In Vitro Growth Inhibition of Human Malignant Melanoma Cells by Glucocorticoids

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ABSTRACT

The human malignant melanoma cell line, NEL, was found to contain glucocorticoid receptors. When the binding data were analyzed according to the method of Scatchard, results indicated a ligand binding capacity of 247 fmol/mg protein and a Kd of 1 x 10⁻⁷ M. Additional studies showed that the continuous incubation of NEL cells with triamcinolone acetonide (TA) for 72 hr results in a 30% inhibition in cell growth. To ascertain the mechanism by which glucocorticoids inhibit the growth of NEL cells, uptake and incorporation studies were carried out using various ³H precursors. Results indicate that, after 4 hr of TA treatment, a modest inhibition in [³H]thymidine uptake was observed, while stimulation of [³H]thymidine incorporation was noted at all steroid concentrations tested. However, cells incubated for 18 hr with TA (concentration, >10⁻⁸ M) showed a 30% decrease in the amount of [³H]thymidine incorporated into DNA. TA had no effect on [³H]leucine or [³H]glucose uptake after 4 hr of treatment but did inhibit [³H]glucose (42%) uptake after 18 hr of treatment. A slight stimulation (9%) in [³H]leucine incorporation was observed at this time point. When NEL cells were incubated with TA and the antiguocorticoid, progesterone, the inhibition in [³H]thymidine incorporation was negated. These findings indicate that glucocorticoids exert some influence on the growth of human melanoma cells, and this effect is mediated through the glucocorticoid receptor.

INTRODUCTION

Demonstration of the presence of intracellular steroid receptors is a prerequisite for a tissue to be classified as a target site for hormonal action (2). Recently, it has been shown that biopsy samples taken from patients with malignant melanoma contain glucocorticoid receptors (3, 5, 13). These findings suggest that human melanoma cells should respond in some manner to glucocorticoid treatment.

In addition to the above reports concerning the identification of glucocorticoid receptors in human melanoma extracts, others have reported the presence of glucocorticoid receptors in the B16 murine melanoma cell line (4), the Syrian hamster melanoma cell line, RPMI 3460 (8), and the hamster malignant melanoma, MMI (16). With respect to the B16 and RPMI 3460 melanoma cell lines, studies have shown that exposure to glucocorticoids results in a significant inhibition in cellular growth (1, 4, 9). However, another study using the hamster malignant melanoma, MMI, has shown that glucocorticoid treatment enhanced tumor growth (15). This same report also demonstrated that bilateral adrenalectomy retarded tumor growth.

To our knowledge, there have been only 2 reports concerning the response of human malignant melanoma to glucocorticoid treatment. Johnson et al. (10) reported that a steroid with glucocorticoid activity produced objective tumor regression in 5 of 44 patients, while Chaudhuri et al. (5) reported that glucocorticoid treatment induced exacerbation of the disease. Since the studies are not in agreement concerning the response of the tumor to glucocorticoid treatment, the therapeutic value of glucocorticoids in human malignant melanoma is uncertain. Therefore, our laboratory has undertaken an investigation to determine what effects glucocorticoids have on a human malignant melanoma cell line which we have established recently in culture. The report which follows indicates that this cell line contains a specific glucocorticoid receptor, and the biological response of the cell to the hormone is a decrease in cellular growth.

MATERIALS AND METHODS

Materials. [1,2,4-³H]TA (26 Ci/mmol), [5'-³H]thymidine (15 Ci/mmol), o-[2-³H]glucose (12 Ci/mmol), and L-[4,5-³H]leucine (147 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, Ill. TA, cortisol, progesterone, 17ß-estradiol, and dihydrotestosterone were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of reagent grade.

Cell Culture. Human malignant melanoma cells were obtained from a biopsy of an i.d. metastatic melanoma in a white male patient. The established melanin-producing cell line, designated NEL, was maintained in MEM supplemented with 10% fetal bovine serum, gentamicin (50 µg/ml), and 2 mM glutamine (pH 7.4). Cells were grown in Corning T-75 sq cm flasks and maintained at 37° in a humidified atmosphere of 5% CO₂ in air. All cell culture media and sera were purchased from M. A. Bioproducts, Walkersville, Md.

Growth Curve. To evaluate the effects of glucocorticoids on the growth of NEL cells, the following protocol was established. Cells were trypsinized off the substratum and counted in a hemacytometer in the presence of trypan blue. Only cells excluding the dye were counted as viable cells. NEL cells (0.5 x 10⁶ cells/flask) were then plated in T-75 sq cm flasks and allowed to stabilize for 24 hr. After this time period, the media were removed and replaced with fresh media containing either ethanol (control) or TA. Twenty-four hr later, 3 flasks from each group were removed, and cell counts were taken on each flask. Media were removed from the remaining flasks and replaced with fresh MEM containing either ethanol or TA. This procedure was repeated at every time point.

³H Precursor Uptake and Incorporation Studies. NEL cells (0.5 x 10⁶ cells/flask) were plated in T-25 sq cm flasks. Twenty-four hr after the cells were plated, media were removed from the flasks and replaced with serum-free MEM. The replacement of complete medium (MEM plus serum) with serum-free medium allows us to investigate the biological effects of TA on NEL cells without interference from growth factors or hormones which are present in fetal bovine serum. The serum-free medium slows the growth of NEL cells over a 48-hr period by 38%
compared to that of cells growing in complete medium. However, this reduction in cell growth is due to the cell undergoing fewer population doublings and not to any cytotoxic effects of the serum-free medium.

The serum-free MEM cultures were incubated for a total of 24 hr after which the media was removed and replaced with fresh serum-free MEM containing various concentrations of TA and then incubated for 4 hr. Media were removed and replaced with serum-free MEM containing [3H]thymidine (5 μCi/flask), [3H]glucocorticoid (10 μCi/flask), or [3H]glucose (25 μCi/flask) and were incubated for 1 hr. After this last incubation period, the media were removed, and each flask was washed 3 times in phosphate-buffered saline (CaCl2, 0.88 mM; MgCl2, 0.5 mM; KCl, 2.68 mM; KH2PO4, 1.5 mM; NaCl, 136.7 mM; Na2HPO4, 8.8 mM). The cells were trypsinized off the substrate and placed into 15-ml conical tubes containing 4 ml of complete media (MEM plus serum). The tubes were centrifuged, and the supernatant fraction was discarded. One ml of cold 10% trichloroacetic acid was added to the cell pellet and then kept on ice for 15 min. The tubes were centrifuged, and the supernatant fraction was taken and placed into scintillation vials containing 10 ml of scintillation fluid (Scinti Verse II; Fisher Scientific Co.). The vials were then counted in a scintillation counter.

For the measurement of the amount of [3H]thymidine incorporated into DNA, the acid-insoluble fraction was collected on Millipore filters (0.45 μm). The filters were washed with 10 ml of cold 10% trichloroacetic acid and then placed into scintillation vials and counted as described above.

Steroid Binding. The detection of glucocorticoid receptors in NEL cells was performed as follows. Twenty-four hr before experimentation, NEL cells were placed into serum-free MEM. On the day of the experiment, cells were trypsinized off the substratum and plated into conical tubes containing complete media. The tubes were centrifuged and the cell pellet was washed twice with cold phosphate-buffered saline. Three ml of cold buffer (10 mM Tris-HCl, 1.5 mM EDTA, 10 mM monothioglycerol, and 10% glycerol, pH 7.8 at 4°C) were added to the cell pellet, mixed, and then sonicated (three 10-second bursts using a Virsonic cell disruptor). The broken cell suspension was then centrifuged in a Beckman L5-50B refrigerated ultracentrifuge at 4°C, 105,000 x g for 1 hr. The resulting supernatant fraction (105,000 x g fraction) was then incubated with 30 nM [3H]TA for 18 hr. The dextran-coated charcoal assay was used to separate the bound from the free steroid.

The binding of [3H]TA to the glucocorticoid receptor present in melanoma cytosol was determined using the method of Scatchard (14). Results are depicted in Chart 1. To determine if the human malignant melanoma cell line, NEL, contained specific glucocorticoid receptors, such experimentation showed the presence of a saturable glucocorticoid receptor (data not shown). These data were then analyzed according to the method of Scatchard (14) and are depicted in Chart 1. Results indicate a ligand binding capacity of 247 fmol/mg protein and a Kd of 1 x 10^-9 M.

Studies aimed at elucidating the specificity of the ligand binding site show that TA, progesterone, and cortisol were the most effective competitors. Dihydrotestosterone and 17β-estradiol were weak competitors (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Competitor</th>
<th>% of competition by a 100-fold excess of steroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>100</td>
</tr>
<tr>
<td>Progesterone</td>
<td>95</td>
</tr>
<tr>
<td>Cortisol</td>
<td>88</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>13</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>6</td>
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</tbody>
</table>

RESULTS

Binding Studies. Initial experiments were carried out to determine if the human malignant melanoma cell line, NEL, contained specific glucocorticoid receptors. Such experimentation showed the presence of a saturable glucocorticoid receptor (data not shown). These data were then analyzed according to the method of Scatchard (14) and are depicted in Chart 1. Results indicate a ligand binding capacity of 247 fmol/mg protein and a Kd of 1 x 10^-9 M.

Studies aimed at elucidating the specificity of the ligand binding site show that TA, progesterone, and cortisol were the most effective competitors. Dihydrotestosterone and 17β-estradiol were weak competitors (Table 1).

Cell Growth Curve. Our data indicate that NEL cells contain specific glucocorticoid receptors. To date, numerous investigations have demonstrated this same finding both in animal and human melanoma cell lines. However, as stated previously, the biological response to glucocorticoid treatment has not always been the same. Therefore, it was important to determine if the glucocorticoid receptor present in NEL cells was biologically active and, if so, what the biological response of NEL cells was to glucocorticoid treatment. An experiment to determine the biological response of NEL cells to TA is shown in Chart 2. When...
NEL cells were treated continuously with TA (1 × 10⁻⁷ M) over a 72-hr period, a 30% growth inhibition was observed.

**³H Precursor Uptake and Incorporation Studies.** To understand the mechanisms by which TA regulates the growth of NEL cells, uptake and incorporation studies were carried out using [³H]thymidine, [³H]leucine, and [³H]glucose. NEL cells were incubated with various concentrations of TA for either 4 or 18 hr and then pulsed for 1 hr with the tritiated precursors. The effect of TA on [³H]thymidine uptake is shown in Chart 3A. Inhibition in [³H]thymidine uptake at 4 and 18 hr was observed when cells were incubated with TA ranging from 10⁻⁸ M to 10⁻⁶ M. At TA concentrations of less than 10⁻⁸ M, there were either no effects or slight increases in [³H]thymidine uptake. With respect to [³H]thymidine incorporation, the results shown in Chart 3B indicate clearly that TA, at all concentrations tested, stimulated the incorporation of [³H]thymidine after the 4-hr incubation period. However, at the 18-hr time point, TA concentrations greater than 10⁻⁹ M caused a significant inhibition in [³H]thymidine incorporation. A stimulation in [³H]thymidine incorporation was still observed at TA concentrations of 10⁻⁸ M and 10⁻¹⁰ M.

Analogous studies to ascertain if TA had any effect on leucine or glucose uptake and incorporation into NEL cells revealed that cells incubated with 10⁻⁸ M TA for 4 hr had no effect on either the transport or incorporation of [³H]leucine or [³H]glucose (data not shown). However, NEL cells incubated for 18 hr with 10⁻⁸ M TA showed an 8% inhibition in [³H]leucine uptake and a 9% increase in incorporation (Table 2). A more significant inhibition in [³H]glucose uptake was observed at this time point. NEL cells incubated with 10⁻⁸ M TA for 18 hr show a 42% decrease in the amount of [³H]glucose transported into the cell (Table 2). The incorporation of [³H]glucose into the acid-insoluble fraction was inhibited by only 10%.

**Antiglucocorticoid Effects of Progesterone.** Initial experimentation showed that NEL cells contained specific glucocorticoid receptors. To help ascertain if the inhibition of [³H]thymidine incorporation observed after TA treatment was mediated through the receptor mechanism, experimentation was carried out in the presence or absence of the antiglucocorticoid progesterone (11). The data shown in Table 3 show that, when NEL cells were incubated with 1 × 10⁻⁸ M TA, there was a 21% inhibition of [³H]thymidine incorporation into DNA. However, when NEL cells were incubated with TA and a 100-fold excess of progesterone, the inhibition of [³H]thymidine incorporation was negated. Progesterone by itself had no effect on [³H]thymidine incorporation.

**DISCUSSION**

The data presented in this study demonstrate that the human malignant melanoma cell line, NEL, contains a specific glucocorticoid receptor. Additional experimentation showed that the continued presence of 10⁻⁶ or 10⁻⁷ M TA during a 72-hr growth period resulted in approximately 30% inhibition in cellular growth compared to that of the control group. In an attempt to elucidate the mechanism by which glucocorticoids inhibit cell growth, we examined the effect of glucocorticoid treatment on the uptake and incorporation of various [³H]precursors into the cell. Our results indicate that high concentrations of TA (≥ 10⁻⁸ M) inhibit the uptake of [³H]thymidine, while more physiological steroid

<table>
<thead>
<tr>
<th>Steroid</th>
<th>[³H]Thymidine incorporation (dpm/flask) (%)</th>
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<tbody>
<tr>
<td>None</td>
<td>26,002 ± 316³</td>
</tr>
<tr>
<td>TA (10⁻⁷ M)</td>
<td>20,681 ± 877 79</td>
</tr>
<tr>
<td>Progesterone (10⁻⁸ M)</td>
<td>26,527 ± 918 102</td>
</tr>
<tr>
<td>TA (10⁻⁸ M) + Progesterone (10⁻⁸ M)</td>
<td>26,519 ± 2,779 102</td>
</tr>
</tbody>
</table>

³ Mean ± S.D.

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>³H precursor</th>
<th>Uptake (dpm/flask)</th>
<th>Treated/control (%)</th>
<th>Incorporation (dpm/flask)</th>
<th>Treated/control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Leucine</td>
<td>12,122 ± 342³</td>
<td>92</td>
<td>20,718 ± 1,159</td>
<td>109</td>
</tr>
<tr>
<td>TA</td>
<td>Leucine</td>
<td>12,119 ± 141</td>
<td>98</td>
<td>22,542 ± 1,811</td>
<td>109</td>
</tr>
<tr>
<td>Control</td>
<td>Glucose</td>
<td>63,038 ± 3,633</td>
<td>58</td>
<td>9,054 ± 350</td>
<td>90</td>
</tr>
<tr>
<td>TA</td>
<td>Glucose</td>
<td>36,328 ± 1,018</td>
<td>58</td>
<td>8,172 ± 669</td>
<td>90</td>
</tr>
</tbody>
</table>

³ Mean ± S.D.
concentrations stimulate \(^{3}H\)thymidine uptake. However, the
effect of TA on \(^{3}H\)thymidine incorporation appears to be some-
what more complicated. When NEL cells were incubated for 4
hr with TA, there was a stimulation in the incorporation of
\(^{3}H\)thymidine at all steroid concentrations tested. At the present
time, we can only speculate that this increase in \(^{3}H\)thymidine
incorporation by TA is due to the activation of certain genes
which ultimately code for proteins involved in the growth regu-
lization of NEL cells. The results obtained after the 18-hr incubation
support this supposition. The data depicted in Chart 3B clearly
show a 30% inhibition in \(^{3}H\)thymidine incorporation into DNA
after treatment with steroid concentrations \(\geq 10^{-8}\) m. Since TA
had little effect on the growth rate of NEL cells during the first
24 hr of treatment (Chart 2), the reduction in \(^{3}H\)thymidine
incorporation cannot be due to a decrease in cell number and,
therefore, must be due to glucocorticoid regulation of DNA
synthesis.

Another point that merits discussion is the lack of an early
steroid effect on either leucine or glucose uptake and incorpo-
ration into NEL cells. It was only after an 18-hr incubation with
TA that a significant inhibition in \(^{3}H\)glucose uptake was ob-
served. Protein synthesis showed a 9% increase at this point.
These findings deviate slightly from observations made on the
effect of glucocorticoids on thymic lymphocytes (7). In those
studies, glucocorticoids caused an early inhibition in glucose
metabolism and protein synthesis. Thus, it may be that the
mechanism of cell regulation in melanomas is different than that
observed in lymphocytes.

As stated above, the treatment of NEL cells with TA produced
a 30% growth inhibition. This growth inhibition appears to be
due to a slight increase in the population doubling time (14 hr for
controls compared to 17 hr for TA-treated) and not to any
cytotoxic effects. Similar results have been reported by Horn
and Buzard (9) on the RPMI melanoma cells. Taken together,
these findings show a striking similarity to a report by Loeb et al. (12)
on the in vitro suppression of DNA synthesis in hepatoma
cells by glucocorticoids. These researchers found that treatment
of hepatoma cells in culture with glucocorticoids suppressed
thymidine incorporation into DNA but did not cause any cytolytic
effects. Additional experimentation demonstrated that RNA syn-
thesis proceeds with little alteration even when DNA synthesis
is inhibited by 90%. Thus, it appears that the mechanism of
action of glucocorticoids in hepatoma cells is similar to the action
observed in melanoma cells. However, additional experimenta-
tion is needed to delineate the exact mechanism by which
glucocorticoids regulate the growth of melanoma cells.

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