Inhibition of Prostate Cancer Metastasis in Vivo: A Comparison of 1,25-Dihydroxyvitamin D (Calcitriol) and EB1089

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Abstract

The steroid hormone 1,25-dihydroxyvitamin D [1,25(OH)2D, also known as calcitriol] is known to inhibit the proliferation and to promote the differentiation of human prostate cancer cells. Additionally, we showed that 1,25(OH)2D markedly inhibits the invasiveness of human prostate cancer cells in vitro (G. G. Schwartz et al., Cancer Epidemiol. Biomark. Prev., 6: 727–732, 1997). These properties support the use of 1,25(OH)2D as differentiation therapy in prostate cancer. However, the use of 1,25(OH)2D in vivo is limited by the risk of hypercalcemia. We therefore compared the effects of 1,25(OH)2D and of EB1089, an analogue of 1,25(OH)2D with reduced calcemic effects, in an in vivo model of androgen-insensitive metastatic prostate cancer, the rat Dunning MAT LyLu prostate cancer model. Tumor growth and metastasis were studied using Copenhagen rats given s.c. injections of MAT LyLu cells. Fifty male rats were divided into five groups of 10 rats each. Four experimental groups received i.p. injections of low and high doses of 1,25(OH)2D and EB1089 (0.5 and 1.0 μg/kg, low and high, respectively). A control group received injections of vehicle only. Tumor volumes were measured three times per week. Rats were weighed weekly. The number of metastases to the lungs and the extent of hypercalcemia were evaluated. Compared with controls, tumor volumes were significantly smaller in all experimental groups. Similarly, the number of lung metastases (number of foci/lung) was reduced markedly by both 1,25(OH)2D and EB1089. Control rats developed 22.7 (± 1.98 SE) tumor foci per lung. Rats treated with 1,25(OH)2D and with EB1089 (1.0 μg/kg) developed 10.4 (± 2.81) and 7.70 (± 1.29) tumor foci, respectively (P < 0.001 and P < 0.0001, respectively; drug versus control). Compared with controls (10.79 ± 0.1 mg/dl), serum calcium levels were significantly elevated in both 1,25(OH)2D and EB1089-treated rats (P < 0.001). However, EB1089 was significantly less calcemic than 1,25(OH)2D (12.59 ± 0.21 mg/dl versus 14.47 ± 0.46 mg/dl; 1.0 μg/kg; P < 0.001). Rats treated with 1,25(OH)2D showed marked weight loss: 20.0 ± 1.9% and 26.3 ± 1.7% of their initial weight (low and high doses, respectively, P < 0.001). Weight loss was significantly lower in rats treated with EB1089 at the high dose 8.4 (± 2.9) %. Moreover, rats treated with low-dose EB1089 gained 5.2 (± 3.7) % of their initial weight. In conclusion, 1,25(OH)2D and EB1089 showed marked and equivalent inhibition of prostate cancer metastasis in vivo. EB1089 was significantly less calcemic than 1,25(OH)2D and did not induce severe weight loss. This is the first report of a vitamin D analogue that significantly inhibits prostate cancer metastasis in vivo and that does so without producing cachexia or unacceptable hypercalcemia.

Introduction

Prostate cancer is the most commonly diagnosed (nonskin) cancer and the second leading cause of cancer deaths among United States men, accounting for more than 184,000 new cases and 41,000 deaths in 1998 (1). Few modifiable risk factors for prostate cancer have been identified consistently by epidemiological studies. Pending the identification of such factors, reductions in prostate cancer mortality must come from earlier diagnosis and/or the development of more effective therapies. Despite success in earlier diagnosis, ~50% of the prostate cancers diagnosed annually in the United States and the United Kingdom are locally or distantly advanced at the time of diagnosis and are thus incurable (2). Although palliative therapies (e.g., androgen withdrawal) may control advanced cancers, most men with advanced prostate cancer will develop androgen-independent cancer, the median survival of which is <2 years (3).

There are no effective therapies for androgen-independent prostate cancer. Cytotoxic therapies have yielded low response rates and have not produced increases in survival (4). Thus, new therapeutic approaches are urgently needed. One alternative to cytotoxic drugs is drugs that induce neoplastic cells to mature to a more normal phenotype, i.e., differentiation therapy (5). An especially promising class of differentiating agents is vitamin D compounds (6). For example, 1,25(OH)2D3, the hormonal form of vitamin D, is known to inhibit the proliferation and to induce the differentiation of many normal and neoplastic cells, including prostatic cells (reviewed in Ref. 7).

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3 The abbreviations used are: 1,25(OH)2D, 1,25-dihydroxyvitamin D; VDR, vitamin D receptor; EB1089, 1α,25-dihydroxy-22,24-diene-24,26,27-triiodovitamin D3; CMV, cytomegalovirus; MOP, mouse osteopontin; tk, thymidine kinase; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; 6760, 1,25-dihydroxy-16-ene-23-yne-26,27-hexafluoro-19-nor-cholecalciferol; PSA, prostate-specific antigen.

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A role for vitamin D in the natural history of prostate cancer was first proposed by Schwartz and Hulka (8), who observed that the epidemiology of prostate cancer resembles that of adult vitamin D insufficiency (8). In 1992, Hanchette and Schwartz (9) demonstrated that United States prostate cancer mortality rates were inversely related to the availability of UV radiation, the major source of vitamin D. They interpreted these data to suggest that 1,25-dihydroxyvitamin D maintained the differentiated phenotype of prostatic cells and that low levels of vitamin D permitted the progression of preclinical prostate cancer to clinical disease (10).

Laboratory studies have confirmed an important regulatory role for 1,25(OH)2D in prostatic cells. High-affinity binding sites for 1,25(OH)2D (commonly called VDRs) were demonstrated in the human prostate cancer cell line LNCaP by Miller et al. (11), who showed that exposing these cells to 1,25(OH)2D stimulated their differentiation. Subsequently, VDRs were demonstrated in other well-characterized human prostate cancer cell lines (12), and physiological levels of 1,25(OH)2D were shown to inhibit the proliferation of these cells (13–15). Similarly, physiological doses of 1,25(OH)2D were shown to inhibit the proliferation of human prostate cells in primary culture and to inhibit the invasiveness of a human prostate cancer cell line (16, 17). The addition of 1,25(OH)2D to normal rats results in an increase in the differentiation of the normal prostatic epithelium (18; reviewed in Ref. 19). Finally, Schwartz et al. (20) demonstrated that normal and cancerous human prostatic cells in vitro synthesize 1,25(OH)2D from its precursor, 25-hydroxyvitamin D. This suggests that 1,25(OH)2D plays a fundamental autocrine/paracrine role in prostatic cells, whereby the hormone that is synthesized endogenously acts locally to regulate prostatic growth and differentiation.

These data support the use of 1,25(OH)2D as differentiation therapy in prostate cancer. However, the clinical use of 1,25(OH)2D is limited by the risk of hypercalcemia. Consequently, many analogues of 1,25(OH)2D have been synthesized with the goal of retaining its antiproliferative and prodifferentiating effects while reducing its calcemic effects (21). For example, EB1089 is a 1,25(OH)2D analogue that is 2-fold more potent than 1,25(OH)2D in its affinity for the human VDR and 3-fold more potent in inhibiting the proliferation of LNCaP cells (22). EB1089 has been reported to be less calcemic than calcitriol in vivo in rats implanted with Leydig cell tumors (23). However, the effects of EB1089 on serum calcium levels in rats with prostate tumors are unknown. The present study compared these determinations were made without knowledge of the treatment group. Tumor growth was measured using calipers, and the volume was approximated to an ellipsoid (i.e., length × width × height × 0.5236). Differences in tumor volumes between groups were evaluated at 12, 15, 17, and 19 days using ANOVA and Tukey tests. Statistical analyses were performed with the INSTAT statistical programs (Ravitz Software, San Diego, CA) and STATISTIX (Analytical Software, Tallahassee, FL).

Measurement of Serum Calcium

About 2 to 5 ml of heart blood was withdrawn at the time of euthanasia. Sera were obtained and analyzed immediately for serum calcium, as described previously by Schwartz et al. (25). Rats were being treated with drugs until 2 days before euthanasia. Thus, the maximum interval between drug treatment and blood collection was 48 h.

VDR Methods in Vitro

Because the antiproliferative effects of 1,25(OH)2D depend, at least in part, on the expression of functional VDRs, we sought to examine the mechanism for the antitumor effect of these drugs by quantitating VDR expression in MAT LyLu cells.

Materials. [26,27-methyl-3H]1,25(OH)2D and 14C-chloraphenicol were purchased from Du Pont NEN (Boston, MA). Radiolabeled 1,25(OH)2D Binding Assay. Assays were performed as described previously (26). Briefly, soluble cell fractions (cytosols) were prepared, and 200-μl aliquots containing 1 mg/ml protein were incubated with increasing concentrations of [3H]-1,25(OH)2D with or without 500-fold excess of radioinert 1,25(OH)2D. Bound and free 1,25(OH)2D were separated by the hydroxyapatite method (27). The protein concentration was determined by the method of Bradford. Bmax
and $K_r$ were estimated from Scatchard analysis using Prism software (GraphPad, Inc. San Diego, CA).

**Recombinant Plasmids, Transfection, and Chloramphenicol Acetyltransferase Assay.** The VDR cDNA expression vector pRC-CMV-VDR (henceforth CMV-VDR), provided by Dr. Leonard Freedman (Memorial Sloan-Kettering Cancer Center), consists of the full-length human VDR coding region driven by the CMV promoter (28). The reporter plasmid MOPVDRECAT was constructed as described previously by Zhuang et al. (26) and consists of two copies of the VDRE of the MOP gene (29) linked 5' to the tk promoter and CAT gene of the vector pBLCAT2 (30).

For transfections, cells were passaged 16–20 h before transfection, and the medium was changed to DMEM 1 h before transfection. The calcium phosphate method was used in which cells were incubated with DNA precipitates for 6–8 h at 37°C, followed by a 1-min glycerol shock (15% glycerol in DMEM; 31). Cells were cultured in the presence or absence of 10 nM 1,25(OH)$_2$D in RPMI containing 10% FBS. Cells were harvested ~40 h after transfection, and cell extracts were prepared for analysis of $\beta$-galactosidase and CAT activity. The cell lines were transfected with MOPVDREtkCAT and CMV-$\beta$-gal, which encodes the $\beta$-gal gene driven by the CMV promoter. Cell extracts containing equivalent amounts of $\beta$-gal activities were used for analysis of CAT using an adaptation of the method of Gorman et al. (32). The percentage of conversion of $[^{14}$C]chloramphenicol to acetylated forms on thin-layer chromatograms was quantified using a Molecular Dynamics Phosphorimager and ImageQuant software (Sunnyvale, CA). The effect of experimental conditions was tested by ANOVA and t-tests.

**Results**

**Effect of 1,25(OH)$_2$D and EB1089 on Growth and Metastasis of Dunning MAT LyLu Tumors.** All 50 rats (100%) injected with MAT LyLu cells developed tumors. Tumor volumes of rats treated with calcitriol and EB1089 were significantly smaller than tumor volumes of control rats (Fig. 1). These differences emerged starting day 15 after tumor implantation. By day 19, the mean tumor volume in the control group (15.0 ± 2.1 cm$^3$) was nearly four times that seen in the groups treated with calcitriol (4.3 ± 1.3 and 4.6 ± 0.6 cm$^3$, low and high doses, respectively). Tumor volumes in the rats treated with EB1089 were intermediate between control and calcitriol groups. By day 19, the tumors in the calcitriol-treated rats were significantly smaller than those of rats treated with EB1089 at high dose. The rats in the control group were sacrificed on day 19 because the tumor volumes all exceeded 10 cm$^3$.

Tumor cell foci in the lungs were observed in all animals (Fig. 2). Metastatic tumor foci appeared as small circular eruptions on the surface of the lungs. Histological sectioning confirmed that most of the tumor foci were located on the lung periphery. This finding made it possible to evaluate response by inspection of the lung surface. The smallest tumor focus was ~0.5 mm in diameter, and the largest was ~2 mm. Both 1,25(OH)$_2$D and EB1089 caused significant inhibition of tumor foci in the lungs ($P < 0.00001$).

The mean number of tumor foci in the control group was 22.7 ± 1.98 SEM versus 10.4 ± 2.81 for rats treated with 0.5 μg/kg 1,25(OH)$_2$D and 9.6 ± 1.97 SEM for rats treated with 1.0 μg/kg 1,25(OH)$_2$D. Rats treated with EB1089 showed similar decreases in the number of metastatic foci in the lungs. The mean number of tumor foci in the EB1089-treated rats was 9.7 ± 2.18 and 7.7 ± 1.29 for low and high doses, respectively. 1,25(OH)$_2$D and EB1089 did not differ significantly from one another in their inhibition of lung metastases (Fig. 3).

**Serum Calcium.** Rats treated with calcitriol showed significant elevations in serum calcium. Compared to the serum calcium level of 10.8 mg/dl ± 0.11 SEM in the control group, serum calciums were 14.65 ± 0.59 and 14.44 ± 0.31 mg/dl in the calcitriol-treated groups (low and high dose, respectively). Rats treated with EB1089 also showed significant elevations in serum calcium: 12.74 ± 0.28 and 12.59 ± 0.23 mg/dl (low and high doses; Fig. 4). However, these values were significantly lower than those for rats treated with calcitriol ($P < 0.001$).

**Body Weight.** Compared with their initial weights, the mean weight of rats in the control group increased by 8.1% during the 22 days of the experiment (Fig. 5). Rats treated with calcitriol exhibited significant and dose-dependent weight loss (20.0 ± 1.9% and 26.3 ± 1.7% of initial weight, low and high doses, respectively, $P < 0.0001$, both groups compared with control). Additionally, calcitriol-treated rats appeared lethargic and exhibited a decreased tolerance to handling. Two rats treated with the high dose of 1,25(OH)$_2$D showed reduced mobility. Conversely, rats treated with EB1089 showed no signs of lethargy or irritability. Although rats treated with EB1089 at high doses also showed significant weight loss (8.4 ± 2.9% of their body weight) ($P < 0.001$, compared with control), the weight loss seen in these animals was significantly lower than the weight loss shown by rats in either 1,25(OH)$_2$D-treated group ($P < 0.0001$). Moreover, rats treated with low dose of EB1089 gained an average of 5.2 ± 3.7% of their initial weight. The weight gain in the rats treated with low dose EB1089 did not differ significantly from the weight gain in the control group (8.1%; Fig. 5).

**VDRs.** To determine whether functional VDR were expressed in MAT LyLu cells, we evaluated the effect of 1,25(OH)$_2$D in cells transfected with a VDRE-containing reporter gene. We used the reporter plasmid MOPVDREtkCAT, which contains...
Fig 2. Representative lung metastases of MAT LyLu tumors in control, 1,25(OH)$_2$D$_3$- and EB1089-treated rats. A, control rat; B, 1,25(OH)$_2$D$_3$-treated rat (1 μg/kg); C, EB1089-treated rat (1 μg/kg).
two copies of the VDRE found in the MOP gene. We chose this plasmid because our previous studies in four other well-characterized prostate cancer cell lines indicated that VDR transcriptional activity could be detected using this VDRE-containing reporter, even in cell lines that expressed extremely low levels of VDR (26). No hormone-inducible CAT activity was detected in MAT LyLu cells, suggesting that functional VDRs are not expressed in these cells. Transfection of a VDR cDNA resulted in 1,25(OH)2 D-inducible CAT activity, suggesting that other components required for 1,25(OH)2 D responsiveness (e.g., the retinoid X receptor) are likely to be present and that 1,25(OH)2 D was not metabolized rapidly.

Because the reporter gene assay described above detects only receptors that have the capacity for transcriptional activity, we also tested whether VDRs capable of specific hormone binding were present. We performed radiolabeled 1,25(OH)2 D binding assays. These tests did not detect a factor with the capacity for saturable and high-affinity ligand binding as is observed in prostate cancer cell lines expressing VDRs. As a positive control, we used the human prostate cancer cell line ALVA 31, which is known to express VDR (26). Thus, the VDR was not detectable in MAT LyLu cells, with the exception of some binding sites with extremely low affinity (Fig. 6).

**Discussion**

Both 1,25(OH)2 D and EB1089 significantly decreased tumor volumes in treated rats. These results are similar to those reported previously by us using the (nonmetastatic) PC-3 human prostate cancer cell line xenografted into nude mice (25). Because factors other than cancer cell proliferation (e.g., local inflammation and tumor necrosis at the site of the primary tumor) are known to influence "tumor" volumes, measurements of tumor volume (unlike measurements of tumor weight) are not definitive evidence of an antiproliferative effect on tumor cells. Our findings with respect to tumor volume, however, are similar to those of Getzenberg et al. (33), who recently reported that calcitriol and EB1089, may inhibit the capacity of cells to invade through a basement membrane and/or these drugs may inhibit the secretion of collagenses by prostatic cells, enzymes which aid in the dissolution of the basement membrane, an early step in the metastatic cascade (17). Calcitriol may also act at later steps in this cascade. For example, the growth of prostate tumors in vivo is the result of numerous physiological processes, including the formation of new blood vessels that are required to support tumor growth (neovascularization; Refs. 34 and 35). Calcitriol has been shown to exert significant antiangiogenic effects in vivo in several tumors, e.g., rat mammary tumors and murine retinoblastomas (36, 37).

The apparent absence of functional VDRs in MAT LyLu cells. Our findings with respect to tumor volume, however, are similar to those of Getzenberg et al. (33), who recently reported that calcitriol and EB1089, may inhibit the capacity of cells to invade through a basement membrane and/or these drugs may inhibit the secretion of collagenses by prostatic cells, enzymes which aid in the dissolution of the basement membrane, an early step in the metastatic cascade (17). Calcitriol may also act at later steps in this cascade. For example, the growth of prostate tumors in vivo is the result of numerous physiological processes, including the formation of new blood vessels that are required to support tumor growth (neovascularization; Refs. 34 and 35). Calcitriol has been shown to exert significant antiangiogenic effects in vivo in several tumors, e.g., rat mammary tumors and murine retinoblastomas (36, 37).
Calcitriol Inhibits Prostatic Metastases

Because metastatic disease is the ultimate cause of death in prostate (and many other) cancers, the inhibition of metastasis is a rational goal for anticancer therapy (43). This point is especially significant in prostate cancer because the prevalence of microscopic, so-called “latent” prostate cancer, is extremely high. As many as 60% of men over the age of 60 have evidence of microscopic prostate cancers, and the prevalence of these cancers increases further with age (44). The identification of factors that determine which of these “latent” tumors will remain latent is a critical research goal. We have suggested that vitamin D is one such factor. This view is consistent with the geographic distribution of prostate cancer mortality in the U.S., which is correlated inversely with the geographic distribution of UV radiation, the principal source of vitamin D, and with results of some (45) but not all (46) prospective epidemiological studies of prostate cancer and serum vitamin D metabolites. The recent demonstrations that men with genetic variants of the VDR are at 3–5-fold increased risk of prostate cancer and that prostate cells synthesize 1,25(OH)2D from its substrate, 25-hydroxyvitamin D, further underscore the importance of vitamin D in the natural history of prostate cancer (8, 47, 48).

The greatest impediment to the clinical use of vitamin D-based therapies in cancer is hypercalcemia. In the present study, EB1089 was as effective as 1,25(OH)2D in inhibiting metastasis in vivo but was significantly less calcemic. This is in contrast to the study by Getzenberg et al. (33) in which 5 μg of 6760 (the only effective dose) was as calcemic as 1 μg of calcitriol.

The 19% mean elevations in serum calcium in rats treated with EB1089 may approximate a clinically acceptable range of toxicity in humans, considering that serum calcium levels can be controlled by several methods, including the use of calcium-binding agents and calcium-restricted diets. For example, in a recent trial of the vitamin D analogue, alfacalcidol, in myelodysplastic syndrome, oral doses of 6 μg daily achieved a sustained response in three of six patients. In that study, alfacalcidol was administered with the use of calcium-lowering agents (49). It is noteworthy that the antimetastatic effects that we observed were obtained using a relatively high calcium diet (0.95% calcium). It would be valuable to investigate whether these antimetastatic effects can be increased using some restriction in dietary calcium. For example, the use of a 0.5% calcium diet has been shown to have no growth-retarding effects in rats but permits up to 10 times the amount of 1,25(OH)2D without inducing hypercalcemia (50).
Rats treated with 1,25(OH)2 D in our study showed marked weight loss: 20.0 ± 1.9% and 26.3 ± 1.7% of their initial body weight. Similar findings were observed by Getzengen et al. (33) for rats treated with either 1 µg of 1,25(OH)2 D and 5 µg of 6760, who experienced weight loss of 30–34% of their initial body weight. In contrast, rats treated with EB1089 at the low dose gained weight comparable with that of the control group. To our knowledge, this is the first report of a 1,25(OH)2 D analogue that is significantly effective against prostate cancer metastasis in vivo and that is effective without causing cachexia or unacceptable hypercalcemia.

These findings may be rapidly translatable to clinical trials. For example, Osborne et al. (51) reported the first trial of (oral) 1,25(OH)2 D in prostate cancer. These authors treated 14 men with advanced, androgen-insensitive prostate cancer (stage D; mean PSA at entry, 266; range, 20–950). Two patients showed decreases of 25 and 45% in serum PSA. Dose escalation was limited by hypercalcemia. These findings suggested that 1,25(OH)2 D might be effective if treatment were initiated earlier in the course of disease. Accordingly, Gross et al. (52) recently treated men with early recurrent prostate cancer (i.e., men with detectable PSA after definitive treatment) with oral 1,25(OH)2 D. A significant decrease in the rate of rise of the PSA with detectable PSA after definitive treatment) with oral 1,25(OH)2 D analogues (EB1089) in primary cultures of human prostatic cells. Cancer Epidemiol. Biomark. Prev., 6: 727–732, 1997.


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