.4 for epidermoid and 58.6–167.1 fmol/mg protein for large cell carcinomas).

The dissociation constants for dexamethasone binding in SCLC and NSCLC cells were in the range of values obtained by others for lung cancer biopsies and cell lines [10–13]. When comparing the dissociation constants of whole cell and cytosolic assays, there was a tendency to higher values for the dissociation constants in the whole cell assays, which may be due to the different incubation conditions used.

The growth of approximately one third of the NSCLC cell lines tested was inhibited by the synthetic glucocorticoid dexamethasone. The present study confirms the marked antiproliferative effect of dexamethasone on A-549 cells [12, 14, 15]. The growth inhibition of the cell lines EPLC-32M1, LCLC-97TM1, NCI-H157 and MSTO-211H was less pronounced, but still highly significant. It is conceivable that heterogeneity of the tumour cell populations, also observed in the immunocytochemical GR analysis, could be responsible for the partial resistance to growth inhibition in these cell lines, but other explanations are also possible.

RU-486 belongs to a new class of synthetic antigestagenic compounds and is known, in addition, to possess strong antiglucocorticoid activity [33]. The simultaneous addition of RU-486 was able to block the antiproliferative effects of dexamethasone in the glucocorticoid-sensitive cell lines in a dose-dependent manner, consistent with the competition of both substances at the receptor level.

All cell lines sensitive to dexamethasone had high GR concentrations ($\geq$ 164 fmol/mg cytosolic protein). It may, therefore, be speculated that high receptor concentrations are a prerequisite for the growth inhibition induced by dexamethasone. This hypothesis is supported by the observation that growth inhibitory effects of hydrocortisone on two human lung adenocarcinoma xenografts were correlated to the GR tissue concentrations [11]. A correlation between GR content and dexamethasone sensitivity has also been shown for other solid tumour models [4]. Moreover, it has been reported that the disease-free survival of patients who have undergone surgery for squamous cell lung carcinoma tended to be more favourable for patients with GR-rich tumours [34], an observation that may indicate inhibitory effects of physiological concentrations of glucocorticoids on NSCLC tumour progression.

The molecular mechanisms of the growth inhibition of NSCLC cells by glucocorticoids remain to be clarified. Some NSCLC cell lines of this study contained large amounts of GR and did not respond to dexamethasone; this observation may indicate defective growth inhibitory mechanisms at the receptor or postreceptor level. In a variety of tumours which are growth regulated by steroids, mutations in receptor genes have been found which may lead to steroid resistance. Alterations of the androgen receptor in cell lines stemming from prostate carcinoma [35] as well as of the oestrogen receptor in breast cancer [36] have been described. However, in leukaemia cell lines the vast majority of genetic alterations leading to glucocorticoid resistance could be attributed to loss of GR mRNA expression [37].

SCLC cell lines had comparatively lower amounts of GR. Prior to this investigation, cytosolic receptor assays performed with SCLC biopsies have indicated the complete absence of GR [32]. To our knowledge, only one SCLC cell line (COR L103) has previously been described as positive for GR [13]. Therefore, the frequent presence of small amounts of GR in SCLC cell lines may be of some interest. The growth of all SCLC cell lines used in this study was insensitive to dexamethasone. Clark and associates [13] found that in the ACTH (adrenocorticotrophic hormone)-producing SCLC cell line COR L103, regulation of the pro-opiomelanocortin, the tyrosine amino transferase and the GR genes by glucocorticoids, as known from other cell systems, was defective. The functions of GR in SCLC thus remain to be identified. In view of the small receptor concentrations, assays aimed to detect GR functions in SCLC may need extremely high sensitivity. Further efforts should elucidate the relevance of GR to this type of lung cancer.

Glucocorticoids may be administered to patients with NSCLC with dose-dependent, tolerable side-effects. A treatment of NSCLC of high GR content with dexamethasone or other synthetic glucocorticoids could form a strategy in influencing a disease with low sensitivity to conventional chemotherapy.

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