Effects of 1,25-Dihydroxyvitamin D3 [1,25(OH)2D3] and Its Analogues (EB1089 and Analog V) on Canine Adenocarcinoma (CAC-8) in Nude Mice

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The aim of this study is to determine the effects of 1,25(OH)2D3 and its analogues on tumor growth and body weight, changes in plasma ionized calcium, parathyroid hormone-related protein (PTHrP) production, bone resorption, and the distribution of the 1,25(OH)2D3 receptor (VDR) on tumors in nude mice-bearing the canine adenocarcinoma (CAC-8). Thirty-seven nude mice were implanted subcutaneously with CAC-8. Two weeks after implantation, the mice were divided into 5 groups and injected intraperitoneally 3 times/week for 4 weeks with 5 different substrates. Group I (nontumor-bearing mice) were injected with vehicle. Groups II through V were CAC-8-bearing mice injected with the following: Grp. II, vehicle; Grp. III, analog V; Grp. IV, 1,25(OH)2D3; and Grp. V, EB1089. Our results showed that mice body weight (% change) of CAC-8-bearing mice was significantly lower than those of nontumor-bearing mice (p < 0.05). CAC-8-bearing mice treated with analog V maintained their body weight better than CAC-8-bearing mice treated with either vehicle, 1,25(OH)2D3, or EB1089. A reduction of tumor growth was observed in CAC-8-bearing mice treated with 1,25(OH)2D3 and its analogues; however, the reduction was not statistically significant compared to the vehicle-treated CAC-8-bearing mice. All CAC-8-bearing mice increased osteoclastic bone resorption and hypercalcemia. Immunohistochemical staining of CAC-8 with VDR antibody demonstrated a positive reaction in nuclei of tumor cells. In conclusion, CAC-8-bearing mice treated with analog V were more active and maintained their body weight better than other CAC-8-bearing groups. Analog V-treated mice also showed no toxic side effects of hypercalcemia despite an increase in plasma ionized calcium comparable to nontumor-bearing mice. Tumor volumes of CAC-8-bearing mice treated with 1,25(OH)2D3 and its analogues were smaller than vehicle-treated CAC-8-bearing mice. This finding suggested an inhibitory effect on tumor cell growth.

Key words 1,25-dihydroxyvitamin D3; vitamin D analogue; parathyroid hormone-related protein; canine adenocarcinoma (CAC-8); EB1089; 1,25-dihydroxy-16-ene-23-yne-vitamin D3; vitamin D analogue

Recently the active form of vitamin D, 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3] and its synthetic analogues have been shown to exert anti-tumor effects both in vitro and in vivo.1–5) As summarized by Johannes et al,6) many investigations have reported that 1,25(OH)2D3 and its analogues affect tumor cells due to: a) blockade of the cells in the G1/G0 phase and reduction of the number of cells in S phase of cell cycle progression,7–9) b) induction of apoptosis,10,11) c) expression and regulation of oncogenes and tumor suppressor genes,12–14) d) interaction with tumor- or stroma-derived growth factors for growth inhibition [e.g. transforming growth factor (TGFβ), insulin-like growth factor (IGF)],15–17) and e) induction of differentiation.18,19) However, most studies have been completed in human tumors with little information available in other species on the effects of 1,25(OH)2D3 and its analogues.

This study examined the effects of 1,25(OH)2D3 and its analogues [22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D3 (EB1089) and 1,25-dihydroxy-16-ene-23-yne-vitamin D3 (analog V)] on the canine adenocarcinoma (CAC-8) model of humoral hypercalcemia of malignancy (HHM) in nude mice. Canine adenocarcinoma derived from apocrine glands of the anal sac is commonly associated with cancer-associated hypercalcemia.20–23) HHM is a paraneoplastic syndrome in dogs that significantly contributes to the morbidity and mortality of cancer patients. It is well documented that an autonomous overproduction of parathyroid hormone-related protein (PTHrP) is the principal humoral factor that causes hypercalcemia in dogs with this carcinoma due to increased release of calcium from bone and enhanced reabsorption of calcium in the kidney.22,23) The inhibitory effect of 1,25(OH)2D3 and its analogues on PTHrP production thus far has been reported on both normal human cells24,25) and human tumor cells.26–30) In order to achieve the inhibitory effect of 1,25(OH)2D3, its analogues on PTHrP production, high doses and/or prolonged treatment with this potent active vitamin D metabolite most likely will be needed, which has the potential to enhance the hypercalcemia. 1,25(OH)2D3 itself is also an important regulator of bone development, bone metabolism, calcium homeostasis and increases blood calcium.31) In order to minimize this potential toxic effect, vitamin D analogues have been synthesized with noncalcemic effect. Among these noncalcemic analogues, EB1089 and analog V, have been studied on tumor growth.1,2,32) The effects of these two analogues (EB1089 and analog V) on canine adenocarcinoma model of HHM in nude mice were evaluated in this study. The specific objectives were to determine the effects of 1,25(OH)2D3 and its analogues on tumor growth and body weight, changes in plasma ionized calcium, PTHrP production, bone resorption, and the distribution of the 1,25(OH)2D3 receptor (VDR) on tumors in CAC-8-bearing mice.

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MATERIALS AND METHODS

Vitamin D and Its Analogues  1,25(OH)2D3 and analog V were a generous gift from Dr. S. G. Reddy (School of Medicine, Brown University, RI, U.S.A.). EB1089 was provided by Dr. L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). The stock solution of 1,25(OH)2D3 and its analogues were suspended in absolute ethanol at 1×10−3 M (1.2 ml = 500 μg) and protected from light. In this experiment, each compound was dissolved in vegetable oil (Wesson®) containing 3% (vol/vol) ethanol.

Animals Thirty-seven male nude mice (HSD: Athymic nude, 4 weeks-of-age) were purchased from laboratory (Harlan Sprague-Dawley Inc., Indianapolis, IN, U.S.A.). The mice were fed sterilized rodent chow (Harlan Teklad, Madison, WI, U.S.A.) and housed under barrier conditions for 7 d prior to injection of tumor tissue.

Experimental Design Six mice were used as a control nontumor-bearing group. Thirty-one mice were implanted subcutaneously between the scapulae with approximately 6—10 mm3 cryopreserved CAC-8, passage 30 tissue. Two weeks after implantation, mice were divided into 5 groups (Grps.) and injected intraperitoneally 3 times per week with 5 different substrates. Grp. I (n = 6) was nontumor-bearing mice injected with vehicle (vegetable oil) (0.1 ml/mouse). Grps. II to V were all tumor-bearing mice injected with the following substances: Grp. II (n = 6), vehicle (0.1 ml/mouse); Grp. III (n = 8), analog V (1.5 μg/mouse); Grp. IV (n = 8), 1,25(OH)2D3 (0.05 μg/mouse); and Grp. V (n = 9), EB1089 (0.01 μg/mouse) for up to 4 weeks. Doses of 1,25(OH)2D3 and its analogues (EB1089 and analog V) were determined based on previous studies1,2,4,31) and our preliminary study on CAC-8-bearing mice (data not shown). Body weight and tumor volume were determined 3 times per week. Tumor volume was measured by vernier calipers and calculated using the formula: 1/2(length×width×height).33) After the mice were treated with vehicle, 1,25(OH)2D3 and its analogues for 4 weeks, they were sacrificed according to the Institutional Laboratory Animal Care and Use Committee (ILACUC) protocol of The Ohio State University. Approximately 300—500 μl of blood was obtained from femoral vein by using anaerobic collection to prevent changes in the pH of the sample in lithium heparin-containing tubes (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) for measurement of plasma ionized calcium and PTHrP production.

Plasma Ionized Calcium Measuring plasma ionized calcium at the same pH as the blood at the time of collection is important for the most biologically relevant value.34) Plasma ionized calcium was immediately measured using a Nova 8 analyzer (Nova biomedical, Waltham, MA, U.S.A.) after collected from mice.

PTHrP Immunoradiometric Assay (IRMA) PTHrP was measured using IRMA kit (DiaSorin Corp., Stillwater, MN, U.S.A.) with human recombinant PTHrP 1—84 for standards and controls. The IRMA contained human anti-PTHrP 1—40 antibody bound to polystyrene beads and anti-PTHrP 57—80 antibody labeled with 125I. Samples were incubated with the antibodies, and the polystyrene beads were washed to remove unbound-labeled antibody. The radioactivity remaining from the bound-labeled antibody was measured using a gamma counter. PTHrP content was analyzed with GraphPad Prism™ (GraphPad software Inc., San Diego, CA, U.S.A.).

Histopathology Tissues and bone were collected at time of sacrifice. Transplanted CAC-8 carcinomas, liver, kidney, heart and lungs were examined from control nontumor-bearing and CAC-8-bearing mice. Tissues were fixed and embedded for histologic and immunohistochemical evaluations. Lumbar vertebrae from all control nontumor-bearing and CAC-8-bearing mice were cut and fixed in 10% neutral-buffered formalin for histomorphometric evaluation as described in bone histomorphometry.

Immunohistochemistry Tumor tissues from all CAC-8-bearing mice treated with vehicle, 1,25(OH)2D3, and its analogues were fixed and processed by the freeze substitute technique for immunohistochemical localization of VDR and histopathology.35) The staining was completed by incubating tissue sections with 5% normal goat serum (NGS) in phosphate buffered saline (PBS, pH 7.4) for 30 min: primary antibody, rat monoclonal antibody VDR (Chemicon International Inc., Temecula, CA, U.S.A.) 1:50 at 4°C overnight; secondary antibody, goat anti-rat IgG (Chemicon International Inc., Temecula, CA, U.S.A.) 1:20 in PBS for 30 min; rat peroxidase anti-peroxidase (PAP) 1:100 in 1% NGS in PBS for 30 min; and 0.05% dimethylaminobenzobenzene (DAB) and 0.01% hydrogen peroxide in 0.05% Tris–HCl buffer for 5 min. Slides were washed between each step with PBS, dehydrated, mounted with aqua-Mount (Lerner Laboratories, Pittsburgh, PA, U.S.A.), and visualized by light microscopy.

Bone Histomorphometry Histomorphometric evaluation of bone was performed in all 5 groups of mice. Bone resorption parameters were evaluated using lumbar vertebrae. The vertebrae were trimmed off musculature and lateral vertebral processes and fixed in 10% buffered formalin at 4°C. The vertebrae were decalcified in 10% EDTA (pH 7.4) for 2 d, directly infiltrated in 3 changes of JB4 glycol methacrylate (Polysciences Inc., Warrington, PA, U.S.A.) for 6 d, and embedded at 4°C as described by Brinn and Pickett.36) Midline sections were cut at 5 μm, stained for tartrate-resistant acid phosphatase (Sigma Diagnostics, St. Louis, MO, U.S.A.), and counterstained with hematoxylin. The total endosteal perimeter of a single section of a lumbar vertebra was measured with the Zeiss Interactive Digital Analysis System (ZIDAS) at a magnification of 200×. Acid phosphatase-positive cells were defined as cells (osteoclasts) on the bone surface, which stained intensely red or contained numerous red granules in the cytoplasm. The endosteal perimeter lined by osteoclasts and the numbers of osteoclasts were counted. The data were expressed as percent resorptive perimeter (percent of the endosteal surface lined by osteoclasts) and number of osteoclasts/mm endosteal surface.

Statistical Analysis The results were expressed as the mean (standard error of the mean (S.E.M.) and were analyzed by one-way analysis of variance using an ANOVA37) and Turkey’s multiple comparisons test using Instat program (GraphPad Software Inc., San Diego, CA, U.S.A.).

RESULTS

Histopathology Histopathologic evaluation of CAC-8 tumors revealed no differences between groups of animals.
Mineralization at the corticomedullary region of the kidney was present in some mice, but was not limited to any particular group. Microscopic lesions or tumor metastases were not found in the liver and lung, or elsewhere. During the experiment some CAC-8-bearing mice developed life-threatening clinical signs related to the hypercalcemia (weight loss, anorexia, depression, and dehydration) had to be sacrificed before the scheduled termination of the experiment at days 11, 14, 18 (two mice from Grp. II); at day 26 (one mouse from Grp. III); at days 14, 18 (three mice from Grp. IV); and at days 11, 14, 18 (five mice from Grp. V).

**VDR Distribution in the CAC-8 Adenocarcinoma** Immunohistochemical staining of CAC-8 tumor tissue with VDR-antibody demonstrated positive peroxidase reaction in nuclei of carcinoma cells (Figs. 1A, B).

**Tumor Volume** There were no significant differences in the tumor volumes of CAC-8-bearing mice treated with 1,25(OH)_{2}D_{3} and its analogues as compared to control CAC-8-bearing mice treated with vehicle.

**Body Weight** The body weights (% change) of CAC-8-bearing mice were significantly lower than those of nontumor-bearing mice (p<0.05) (Fig. 2). There was a significant difference in body weight gain between control CAC-8-bearing mice treated with vehicle and CAC-8-bearing mice treated with EB1089 at day 14 (Fig. 2). CAC-8-bearing mice treated with analog V maintained their body weight better than CAC-8-bearing mice treated with either vehicle, 1,25(OH)_{2}D_{3}, or EB1089 (Fig. 2).

![Fig. 1. (A) Immunoperoxidase Labeling of 1,25(OH)_{2}D_{3} Receptor (VDR) from CAC-8 Adenocarcinoma Transplanted Mouse Treated with Vehicle. The tumor section, stained with monoclonal anti-VDR, demonstrated a positive labeling in all nuclei of tumor cells (arrowheads). (B) The Control Section Reacted with Non-specific Antiserum in Place of a Specific Primary Antibody. The absence of reaction product in the nuclei of tumor cells (arrowheads) indicates the reaction observed with anti-VDR is due to the presence of authentic VDR. Magnification ×300.](image)

![Fig. 2. Percent Change in Body Weight Gain in Mice Administered 1,25(OH)_{2}D_{3} and Its Analogues (EB1089 and Analog V)].(mean±S.E.M.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma ionized calcium (mg/dl)</th>
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<tbody>
<tr>
<td>Nontumor-bearing mice + vehicle</td>
<td>4.89±0.14*</td>
</tr>
<tr>
<td>CAC-8-bearing mice + vehicle</td>
<td>8.99±0.87*</td>
</tr>
<tr>
<td>CAC-8-bearing mice + analog V</td>
<td>9.02±1.38*</td>
</tr>
<tr>
<td>CAC-8-bearing mice + 1,25(OH)<em>{2}D</em>{3}</td>
<td>9.34±1.64*</td>
</tr>
<tr>
<td>CAC-8-bearing mice + EB1089</td>
<td>9.00±1.41*</td>
</tr>
</tbody>
</table>

There were significant differences in plasma ionized calcium levels in CAC-8-bearing mice treated with 1,25(OH)_{2}D_{3} and its analogues as compared to control nontumor-bearing mice (p<0.001). No significant differences in plasma ionized calcium levels in CAC-8-bearing mice treated with 1,25(OH)_{2}D_{3} and its analogues as compared to vehicle-treated CAC-8-bearing mice. (Grp. I; n=6, Grp. II; n=4, Grp. III; n=7, Grp. IV; n=5, Grp. V; n=4).

**Hypercalcemia** The plasma level of ionized calcium [Ca^{2+} (mg/dl)] was greater in all CAC-8-bearing mice (Grp. II=8.99±0.87, Grp. III=9.02±1.38, Grp. IV=9.34±1.64, Grp. V=9.00±1.41) as compared to nontumor-bearing mice (Grp. I=4.89±0.14) (Table 1). There were no significant differences in plasma ionized calcium in CAC-8-bearing mice treated with 1,25(OH)_{2}D_{3} and its analogues as compared to vehicle-treated CAC-8-bearing mice (Table 1).

**PThrP Production** By day 28, all CAC-8-bearing mice treated with vehicle, analog V, 1,25(OH)_{2}D_{3}, or EB1089 (Grps. II to V) did not inhibit PThrP production. The levels of PThrP secretion (psu) were greater in all CAC-8-bearing mice (Grp. II=40.74, Grp. III=38.70, Grp. IV=45.38, Grp. V=40.31) compared to the nontumor-bearing mice (Grp. I<1 psu). Each value was represented per group because individual samples were pooled together in each group to get adequate amounts of samples.

**Bone Resorption** Histomorphometric evaluation of lumbar vertebrae stained for tartrate-resistant acid phosphatase
revealed a significant increase in bone resorption and number of osteoclasts/mm of endosteal surface in CAC-8-bearing mice as compared to nontumor-bearing mice \( (p<0.001) \) (Table 2, Figs. 3A, B). There were no significant differences in resorption perimeter and number of osteoclasts/mm of endosteal surface in CAC-8-bearing mice treated with 1,25(OH)\(_2\)D\(_3\) and its analogues as compared to control CAC-8-bearing mice treated with 1,25(OH)\(_2\)D\(_3\) and its analogues as compared to control vehicle-treated CAC-8-bearing mice. (Grp. I; \( n=6 \), Grp. II; \( n=4 \), Grp. III; \( n=7 \), Grp. IV; \( n=5 \), Grp. V; \( n=4 \)).

**DISCUSSION**

This study examined the effects of 1,25(OH)\(_2\)D\(_3\) and its low calcemic analogues (EB1089 and analog V) on tumor growth, body weight, blood calcium, bone resorption, and VDR distribution in nude mice with the canine adenocarcinoma (CAC-8) model of humoral hypercalcemia of malignancy. The active form of 1,25(OH)\(_2\)D\(_3\) and its analogues have been reported to inhibit cell growth and induce differentiation in both normal and malignant cell types.\(^{38–42}\) The results of the current study demonstrated a numerical reduction in tumor volume in CAC-8-bearing mice treated with 1,25(OH)\(_2\)D\(_3\) and its analogues (EB1089 and analog V). CAC-8-bearing mice treated with analog V maintained body weight better than other CAC-8-bearing groups. These mice also were more active than other CAC-8-bearing groups and did not have toxic side effects of hypercalcemia despite an increase in plasma ionized calcium as compared to nontumor-bearing mice (Table 1). In addition, only a single mouse from the analog V-treatment group (Grp. III) became clinically ill and had to be sacrificed before the scheduled-time because of weight loss, anorexia, depression, and dehydration. In contrast, mice from other CAC-8-bearing groups treated with vehicle and other substrates had to be sacrificed at days 11 (from Grps. II, V) and at days 14 and 18 (from Grps. IV, V). A previous study reported that analog V \( (1.6 \mu g/mouse) \) treated mice with myeloid leukemia had a significantly longer survival time than those treated with 1,25(OH)\(_2\)D\(_3\) \( (0.1 \mu g/mouse) \) with a diminished hypercalcemic effect.\(^{11}\) The same study reported that 1,25(OH)\(_2\)D\(_3\) and its analogues also inhibited leukemia cell growth and differentiation in vitro.\(^{11}\) Analog V may need to be administered for a longer period and/or at a higher dose in order to achieve a significant reduction in tumor volume in these CAC-8-bearing mice.

A number of vitamin D analogues, such as EB1089 and analog V, have been synthesized to retain the antiproliferative properties of 1,25(OH)\(_2\)D\(_3\) with less hypercalcemic effects. In the present study, EB1089 and analog V failed to lower plasma ionized calcium concentration. In contrast, a previous study reported that treatment with EB1089 \( (0.05 \mu g/(kg \cdot d)) \) in rats with nitrosomethylurea-induced tumors significantly inhibited tumor growth without changing serum calcium levels. In addition, treatment with the same dose of 1,25(OH)\(_2\)D\(_3\) did not affect tumor growth and caused hypercalcemia. EB1089 has also been reported to have antiproliferative activity for breast cancer cell lines both in vivo and in vivo.
vitro). Therefore, it appears that effective treatment of neoplasms with noncalcemic vitamin D analogues may rely on some factors, such as type of tumor, duration of treatment, and species difference. The specific mechanism by which 1,25(OH)2D3 and its analogues inhibit tumor growth at present is unclear. Therefore, it is possible that feeding a low calcium diet during the experimental period in future studies will permit the use of vitamin D analogues for longer period and obtain a greater inhibitory effect on tumor growth.

Canine adenocarcinoma is a cancer associated with hypercalcemia. An autonomous overproduction of PTHrP is the principal humoral factor that causes hypercalcemia in dogs with this carcinoma. Levels of PTHrP secretion increased in all CAC-8-bearing mice treated with vehicle, 1,25(OH)2D3, analog V, or EB1089 in this study. These findings indicated that 1,25(OH)2D3 and its analogues did not inhibit PTHrP production and caused high levels of plasma ionized calcium. Similarly, our in vitro studies in a different canine tumor, squamous cell carcinoma cell lines (SCC2/88), showed that these compounds increased PTHrP production and PTHrP mRNA expression. In human tumors, 1,25(OH)2D3 has been shown to inhibit PTHrP production, suppress PTHrP gene transcription, and prevent the development of the HHM syndrome. With this evidence, it indicates that the effect of 1,25(OH)2D3 on PTHrP production in canine tumors may differ from human tumors. However, the exact mechanisms are still unknown at the present. At the level of PTHrP transcription, it demonstrated that cell- and tissue-specific transcription factors and promoter utilization were important for the regulation of PTHrP gene expression. All together, it is possible that species- and cell type-specific differences in 1,25(OH)2D3 on the regulation of PTHrP production and PTHrP gene expression may be due to tissue-specific transcription factors and tissue-specific promoter utilization.

Humoral hypercalcemia of malignancy has been reported to be associated with increased osteoclastic bone resorption and hypercalcemia. In the canine adenocarcinoma (CAC-8) model of HHM in nude mice, it was found that increased osteoclastic bone resorption and increased bone formation resulted in the overall loss of bone volume and bone-remodeling balance. In our study we also found a significant increase in percent resorption perimeter and number of osteoclasts/mm of endosteal surface in lumbar vertebral tumor-bearing mice with hypercalcemia as compared to nontumor-bearing controls (p<0.001) (Table 2). In cancer-associated hypercalcemia humoral factors produced by tumor cells, including PTHrP and cytokines, bind to receptors on osteoblasts and subsequently enhance the activity of osteoclasts resulting in increased bone resorption. Nevertheless, the degree to which osteoclastic bone resorption contributes to the hypercalcemia of HHM is unknown. Use of antitumor drugs that inhibit bone resorption and induce hypocalcemia for the treatment of cancer-associated hypercalcemia patients has been studied. The results of our study revealed that 1,25(OH)2D3 and its noncalcemic analogues did not decrease osteoclastic bone resorption on CAC-8-tumor-bearing mice. This finding suggested that 1,25(OH)2D3, EB108,9 and analog V did not inhibit bone resorption. It is possible that CAC-8-bearing mice treated with 1,25(OH)2D3 and its noncalcemic analogues combined with inhibiting bone resorption agents in further studies may be more effective in reduction of tumor growth without hypercalcemia.

In conclusion, this study demonstrated that CAC-8-bearing mice treated with analog V were more active and maintained their body weight better than other CAC-8-bearing groups. Analog V-treated mice also showed no toxic side effects of hypercalcemia despite an increase in plasma-ionized calcium comparable to nontumor-bearing mice. Tumor volumes of CAC-8-bearing mice treated with 1,25(OH)2D3 and its analogues were smaller than vehicle-treated CAC-8-bearing mice. This finding suggested an inhibitory effect on tumor cell growth.

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