Enhancement of 1,25-Dihydroxyvitamin D₃-Mediated Antitumor Activity With Dexamethasone

Wei-Dong Yu, Mariann C. McElwain, Ruth A. Modzelewski, Derick M. Russell, David C. Smith, Donald L. Trump, Candace S. Johnson*

Background: The active metabolite of vitamin D, i.e., 1,25-dihydroxycholecalciferol (1,25-D₃), inhibits the growth of murine SCCVII/SF squamous cell carcinoma cells, both in vitro and in vivo. However, in vivo use of 1,25-D₃ is hampered as a result of hypercalcemia (i.e., elevated levels of calcium in the blood). Glucocorticoids, such as dexamethasone, affect calcium absorption and modulate vitamin D receptor binding and have been used to treat hypercalcemia. In this study, we examined the effect of dexamethasone on tumor growth inhibition by 1,25-D₃. Methods: The effects of 1,25-D₃ and dexamethasone, alone and in combination, on the growth of SCCVII/SF cells in in vitro culture or in vivo in female C3H/HeJ mice were determined by clonogenic tumor cell assay and/or by actual changes in tumor volume. Vitamin D receptor–ligand-binding activities in whole-cell extracts from cells (in culture), tumors, and normal tissues were assayed by single-point saturation analysis and equilibrium binding. Results: Treatment of cultured SCCVII/SF cells with 500 nM dexamethasone for 24 hours before addition of 1,25-D₃ reduced their survival. The growth of SCCVII/SF tumors was inhibited in mice treated simultaneously with dexamethasone and 1,25-D₃ (as compared with no treatment or single-agent treatment); hypercalcemia was also reduced. Total vitamin D receptor content in SCCVII/SF cells was increased after treatment with dexamethasone. Treatment of tumor-bearing animals with dexamethasone (9 μg/day) for 7 days led to increased vitamin D receptor–ligand-binding activities in whole-cell extracts from tumor or kidneys and decreased activity in intestinal mucosa. Conclusions: Dexamethasone may enhance the antitumor effect of 1,25-D₃ by increasing vitamin D receptor–ligand-binding activity. [J Natl Cancer Inst 1998;90:134–41]

The active metabolite of vitamin D, i.e., 1,25-dihydroxycholecalciferol (1,25-D₃), is primarily known for its involvement in the regulation of bone and mineral metabolism (1,2). In addition, 1,25-D₃ can modulate the growth and differentiation of a number of normal and malignant cell types (1–12). 1,25-D₃ inhibits leukemia and tumor cell proliferation in vitro (6,13,14) and leukemia/tumor cell growth in vivo in rodent leukemia and tumor model systems (15,16). We have demonstrated that 1,25-D₃ inhibits the proliferation of murine and human squamous cell carcinoma cells in vitro (17). In an animal model involving the growth of transplanted squamous cell carcinomas in C3H/HeJ mice, 1,25-D₃ treatment effectively inhibits the growth of newly transplanted or established tumors. Although antiproliferative effects have been observed with 1,25-D₃ in this model system, the tumor-bearing animals became severely hypercalcemic when given doses of 1,25-D₃ greater than 0.25 μg/mouse over a 2-week period.

Glucocorticoids, such as dexamethasone, have been shown to inhibit calcium absorption by the intestine and to increase calcium excretion by the kidneys (18,19). Clinically, 1,25-D₃ has been shown to reverse glucocorticoid-mediated suppression of calcium absorption, and glucocorticoids have been used to treat hypercalcemia (20). Glucocorticoids do not appear to compete for the vitamin D receptor-binding site (21), and reports vary on the effect of glucocorticoids on vitamin D receptor binding (22–24). Specific high-affinity receptors for 1,25-D₃ are found in a variety of normal and malignant murine and human cell lines and in fresh tissues (1–3,25–31). The ability of 1,25-D₃ to inhibit the growth of transformed cells appears to be associated with the number(s) of vitamin D receptors per cell (32). Controversy exists on whether the levels of vitamin D receptor increase or decrease as cells dedifferentiate (i.e., progress) toward a more malignant phenotype (12,30).

We examined the effects of the glucocorticoid dexamethasone on 1,25-D₃-mediated antiproliferative effects in vitro and in vivo as well as on 1,25-D₃-induced hypercalcemia. In addition, we examined the effect of dexamethasone on vitamin D receptor–ligand binding in vitro in squamous cell carcinoma cells and in vivo in normal and tumor tissues isolated from dexamethasone-treated, tumor-bearing mice.

Materials and Methods

Tumor Cells and Model System

Murine SCCVII/SF squamous cell carcinoma cells were obtained from K. K. Fu (University of San Francisco, CA). They were transfected as previously described (17,18) in 6- to 10-week-old female C3H/HeJ mice (obtained from The Jackson Laboratory, Bar Harbor, ME). The mice were regularly monitored for the presence of adventitious murine viruses and were housed and cared for in accordance with National Institutes of Health guidelines with no special considerations with respect to diet or light–dark cycle. For in vitro studies, cells were grown in RPMI-1640 medium plus 15% fetal calf serum (HyClone Laboratories, Inc., Logan, UT) and passed only twice before being returned to the animals. In vivo, squamous cell carcinomas were routinely produced by subcutaneous inoculation of 5 x 10⁶ log-phase tissue culture cells in the right flank of each mouse. Studies were initiated 9 days later when the tumors were palpable (approximately 2 x 2 mm in size). Animals were randomly assigned into groups.

*Affiliations of authors: W.-D. Yu, M. C. McElwain, R. A. Modzelewski, D. M. Russell (Department of Otolaryngology), D. C. Smith, D. L. Trump (Department of Medicine), C. S. Johnson (Department of Pharmacology), University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, PA. Correspondence to: Candace S. Johnson, Ph.D., University of Pittsburgh Cancer Institute, 200 Lothrop St., BST E1040, Pittsburgh, PA 15213. E-mail: candacej@vms.cis.pitt.edu

See “Notes” following “References.”

© Oxford University Press
and treated intraperitoneally with 1,25-D₃ three times per week until day 20 (2 weeks or six treatments) and/or with dexamethasone administered daily. Effects on tumor induction or growth were examined by treatment with 1,25-D₃ that was begun on day 0 or on the same day as tumor implantation and continued three times per week until day 19 (3 weeks or nine treatments) with dexamethasone administered daily. Using both approaches, we measured the tumor diameter in the mice with calipers every other day. Tumor volumes were calculated by the following formula: volume = (length × width²)/2. Serum calcium levels were determined at various times after treatment as described by Kaufman and Tietz (34).

1,25-D₃ and Dexamethasone
The active metabolite of vitamin D₃, i.e., 1,25-D₃, was provided by Milan Uskokovic (Hoffmann-La Roche Inc., Nutley, NJ). 1,25-D₃ was dissolved in 100% ethyl alcohol at 1 mg/mL and stored in the dark under a layer of nitrogen gas at −70 °C. Dilutions were made in sterile saline just before in vitro or in vivo use. All handling of 1,25-D₃ was done under indirect lighting, and tubes were protected from light by covering them with aluminum foil to prevent inactivation of the compound. Just before use, dexamethasone (Sigma Chemical Co., St. Louis, MO) was diluted in sterile saline.

In Vitro Antiproliferative Effects
To examine the in vitro antitumor effects of 1,25-D₃ and dexamethasone, we determined the ability of cells to survive treatment as measured in a tumor cell clonogenic (i.e., cloning of cells grown and treated in tissue culture) assay. Various dilutions of tumor cells were incubated for 24 hours in six-well tissue culture plates (Corning Costar Corp., Cambridge, MA) with or without 500 nM dexamethasone. Cells were then either left untreated or treated with various concentrations of 1,25-D₃ for an additional 24 hours. After a 7-day incubation at 37 °C in a humidified atmosphere containing 5% CO₂, cell monolayers were washed with saline, fixed with 100% methanol, and stained with 10% Giemsa, and colonies were counted with the use of a light microscope. To calculate the surviving fraction, we divided the cloning efficiency of treated cells by the cloning efficiency of untreated, control cells.

In Vivo Clonogenic Assay
The in vivo effect of 1,25-D₃ with and without dexamethasone on clonogenic tumor cells was determined by a modification of the in vivo clonogenic (i.e., cloning of dissociated cells of surgically removed tumors from treated and untreated animals) cell survival assay as described previously (35,36). Brieﬂy, mice with 9-day squamous cell carcinomas (three to ﬁve animals per treatment group) were treated with 1,25-D₃ at varying doses and/or dexamethasone at 9 μg per mouse daily for 3 days. Twenty-four hours after the last injection, the animals were killed, and their tumors were removed. Aliquots of minced tumor were enzymatically dissociated for 60 minutes at room temperature with a mixture of type I collagenase (37.5 mg/mL; Sigma Chemical Co.), deoxyribonuclease (5 mg/mL; Sigma Chemical Co.), and EDTA (1 mg/mL; Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). Viable tumor cells were then plated at various dilutions. After incubation for 7 days, colonies were counted and the numbers of clonogenic cells per gram of tumor were counted. The mean values (95% conﬁdence interval [CI]) for cell yield, cloning efﬁciency, and number of clonogenic cells for control, untreated tumors were 143.8 × 10⁶ viable tumor cells per gram of tumor (95% CI = 127.8–159.8 × 10⁶), 27.1% (95% CI = 24.7–29.5), and 33.9 × 10⁶ clonogenic tumor cells per gram of tumor (95% CI = 28.3–39.5 × 10⁶), respectively. The surviving fraction per gram of tumor is deﬁned as the number of clonogenic tumor cells per gram of control, untreated tumor. We have previously shown that the in vivo excision clonogenic assay is an accurate measure of in vivo antitumor activity and that a surviving fraction of less than 0.1 was always paralleled by an actual decrease in tumor volume and an increase in tumor regrowth delay (35–38).

Receptor Binding Assay
Analysis of total cellular vitamin D receptor binding was performed as described by Peehl et al. (11). For the preparation of whole-cell extracts from squamous cell carcinoma cells grown in vitro, cells at 90% confluence were either untreated or treated for 24 hours with dexamethasone (500 nM), rinsed twice with ice-cold phosphate-buffered saline, and harvested by being scraped with a rubber policeman. Cell pellets were suspended in 2 mL of KTEDM buffer (i.e., 0.3 M KCl, 10 mM Tris [pH 7.4], 1.5 mM EDTA, 1 mM dithiothreitol, and 10 nM sodium molybdate) containing the protease inhibitor cocktail (10 μg/mL soybean trypsin inhibitor, 1 μg/mL leupeptin, 2 μg/mL pepstatin, and 1 μg/mL aprotinin; all from Sigma Chemical Co.). Cells were disrupted by sonication on ice and centrifuged at 205 000 g for 35 minutes at 4 °C to obtain a soluble extract for the binding studies.

Tissues for vitamin D receptor–ligand binding were processed as described previously by Colston et al. (26). A whole-cell extract preparation from mouse tissues involved removal of tissues after 7 days from tumor implantation in mice that were either untreated or treated intraperitoneally with dexamethasone at 9 μg/20-g mouse for 7 days. Tissues were removed at day 14 after tumor implantation, washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline, chilled immediately on ice, and frozen at −80 °C. Tissue samples were homogenized in KTEDM buffer plus protease inhibitors (4 mL) with a Silverson homogenizer (Fisher Scientific Co., Pittsburgh, PA), by use of three 15-second bursts on low power, with 1-minute cooling intervals. The homogenate was then sonicated and centrifuged at 2000 g for 15 minutes at 4 °C. The supernatant was diluted with an equal volume of KTEDM buffer plus protease inhibitors and centrifuged at 20 000g for 35 minutes at 4 °C.

For single-point saturation analysis, 200 μL of whole-cell extract (1–2 mg of protein/mL) was incubated with 1 nM 1α,25-dihydroxy[26,27-3H]cholecalciferol (specific activity, 102 Ci/mmol; Amersham Life Science, Inc., Arlington Heights, IL) for 16–20 hours at 4 °C with or without a 250-fold excess of unlabelled 1,25-D₃. For equilibrium binding, increasing concentrations (0.02–1.3 nM) of 1α,25-dihydroxy[26,27-methyl-3H]cholecalciferol (specific activity, 177 Ci/mmol) were incubated with whole-cell extracts for 16–20 hours at 4 °C. After incubation, 0.4 mL of hydroxyapatite slurry (70% vol/vol) in 0.05 M Tris–HCl–0.01 M KH₂PO₄ buffer [pH 7.2]) was added to each tube and incubated for 15 minutes. The hydroxyapatite pellets were rinsed three times with 1% Tween 80 in KTEDM buffer. The radioactivity was extracted into 2 mL of 100% ethanol. After centrifugation at 2000 g for 10 minutes at room temperature, 1 mL of supernatant was added to 5 mL of scintillation ﬂuid, and radioactivity was counted in a Beckman 5801 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Nonspeciﬁc binding was subtracted from total binding to determine the speciﬁc binding of the ligand to the 1,25-D₃ receptor. Nonspeciﬁc binding represented less than 5%–10% of the total binding. The 0.3 M KCl buffer has been shown to extract both cytoplasmic and nuclear vitamin D receptors in epithelial cells (39). The resulting data were analyzed by use of equilibrium data Ligand-PC and median dose effect programs of IBM-PC program Dose-Effect Analysis With Microcomputers (Elsevier-Biosoft, Cambridge, U.K.). Scatchard plots were generated by the following equation: B/F = (1/Kd) [B] + Bmax/Kd, where B and F are the bound ligand and free ligand, respectively. Kd is the ligand dissociation constant, and Bmax is the maximal binding of ligand.

Statistical Analysis
For the in vitro and in vivo excision clonogenic assays, the statistical signiﬁcance of differences in surviving fraction for treatment groups was assessed by use of the two-way analysis of variance (ANOVA) (two-sided) because different mice contributed to each mean value. For in vivo experiments in which tumor volumes of the same mice were measured over time, the statistical signiﬁcance of differences between groups was examined by use of repeated-measures ANOVA (two-sided). For receptor studies and serum calcium levels, the statistical signiﬁcance of differences in ligand binding was assessed by use of Student’s t test (two-sided) that assumed unequal variance.

Results
In Vitro Effects of 1,25-D₃ and Dexamethasone
We have shown previously that 1,25-D₃ inhibits in vitro tumor cell proliferation (17). As shown in Fig. 1, in vitro treatment of squamous cell carcinoma cells with dexamethasone at 500 nM for 24 hours before 1,25-D₃ treatment resulted in an increase in 1,25-D₃-mediated growth inhibition in the in vitro clonogenic assay in a dose-dependent fashion. However, when dexamethasone was added to squamous cell carcinoma cells at the same time as 1,25-D₃, antitumor activity was not in-

ARTICLES 135
creased. The antiproliferative activity of 1,25-D₃ alone was similar to previously reported findings (17). Dexamethasone at 500 nM slightly inhibited tumor cell proliferation. The concentration of 500 nM was used because concentrations of dexamethasone higher than 10⁻⁴ nM resulted in greater than 50% growth inhibition of squamous cell carcinoma in vitro (data not shown). Similarly, dexamethasone was shown to enhance 1,25-D₃-mediated antiproliferative in vitro activity by use of the human squamous cell carcinoma cell line PCI-13 (data not shown), which has been shown previously (17) to have a higher IC₅₀ (i.e., the concentration that kills 50% of the tumor cells) and to be more resistant to the effects of 1,25-D₃ alone (data not shown).

Effects of 1,25-D₃ and Dexamethasone In Vivo as Measured by Use of the Excision Clonogenic Assay

We used the in vivo clonogenic assay to examine the effect of dexamethasone on 1,25-D₃-mediated antitumor activity. We have previously demonstrated that an increase in in vivo clonogenic cell kill results in an actual decrease in fractional tumor volume (35–38). Using this assay, we treated tumor-bearing mice (day 9) for 3 days with varying intraperitoneal doses of 1,25-D₃, either with or without 9 μg of daily dexamethasone. Twenty-four hours after the last injection of 1,25-D₃, tumors were removed, dissociated, and plated in the clonogenic cell assay. As shown in Fig. 2, the combination of 1,25-D₃ and dexamethasone resulted in a decrease in surviving fraction as compared with 1,25-D₃ alone. The 1,25-D₃-mediated clonogenic cell kill was enhanced even at the lowest dose of 1,25-D₃ (0.016 mg/kg per day for 3 days). Dexamethasone given alone at a dose of 9 μg per mouse per day for 3 days did not decrease the fraction of cells that survived.

Effect of Dexamethasone on 1,25-D₃-Mediated Antiproliferative Activity In Vivo

To determine whether an increase in clonogenic cell kill was associated with actual differences in tumor volume, we treated tumor-bearing mice (day 9) with either 0.25 μg of 1,25-D₃ three times per week, daily dexamethasone at 9 μg/20-g mouse, or a combination of the two at the same doses and schedules for 2 weeks (six treatments). As shown by analysis of the tumor growth curves in Fig. 3, the combination of 1,25-D₃ and dexamethasone was capable of inhibiting tumor growth as compared with the tumor growth observed in untreated, control mice or in those treated with 1,25-D₃ or dexamethasone alone. As was shown previously (17), treatment of mice with 1,25-D₃ resulted in an antitumor effect as compared with tumor growth in the untreated, control animals. Treatment of mice with dexamethasone alone resulted in a slight tumor-inhibitory effect as compared with the control animals. Animals in the untreated, control group had to be killed at day 19 as a result of tumor burden and because they had become moribund.

To determine the effect of dexamethasone on the antitumor activity of 1,25-D₃, we initiated 1,25-D₃ treatment at the same time as tumor implantation, and the mice were monitored for the
onset of tumor growth during treatment and the effect on tumor growth once the therapy had been terminated. The mice were treated with 0.05 \( \mu \text{g} \) 1,25-D \(_3\) three times per week and with 9 \( \mu \text{g} \) dexamethasone daily. This treatment was initiated on the same day that 5 \( \times \) 10\(^5\) tumor cells were inoculated subcutaneously, and treatment was continued until the 20th day after tumor implantation (nine treatments). As shown in Fig. 4, the combination of 1,25-D \(_3\) and dexamethasone inhibited and delayed tumor growth when compared with either 1,25-D \(_3\) or dexamethasone alone by examination of tumor growth curves. Mice treated with 1,25-D \(_3\) plus dexamethasone had palpable tumors at day 21, whereas those treated with either 1,25-D \(_3\) or dexamethasone alone had palpable tumors at day 16 or day 14, respectively; untreated control mice had tumors at day 12. As was shown previously (17), treatment with 1,25-D \(_3\) alone slightly delayed tumor growth. Treatment with dexamethasone alone resulted in an antitumor effect similar to that of 1,25-D \(_3\) given alone.

When the serum calcium levels in the mice were examined at day 19 or after the last injection, animals treated with dexamethasone plus 1,25-D \(_3\) had lower levels than those treated with 1,25-D \(_3\) alone (Table 1). Animals treated with dexamethasone plus 1,25-D \(_3\) did not become hypercalcemic throughout the treatment period, and their serum calcium levels remained in the normal range. In contrast, mice treated with 1,25-D \(_3\) alone became hypercalcemic (>11.0 mg of Ca\(^{2+}\)/dL of serum) by day 15 or after six treatments with 0.05 \( \mu \text{g} \)/20-g mouse (data not shown). In addition, none of the animals in any treatment groups had a loss in body weight during the experimental period when compared with animals in the control, untreated group. As noted above, the animals in the control group had to be killed on day 19 because of their excessive tumor load and their moribund condition.

**Effect of Dexamethasone on Vitamin D Receptor Binding**

To examine total cellular vitamin D receptor binding, we treated the squamous cell carcinoma cell line for 24 hours with 500 nM dexamethasone. As measured by the single-point saturation assay, maximum 1,25-D \(_3\)-binding capacity in dexamethasone-treated squamous cell carcinoma cells was 33.2 fmol/mg (95% CI 4.30.9–35.5) of protein and was increased as compared with 12.8 fmol/mg (95% CI 4.11.2–14.4) for squamous cell carcinoma treated with dexamethasone (\( P = 4.8 \times 10^{-22} \); two-sided repeated-measures analysis of variance).

**Table 1. Serum calcium levels for treatment of tumors with 1,25-dihydroxycholecalciferol (1,25-D\(_3\)) and/or dexamethasone**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium, mg/dL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.6 (9.3–9.9)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>9.4 (9.0–9.8)</td>
</tr>
<tr>
<td>1,25-D(_3)</td>
<td>11.1† (10.9–11.3)</td>
</tr>
<tr>
<td>1,25-D(_3) plus dexamethasone</td>
<td>9.9‡ (9.3–10.2)</td>
</tr>
</tbody>
</table>

*Values in column = mean serum calcium levels (95% confidence interval) at day 20 after implant, from eight to 10 female C3H/HeJ mice.
†Statistically different from control, untreated group or in the dexamethasone-treated group; \( P = 2.1 \times 10^{-3} \); two-sided Student’s \( t \)-test.
‡Statistically different from animals treated with 1,25-D\(_3\) alone; \( P = 2.5 \times 10^{-4} \) (two-sided Student’s \( t \)-test).
get tissues (intestinal mucosa and kidneys) as well as vitamin D receptor values for a number of tumor cell lines (11,31,40). By equilibrium binding analysis, an increase in ligand binding was observed in squamous cell carcinoma cells treated with dexamethasone as compared with untreated squamous cell carcinoma cells, even at low concentrations of labeled 1,25-D3 (Fig. 5, A). The dose–response saturation plots, which represent only specific binding (nonspecific subtracted out), approached saturation at 0.7 and 1.3 nM, respectively, of [3H]1,25-D3 for animals in the untreated group and those in the dexamethasone-treated group. As shown in the Scatchard plot (Fig. 5, B), linear regression analysis demonstrated a single class of specific, high-affinity receptors with no difference observed in the equilibrium dissociation constant (Kd) from squamous cell carcinoma cells either not treated with dexamethasone (0.13 nM 1,25-D3; 95% CI = 0.12–0.14) or treated with dexamethasone (0.08 nM 1,25-D3; 95% CI = 0.04–0.12).

To determine whether dexamethasone could increase ligand binding in the tumors of animals treated in vivo, we treated mice with 7-day squamous cell carcinoma tumors with 9 µg of dexamethasone. After daily treatment with dexamethasone for 7 days, animals were killed, their tumors were removed on day 14, and whole-cell extracts were prepared for binding analysis. As determined by single-point saturation binding, the vitamin D receptor level (as measured by maximum 1,25-D3-binding capacity per milligram of protein) was significantly higher in tumors from animals treated with dexamethasone (140.6 fmol/mg; 95% CI = 128.5–152.7) than in tumors from untreated, control animals (26.1 fmol/mg; 95% CI = 20.7–31.4) (P = 8.1 × 10−8; two-sided ANOVA). Treatment of tumor-bearing mice with 9 µg of dexamethasone for 3 days also yielded similar results; vitamin D receptor binding in tumor tissue extracts from dexamethasone-treated animals was increased compared with that in untreated, control animals (data not shown). As shown by equilibrium binding (Fig. 6, A) and Scatchard plot (Fig. 6, B) analyses, dexamethasone treatment of mice for 7 days resulted in enhanced vitamin D receptor levels in the whole-cell extracts examined from tumor tissues as compared with tumor cell extracts from untreated animals. Similar to results obtained from analysis of extracts of squamous cell carcinoma cells grown in culture, no difference was observed in the equilibrium binding dissociation constant (Kd) from tumor tissue isolated from animals either untreated (0.03 nM 1,25-D3; 95% CI = 0.01–0.05) or treated (0.07 nM 1,25-D3; 95% CI = 0.03–0.11) with dexamethasone.

Levels of ligand binding were elevated in whole-cell extracts of tumor tissue from dexamethasone-treated animals compared...
Discussion

1,25-D$_3$ is a steroid hormone that is involved in the regulation of calcium and phosphate transport in the intestine and in the mobilization of mineral from the bone (1,2). In addition to these well-recognized actions, 1,25-D$_3$ also inhibits cell growth and induces differentiation in both normal and malignant cell types (1–16). 1,25-D$_3$ arrests human lymphoid cells in G$_1$ phase (41–43). These attributes suggest that 1,25-D$_3$ may have therapeutic potential in the treatment of human cancers. We demonstrated in a murine squamous cell carcinoma model that 1,25-D$_3$ has antitumor activity both in vitro and in vivo (17). These antitumor effects are observed in the treatment of animals with established tumors as well as in the prevention of tumor induction or initiation. Studies presented demonstrate that dexamethasone can enhance 1,25-D$_3$ antiproliferative effects and can decrease 1,25-D$_3$-mediated hypercalcemia.

The mode of action of 1,25-D$_3$ activity appears to depend on binding to the vitamin D receptor, thereby increasing the affinity of the receptor–ligand complex for DNA (44). The vitamin D receptor is a 48- to 55-kd protein with a dissociation constant of approximately 0.1 nM and is part of a superfamily of genes that includes the receptors for estrogen, progesterone, cortisol, thyroxine, retinoic acid, and aldosterone (45,46). The vitamin D receptor appears to be primarily nuclear; however, evidence exists for the presence of cytoplasmic receptors (47). We examined whole-cell extracts that contain both cytoplasmic and nuclear receptors (39). Studies are in progress to examine vitamin D receptor binding and content in nuclear and cytoplasmic preparations from squamous cell carcinoma cells and from tissues isolated from squamous cell carcinoma-bearing mice.

Vitamin D receptors are present in a wide variety of tumor types from tissues including breast, skin, prostate, ovary, thyroid, brain, head and neck, lung, and hematopoietic cells (1–3,25–31). The presence of vitamin D receptor is not always associated with sensitivity to the antiproliferative effects of 1,25-D$_3$; however, receptors must be present in order to see an effect. Shabahang et. al. (12) suggested that, as tumor cells progress toward a more malignant phenotype, they lose responsiveness with the vitamin D receptor either by actual loss or by down-regulation (i.e., reduced expression). If tumor cells lose responsiveness to 1,25-D$_3$, therapeutic interventions with 1,25-D$_3$ could be compromised. One hypothesis for the effect of dexamethasone on numbers of vitamin D receptors in tumors is that dexamethasone is acting to re activate receptors that have been down-regulated (i.e., whose expression and/or activity has been reduced) as a part of the malignant process. Activation of the vitamin D receptor may occur rapidly. As a result, the 24-hour preincubation of cells with dexamethasone may not be required; however, the effects that dexamethasone has on normal murine cells depend on the rate of cell proliferation (48). In preliminary studies, we have observed that messenger RNA levels for the vitamin D receptor (both nuclear and cytoplasmic) are unchanged in tissues from untreated and dexamethasone-treated squamous cell carcinoma-bearing mice and also in squamous cell carcinoma cells grown and treated with dexamethasone in tissue culture (Johnson CS, Hershberger PA, Trump DL: unpublished observations). Therefore, the observed increases in vitamin D receptor levels were not the result of enhanced gene expression. We have initiated studies to examine the time-dependent effects of dexamethasone on the level of vitamin D receptor and related proteins.

We demonstrated that dexamethasone increases ligand bind-

![Fig. 7](image-url)
ing in the tumor but decreases ligand binding in the intestine. These changes occur at the same time that enhanced antitumor activity is observed from the combination of 1,25-D$_3$ plus dexamethasone and may account for the decrease in 1,25-D$_3$-mediated hypercalcemia. The gut plays a central role in calcium absorption (20); while 1,25-D$_3$ was given parenterally in this study, the gut may still be involved in the induction of hypercalcemia. Administration of dexamethasone to tumor-bearing mice also receiving 1,25-D$_3$ brought about an increase in the binding of 1,25-D$_3$ to the vitamin D receptor in tumor cell extracts and an increase in its antitumor activity. In addition, a dexamethasone-induced increase in ligand binding was observed in the kidneys of these tumor-bearing mice. Glucocorticoids increase calcium excretion by the kidneys (19); therefore, the effects of glucocorticoids on calcium excretion in the kidneys may be mediated through the vitamin D receptor.

Glucocorticoids have varied effects on ligand binding to the vitamin D receptor in normal cells and tissues (22–24) and do not compete with 1,25-D$_3$ for binding to the vitamin D receptor (21). In the mouse, dexamethasone treatment results in a decrease in the vitamin D receptors present in cell extracts from the intestine (24). These results agree with what we found in the intestinal mucosa of dexamethasone-treated mice. In contrast, another study (22) demonstrated that, when rats were treated with corticosterone, the levels of ligand binding to the vitamin D receptor in cell extracts of intestinal tissue were increased when compared with those in the same tissue from control, untreated mice. In both of these studies (22,24), however, extremely large doses of glucocorticoids (10–40 μg/20-g mouse and 150–750 μg/150-g rat) were administered daily for 7 days. In experiments reported here, we administered 9 μg of dexamethasone per mouse daily for either 3 or 7 days. This dose in mice is equivalent to a dose of approximately 17.5 mg/day of prednisone in humans, which is a relatively low dose. Dexamethasone also increases vitamin D receptor levels in cultured rat osteoblasts (23). In cell culture, the effects of dexamethasone on vitamin D receptors depend on the rate of cell proliferation, with inhibitory effects observed in cells that are in early log phase or quiescent because of contact inhibition of growth and stimulatory effects in cells in late log phase (49). Therefore, glucocorticoids do appear to play a role in modulation of vitamin D receptor binding even in normal animals.

To examine the mechanism(s) for enhanced antiproliferative activity with the combination of 1,25-D$_3$ and dexamethasone, we determined the effect of dexamethasone on vitamin D receptor–ligand binding both in vitro and in vivo. As described above, we determined that dexamethasone increases ligand binding in the tumor, thereby making tumor cells more sensitive to the effects of 1,25-D$_3$. We focused the receptor studies on dexamethasone alone and not on dexamethasone plus exogenously added 1,25-D$_3$. Glucocorticoids do not compete with 1,25-D$_3$ receptor binding (21); however, 1,25-D$_3$ itself may have an effect on vitamin D receptor binding, which could have an impact on the design of 1,25-D$_3$ and glucocorticoids in the therapy of solid tumors. Therefore, studies in our laboratories are in progress to examine the effect of exogenously administered 1,25-D$_3$ on dexamethasone-induced increase in ligand binding, especially in squamous cell carcinoma-bearing mice.

Glucocorticoids directly lyse leukemic lymphoblasts (49) and are used to treat cancer patients for a variety of indications (i.e., to treat emesis or hypercalcemia) (50). In these studies, dexamethasone alone did not inhibit the growth of squamous cell carcinoma cells in vitro or in vivo. In mice with established tumors, dexamethasone at 9 μg/mouse, given three times per week for six treatments or 2 weeks, only slightly inhibited tumor growth. It was only when dexamethasone was administered to animals early (i.e., starting at the time when tumor cells were injected) to prevent tumor growth that an antitumor effect of dexamethasone (9 μg/mouse, three times per week for nine treatments or 3 weeks) was observed. No difference was seen between animals treated with dexamethasone and those treated with 1,25-D$_3$ alone. In addition, we (51) and other investigators (41–43) have demonstrated that 1,25-D$_3$ can arrest tumor cells in G$_0$/G$_1$ and can decrease the percentage of cells in S phase. Treatment of tumor cells with dexamethasone alone did not result in a differentiating effect, in morphologic changes, or in an alteration of cell cycle status (data not shown). Despite the minimal antitumor activity of dexamethasone, we have not excluded the possibility that 1,25-D$_3$ may be enhancing the activities of dexamethasone. Studies to examine these effects, especially with regard to the role of 1,25-D$_3$ on glucocorticoid receptor levels, are in progress.

In summary, these results demonstrate that dexamethasone can enhance 1,25-D$_3$-mediated antitumor activity and can decrease 1,25-D$_3$-mediated hypercalcemia. The results of our vitamin D receptor–ligand binding studies suggest that the effects of dexamethasone may be mediated through changes in vitamin D receptors.

References

(11) Pechl DM, Skowronsji RJ, Leung GK, Wong ST, Stamey TA, Feldman D.


Notes

Supported by Public Health Service grants R01CA67267 and T32CA60397 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

Present address: D. C. Smith, Department of Medicine, University of Michigan, School of Medicine, Ann Arbor.

We thank Robert H. Getzenberg for his comments and critical review, John W. Wilson for his statistical analysis, and Andrea Piacentini for preparation of the manuscript.

Manuscript received May 5, 1997; revised October 23, 1997; accepted No- vember 7, 1997.