Glucocorticoid-induced Growth Inhibition of Cells from a Human Lung Alveolar Cell Carcinoma

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ABSTRACT

We have studied the effects of dexamethasone on cell growth and DNA synthesis in the A549 cell line, a line that was originally isolated from a human alveolar carcinoma. Dexamethasone at a concentration of 10^{-8} M inhibited growth of these cells. Maximal growth inhibition was seen with 4 \times 10^{-8} M dexamethasone (44\% of control). The population-doubling time of the cells increased from 28.4 hr under control conditions to 66.5 hr with dexamethasone treatment. Dexamethasone also inhibited DNA synthesis within the same concentration range that inhibited cell growth. Decreased [3H]thymidine incorporation was observed as early as 6 hr after steroid addition. Inhibition of growth and DNA synthesis by other steroids was observed and was of the order: dexamethasone > cortisol > corticosterone > deoxycorticosterone. Testosterone and estradiol were not inhibitory. The cells were also examined for presumptive evidence of cytoplasmic [3H]dexamethasone receptors. Specific binding of [3H]dexamethasone was observed, and Scatchard analysis of the data revealed that K_d equaled 7.1 \times 10^{-9} M. The ability of other steroids to decrease the binding of [3H]dexamethasone was related to their ability to inhibit A549 cell growth and their biological activity as glucocorticoids. We feel that the A549 cell line will provide a system in which to study a pattern of glucocorticoid-induced growth inhibition that is not associated with marked catabolic effects and cell lysis and that may provide a model for the study of the relationship of steroid-induced differentiation to growth.

INTRODUCTION

Glucocorticoid effects on the growth of cells in culture have been studied in many cell systems. Corticosteroid stimulation of in vitro growth has been reported in synovial cells (6), lung fibroblasts (35), human skin fibroblasts (7), and HeLa cells (40). Conversely, growth inhibition by glucocorticoids has been associated with skin fibroblasts (13, 18) as well as with several established malignant and nonmalignant cell lines (22) and with cells of lymphoreticular origin (15, 28, 31, 39). The mechanisms by which these steroids can differentially stimulate or inhibit cell growth in vitro are not understood, especially in cells from tissues in which the hormones specifically stimulate a differentiated function. We have begun to study steroid hormone effects on growth and the relationship of growth stimulation and arrest to induction of differentiated function and have chosen lung as the tissue on which the studies will be initiated.

Glucocorticoids accelerate the appearance of pulmonary surfactant in developing mammalian fetal lungs (8, 24, 25) and increase the synthesis of phospholipids important in surfactant function (10, 30). Additional studies have shown that hydrocortisone produces lower lung weight:body weight ratios than those seen in controls (5) and also slows the mitotic rate in fetal rabbit lung (20).

In vitro studies have yielded different results in different systems. Rabbit lung cell cultures show gestational age-dependent variability, with enhancement of growth when lungs are removed at 20 to 22 days of gestation but impairment of growth when the lungs are cultured at 29 days of gestation (36). Early human fetal lung cultures show growth acceleration in response to cortisol (35).

While the specific hormone response of lung is thought to occur as a result of differentiation of the type 2 alveolar cell (3, 19, 21), all studies on growth have been performed with tissue fragments or mixed cell cultures. We have used the A549 tumor cell line as a model for investigation of the effect of glucocorticoids specifically on growth of type 2 alveolar cells. These cells were derived from a human lung alveolar cell carcinoma (12) and have been shown to contain lamellar bodies and to synthesize lecithin (23), both features of type 2 alveolar cells.

MATERIALS AND METHODS

Chemicals and Supplies. DM,^3 hydrocortisone, deoxycorticosterone, corticosterone, 17 /beta-estradiol, testosterone, and all other reagent grade chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Tissue culture medium was purchased from Microbiological Associates, Inc. (Bethesda, Md.). Horse serum was supplied by Grand Island Biological Co. (Grand Island, N. Y.), and FBS was supplied by Irvine Scientific (Irvine, Calif.). [methyl-3H]Thymidine (20 Ci/mmole) and [3H]DM (30 Ci/mmol) were from New England Nuclear (Boston, Mass.). Tissue culture dishes were from Falcon Plastics (Oxnard, Calif.), and multiwell dishes were from Linbro Chemical Co. (New Haven, Conn.). Phosphate-buffered saline was made with NaCl (8 g/liter), KCl (0.2 g/liter), Na_2HPO_4 (1.15 g/liter), and KH_2PO_4 (0.2 g/liter).

Cells. The A549 cell line was generously supplied by Dr. Walter Nelson-Rees. The cells were cultured in Dulbecco's

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modified Eagle’s medium with 12.5% horse serum and 2.5% FBS. Subculturing was done with a solution of trypsin (Difco; 1:250) (0.5 g/liter) with sodium EDTA (0.2 g/liter), NaCl (8 g/liter), KCl (0.4 g/liter), NaHCO₃ (0.85 g/liter), and glucose (1 g/liter).

**Cell Growth Experiments.** For growth experiments, cells were plated at low density in designated medium. After 24 hr, medium was changed or steroids were added. At the time of counting, medium from each sample was centrifuged at 1000 rpm with a CT-1340 head in a Dynac centrifuge for 15 min to collect floating cells. The remainder of the cells were removed from the dishes with buffered trypsin solution and were pooled with the floating cells prior to resuspension and counting. Cells were counted in a Model ZBI Coulter cell counter.

**DNA Synthesis Experiments.** For measurement of DNA synthesis, cells were plated in Linbro 6-well dishes at subconfluent density in serum-containing medium. After cell attachment, medium was changed to serum-free medium for 24 hr prior to addition of test substances. After 24 hr of incubation with steroid, the cells were pulsed for 2 hr with [³H]thymidine (1 μCi/ml). Monolayer cultures were processed for scintillation counting as previously described (17). Maximum initial ethanol concentration (10⁻⁷ M steroid) was 1.7% and had no effect on growth or DNA synthesis.

**Steroid Binding Studies.** For steroid binding studies, confluent plates of cells were scraped in medium and collected by centrifugation. The pellet was washed twice with phosphate-buffered saline and resuspended in 0.01 M Tris with 0.001 M EDTA, pH 7.4. This and all subsequent operations were carried out at 4°C. The cells were homogenized with 3 strokes in a Teflon:glass homogenizer. The homogenate was centrifuged at 105,000 × g for 1 hr. One ml fractions of the high-speed supernatant (cytosol) were incubated with [³H]DM in the presence and absence of a 1000-fold excess of nonradioactive steroid. The unbound steroid was removed at specified times with Dextran-coated charcoal as previously described (1), with the following modifications. After incubation of a 1-ml aliquot of cytosol with [³H]DM for 15 min at 4°C, a 400-μl portion of this was placed in a polypropylene tube with 100 μl of 10% charcoal:1% dextran in Tris:EDTA buffer. Each tube was mixed vigorously for 30 sec, incubated at 0°C for 15 min, and then centrifuged at 12,000 × g for 20 min. One 100-μl sample was counted in a Nuclear Chicago liquid scintillation counter in a mixture of PPO, POPPOP, toluene, and Triton X-100. A second 100-μl sample was analyzed for protein content by the method of Lowry et al. (27). The data are presented as either mol or cpm of [³H]DM bound per mg of protein.

For the correction for the presence of nonspecific binding, cytosols incubated with [³H]DM were simultaneously incubated with a 1000-fold excess of nonradioactive steroid. The [³H]DM bound in the presence of a 1000-fold excess of nonradioactive steroid was subtracted from the total amount of radioactive DM bound to designate the binding as specifically bound steroid.

**RESULTS**

For the establishment that glucocorticoids inhibit the growth of A549 cells and for definition of the concentration ranges that were effective in this inhibition, we studied the effect of increasing concentrations of DM on cell growth (Chart 1). Growth inhibition was concentration dependent over a range from 2 × 10⁻⁹ to 4 × 10⁻⁷ M with little additional inhibition at higher DM concentrations to 10⁻⁶ M. At the end of the growth period, the cell number in 10⁻⁶ M DM was 94% of that in control plates while the cell number in maximally inhibited plates was 44% of that in 10⁻⁶ M DM.

DM also inhibited DNA synthesis in these cells. Chart 2 illustrates that the dose response of this inhibition is similar to that seen with cell growth. Significant depression of [³H]thymidine incorporation into DNA is seen after incubation with 4 × 10⁻⁷ M DM. The inhibition is maximal at 4 × 10⁻⁸ M with no additional inhibition at DM concentrations to 4 × 10⁻⁷ M. Inhibition of DNA synthesis in these cells begins at a DM concentration slightly higher than that reported to inhibit plating of S49 lymphoma cells (34) and lower than that reported to inhibit DNA synthesis of hepatoma cells (26). However, in each case the lowest concentration required for inhibition was between 10⁻⁸ and 10⁻⁷ M.

The pattern of growth arrest produced by glucocorticoids is seen in Chart 3. The cells appear to grow at a rate equal to...
that of the controls for the 24 hr following addition of DM and to slow with a clear difference in growth rate between 2 and 9 days. Population-doubling time in log-phase growth was 24.4 hr in Dulbecco’s modified Eagle’s medium with 10% FBS and 66.5 hr when DM was added at 10^{-8} M.

The effects of DM on DNA synthesis are apparent soon after its addition. In Chart 4 it can be seen that there is a divergence between the curves of control and glucocorticoid-treated plates within 6 hr of exposure. After transfer to serum-free medium, the cells show a transient slowing of [3H]thymidine incorporation into DNA. In contrast to control cells that begin to synthesize DNA in the absence of serum, cells exposed to DM do not demonstrate any significant recovery and continue at a relatively low basal rate. The background levels of DNA synthesis are significant and indicate that some cells are passing through S phase of the cell cycle. This could indicate that DNA synthesis has not been arrested but only slowed or that there are steroid-resistant cells in the population that do not arrest. Growth curves suggest that slowing is occurring, inasmuch as there is a real decrease in growth rate rather than a lowering of the control curve with parallel slope.

These cells continue to synthesize DNA in the absence of serum. The cells appear to survive well in serum-free medium for the period tested with normal morphology and continued attachment. Growth in serum-free medium has been described in other cell strains (37) and tumor lines (9).

We tested the effects of several other steroid hormones to see whether there was glucocorticoid specificity in the inhibition of DNA synthesis. The results are shown in Chart 5. The data here are expressed as percentage of inhibition compared to control cells with no steroid present. DM was the most potent inhibitor, depressing DNA synthesis by 56% at 5 \times 10^{-8} M and by 73% at 5 \times 10^{-7} M. Other steroids that inhibited DNA synthesis, listed in order of decreasing potency, were hydrocortisone, cortisone, deoxycorticosterone, and progesterone. Estradiol and testosterone did not produce significant inhibition. The potency of the steroids as inhibitors of DNA synthesis is similar to their known biological potency as glucocorticoids (16, 32). Testosterone and estradiol did elicit changes at concentrations between 10^{-8} and 10^{-6} M. At these high concentrations, toxic effects and cell death were noted. Fig. 1 shows the increased vacuolization produced by exposure to high concentrations of estradiol for 48 hr. In contrast, DM produces an enlargement and spreading of the cells.

Binding of [3H]DM. The possibility that the inhibitory effects of glucocorticoids in A549 cells are mediated through a hormone receptor was assessed by examining cytosol fractions for specific [3H]DM-binding proteins. When cytosols prepared from homogenates of the cells were incubated with 6 \times 10^{-9} M [3H]DM \pm 6 \times 10^{-6} M nonradioactive DM for 2.5 hr, specific cytosol binding was observed.

The time course of this binding was delineated by incubating the cytosols with [3H]DM \pm 6 \times 10^{-6} M nonradioactive DM for time intervals of up to 48 hr at 0°C. Although specific binding was demonstrated at the earliest time investigated (<15 min), maximal binding was noted after 2 to 4 hr of incubation with [3H]DM. The amount of specific [3H]DM binding at selected times between 2 and 5 hr was.
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Fig. 1. Morphological changes in A549 cells after exposure to different steroids. a, cells grown under control conditions in Dulbecco's modified Eagle's medium with 10% FBS; b, cells grown in the same medium with exposure to \(5 \times 10^{-5}\) M DM for 48 hr; c, cells grown in the same medium with \(5 \times 10^{-5}\) M estradiol for 48 hr.

not significantly different; therefore, cytosols were routinely incubated for 2.5 hr with \[^{3}H\]DM prior to the assay for specific binding.

For the investigation of the affinity of the sites involved in this binding, cytosols were incubated over a 200-fold range of \[^{3}H\]DM (\(3.0 \times 10^{-10}\) M, \(6 \times 10^{-10}\) M, \(.3 \times 10^{-8}\) M) with and without a constant 1000-fold molar ratio of nonradioactive DM. The specific binding of \[^{3}H\]DM was noted at each concentration, and the data were analyzed by the method of Scatchard (33). As shown in Chart 6, it appears that \[^{3}H\]DM binds with only 1 affinity in cytosols prepared from A549 cells. The \(K_d\) of the binding was \(7.1 \times 10^{-9}\) M with a maximum number of binding sites of \(5.7 \times 10^{-13}\) mol/mg of cytosol protein.

Competition Studies. The ability of other steroids to decrease binding of \[^{3}H\]DM was evaluated. After incubation of cytosols with \[^{3}H\]DM, biologically similar (cortisol, corticosterone, deoxycorticosterone) and dissimilar (estradiol, testosterone, progesterone) steroids were also assessed in vitro.

The choice of concentrations of \[^{3}H\]DM used in these studies (\(6 \times 10^{-8}\) M) was based on the Scatchard analysis of the \[^{3}H\]DM binding as a concentration that approximated one-half saturation of the \[^{3}H\]DM sites. Thus, when the cytosols were incubated with \[^{3}H\]DM together with either a 10- or 100-fold molar excess of nonradioactive steroid, it could be assumed that steroids with an appreciable affinity for the same sites would saturate the specific \[^{3}H\]DM sites. The results of this study (Table 1) document the relationship between the ability of various steroids to decrease the amount of \[^{3}H\]DM bound and their potencies as glucocorticoids. When a 100-fold molar excess of nonradioactive steroid was utilized in a competition study, it appeared that, although DM was most effective in decreasing the amount of \[^{3}H\]DM bound, cortisol and corticosterone were also quite effective. Deoxycorticosterone, progesterone, and cortexolone were intermediate in decreasing the \[^{3}H\]DM bound while estradiol and testosterone were virtually without effect. When a 10-fold molar excess of these steroids was incubated in cytosols together with \(6 \times 10^{-8}\) M \[^{3}H\]DM, the pattern of inhibition of \[^{3}H\]DM binding was virtually identical with that found with a 100-fold excess.

DISCUSSION

These studies establish that A549 cells derived from an alveolar cell carcinoma have their in vitro growth and DNA synthesis inhibited by glucocorticoids. This inhibition is dependent on the steroid concentration and occurs with a physiological range of cortisol concentration equivalents. Decreased DNA synthesis can be detected as early as \(6\) hr after the addition of DM, while detectable depression of growth is not apparent until cells have been exposed to DM for 2 days.

One of the difficulties in interpreting growth inhibition in cultures that may have mixed cell populations is that of
deciding whether all or part of the population is affected. Growth curves suggest to us that the steroid-induced growth inhibition is a uniform slowing of growth rate. When a specific treatment produces a cell number lower than those of controls following a given growth period, 1 possible explanation is that some of the cells are killed or arrested while others grow on at an uninhibited rate. In this case, plots of growth rate show parallel curves with the "inhibited" cells tracing the lower curve and intercepting the ordinate at approximately the number of resistant cells originally plated. DM treatment of A549 cells alters the slope of the curve and the doubling time, indicating that most if not all of the cells are affected.

The inhibition of growth and DNA synthesis by steroids in A549 cells appears to be glucocorticoid specific. The potency of the steroids parallels their known pharmacological activity as corticosteroids. The presence of cytosol receptors for [3H]DM and the ability of glucocorticoids to decrease the amount of [3H]DM bound further suggest that these effects are receptor mediated.

Previous studies of the effects of steroid hormones on malignant cells have concentrated on lines derived from hematological and reticuloendothelial neoplasms (14), uterine cancer (29), breast cancer (4), and cancer of the prostate (11). Experimental studies of glucocorticoid effects on hepatoma and neoplastic lymphoid cells have elucidated some of the mechanisms by which these hormones inhibit growth. The 2 major patterns of response are (a) catabolic or cytolytic, in which cells exhibit decreased uptake of precursors, decreased protein and RNA synthesis, inhibition of certain enzyme systems, decreased DNA synthesis and growth, and, in many cases, cell death (34); and (b) growth arrest, in which cells exhibit decreased DNA synthesis and growth but with less decrease in nutrient and precursor uptake and less decrease in RNA and protein synthesis (26).

The inhibitory response in several cell types that respond "catabolically" has been well studied (34). From this work it is apparent that (a) a cytoplasmic steroid receptor and (b) the ability to transmit and localize steroids to the nucleus are important for glucocorticoid-induced cytolyis. Less is known about the inhibitory mechanism in cells that respond with growth arrest but continued metabolic activity. This has been studied in hepatoma cells (26) with results that are similar to those reported here. The hepatoma cells cease DNA synthesis and growth 6 to 12 hr after introduction of hydrocortisone. The A549 cells show a similar pattern of arrest of DNA synthesis but do not completely arrest growth in serum-containing medium. As is the case with A549 cells, hepatoma cells are not killed by hydrocortisone. Fifty % inhibitory concentrations in both systems are remarkably similar.

It is possible that this effect of glucocorticoids is associated with an induction of differentiated function. Glucocorticoids have been shown to be associated with inhibited lung cell growth (5, 20) and induction of phospholipid synthesis (10, 21). This induction of differentiation has not been well characterized, and we know little of the effects of glucocorticoids on mature type 2 alveolar cells. There is variability in other systems. The induction of tyrosine aminotransferase in HTC cells by DM is not associated with inhibition of growth (38). On the other hand, adrenocorticotropic hormone induction of steroid synthesis in adrenal cells is associated with growth-inhibited and similar "anabolic" responses (41) to those seen in the hepatoma cells in response to hydrocortisone (26).

We feel that this is an excellent system in which to study this interesting pattern of glucocorticoid-induced growth inhibition. We also feel that the system may yield many valuable insights into the relationship of steroid-induced differentiation and growth, especially in type 2 lungs where this relationship is particularly important both biologically and clinically.

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REFERENCES

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