Reversal of Hypercalcemia with the Vitamin D Analogue EB1089 in a Human Model of Squamous Cancer

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Abstract

EB1089, an analogue of 1,25 dihydroxyvitamin D with low calcemic activity is a potent inhibitor of parathyroid hormone-related peptide (PTHRP) production in vitro. The purpose of the present study was to determine whether EB1089 could reverse established hypercalcemia in BALB C nude mice implanted s.c. with a human epithelial cancer previously shown to produce high levels of PTHRP in vitro. Total plasma calcium was monitored before and after tumor development and increased steadily when the tumor reached >0.5 cm. When total calcium was >2.85 mmol/liter, animals were treated with a constant infusion of EB1089 or vehicle alone for a period of 2 weeks. A significant and sustained reduction of plasma calcium from 3.2 ± 0.1 to 2.7 ± 0.08 (P < 0.01) mmol/liter was observed during infusion with EB1089. In contrast, calcium levels in vehicle-treated animals continued to rise during the infusion period. Tumor growth velocity also slowed significantly after the administration of EB1089 as compared with vehicle-treated animals. Plasma PTHRP levels measured at the end of the 2 weeks’ infusion period were significantly lower in animals treated with EB1089 as compared with animals treated with vehicle alone (44 ± 8 pg/ml versus 194 ± 35 pg/ml, P < 0.001). These results, therefore, demonstrate that EB1089 can reverse established hypercalcemia in a human model of squamous cancer.

Introduction

Previous studies (1, 2) have clearly demonstrated that 1,25(OH)2D3 is a potent antiproliferative and prodifferentiative agent. These properties have been demonstrated in vitro not only in normal cells but also in cancer cells (3–5). In vivo studies (6) have also produced significant tumor regression in human tumors in nude mice. However, the therapeutic application of 1,25(OH)2D3 is seriously limited by its side effects, which include hypercalcemia and hypercalciuria (7). Although its bio-

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The abbreviations used are: 1,25(OH)2D3, 1,25 dihydroxyvitamin D3; PTHRP, parathyroid hormone-related peptide; MAH, malignancy-associated hypercalcemia; FBS, fetal bovine serum; iPTHRP, immunoreactive PTHRP.

demonstrated that 1,25(OH)2D3 blocks PTHRP production (10). Using a multistep model of epithelial cell carcinogenesis, we demonstrated that the progression from the normal to the malignant phenotype was characterized by a partial resistance to the inhibitory effect by 1,25(OH)2D3 requiring 10- to 100-fold higher concentrations of 1,25(OH)2D3 to achieve the same effects (11, 12). To develop alternative strategies to block PTHRP production in vitro and in vivo, several 1,25(OH)2D3 analogues—known to have low calcemic activities yet to retain strong antiproliferative effects on keratinocytes in vitro (13)—were tested. One such analogue, EB1089 (Leo Pharmaceuticals Ltd, Ballerup, Denmark), has a half-life similar to that of 1,25(OH)2D3 yet is 10 times less potent in promoting hypercalcemia in rats (14). In the tumor progression model, EB1089 was 100 times more potent than 1,25(OH)2D3 in inhibiting PTHRP (14). EB1089 is, therefore, not only a potential inhibitor of PTHRP overproduction in vivo but represents a possible new strategy in hypercalcemia therapy.

Subsequently, an animal model of MAH, the rat Leydig cell tumor H500 (15) was used. The hypercalcemic state associated with this rat testicular cancer has been linked to PTHRP (16, 17). Animals that were implanted with the rat Leydig cell tumor H500 and were treated simultaneously with a constant infusion of EB 1089 maintained normocalcemia and had lower circulating PTHRP concentrations than animals treated with the vehicle alone (18). These results clearly indicated that vitamin D analogues with low calcemic activities can prevent the development of hypercalcemia in an established animal model when administered at the time of tumor implantation. However, for these analogues to be useful clinically, it remains to be determined that they can reverse established hypercalcemia and that they can be applied to human models of MAH. Our present study was designed to closely mimic the clinical situation encountered in patients with MAH. In this experimental design, a human model of squamous cancer-producing PTHRP was used, and animals were treated after the onset of hypercalcemia.

Our present data clearly indicate that EB1089 efficiently blocks PTHRP production and reverses established hypercalcemia in nude mice implanted with human squamous tumors that express high levels of PTHRP.

Materials and Methods

Cell Culture Conditions. The HPK1A cell line was established from normal human keratinocytes by stable transfection with human papillomavirus type 16 (19). Despite acquiring an indefinite life span in culture, these cells retain differentiation properties characteristic of normal keratinocytes (20) and are nontumorigenic when injected into nude mice. These immortalized cells were subsequently transformed into the malignant HPK1A-ras cell line after transfection with a plasmid carrying an activated H-ras oncogene (12, 21). In addition to forming colonies in soft agar, the malignant HPK1A-ras cells produce squamous cell carcinoma when transplanted into nude mice. HPK1A-ras cell line was seeded and grown in DMEM (Life Technologies, Inc.)

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supplemented with 10% FBS (Life Technologies, Inc.) and maintained by serial passaging. Prior to s.c. implantation into nude mice, proliferating cells were trypsinized, washed in DMEM containing 10% FBS, and resuspended in complete medium.

For cell-growth experiments, cells were seeded at a density of 1 × 10^4 cells/well in 24-well cluster plates and grown to 20% confluence. After 24 h in basal conditions (DMEM without serum), fresh medium containing 10% FBS without or with varying concentrations of EB1089 was added to the cultured cells, and incubations were continued for 72 h. For survival assays, cells were treated with increasing concentrations of EB1089 without FBS. Cells were trypsinized and counted in a coulter counter (LKB, Montreal, Quebec, Canada). XTT-Microculture tetrazolium assay for cell growth was done as described previously (12). Cells were trypsinized and counted in a coulter counter (LKB, Montreal, Quebec, Canada). XTT-Microculture tetrazolium assay for cell growth was measured at 490 nm using a Bio-Rad microplate reader. Results were then expressed as percent of FBS-stimulated growth.

**Vitamin D Analogue.** EB1089 was kindly provided by Leo Pharmaceuticals (14). EB1089 has terminal ethyl groups and double binds (at positions 22 and 24) in the side chain. This compound has low calcemic activity (14) and a half-life similar to 1,25(OH)_{2}D_{3} in vivo (Ref. 22; Table 1).

**Animal Protocols.** BalbC nude mice (20 g; female) were implanted s.c. with 10^7 ras-transformed keratinocytes (HPK1Aras) as described previously (21) in 200–300 μl of suspension of complete medium (DMEM and 10% FBS).

All of the animals were examined twice a week for development of a palpable tumor at the site of injection or other s.c. sites. Three-dimensional tumor measurements were done using calipers. Tumor diameters long axis (L) and mean mid axis width (W) were measured to estimate the tumor volume using the following formula:

$$V = \frac{4}{3} \pi \left(\frac{L \times W}{2}\right)^{2}$$

**Plasma Calcium and PTHRP Measurements.** Plasma samples were obtained by orbital bleeding at regular intervals (every 5–7 days), and 50–100 μl were used to measure total calcium and albumin by microchemistry (Kodak Ektachrome).

Animals were killed by cardiac puncture, and 300 μl of plasma were recovered for measurement of plasma calcium and PTHRP. PTHRP was measured using an immunoradiometric assay as described previously (23). The assay recognizes the intact first 86 amino acids of the molecule and has a detection limit of 2 pg/ml.

**Statistical Analysis.** All of the results are expressed as the mean ± SE, and statistical comparisons are made on the basis of Student’s t test or a one-way ANOVA, using a Bonferroni adjustment when appropriate (24).

**Results**

**Effect of EB1089 on Plasma Calcium.** In non-tumor-bearing animals the lowest dose of EB1089 (20 pmol/24 h) did not produce...
hypercalcemia; however, higher doses produced a progressive increase in plasma calcium (Fig. 1). Consequently, hypercalcemic tumor-bearing animals were treated with a constant infusion of 18 pmol/24 h of EB1089.

After tumor cells implantation, a progressive increase in tumor volume was observed that preceded an increase in plasma calcium. Furthermore, these animals reached plasma calcium levels comparable to the calcium levels of non-tumor-bearing mice (Fig. 3).

**Effect on PTHRP Production.** iPTHRP was measured in the plasma of non-tumor-bearing animals (control), untreated tumor-bearing animals, and hypercalcemic tumor-bearing animals treated with EB1089 or vehicle alone. iPTHRP was low and not significantly different between a group of eight non-tumor-bearing animals (controls) and a group of eight normocalcemic tumor-bearing animals (15 ± 4 pg/ml versus 18 ± 5 pg/ml; Fig. 4). Normocalcemic tumor-bearing animals with a tumor volume of <0.5 cm³ were sacrificed 4–6 weeks after tumor implantation. All of the hypercalcemic tumor-bearing animals receiving vehicle alone and bled by cardiac puncture at the time of death had high iPTHRP plasma concentration (194 ± 35 pg/ml), whereas tumor-bearing animals receiving EB1089 and killed 2 weeks after the administration of the analogue had a significant reduction of PTHRP (44 ± 8 pg/ml, P < 0.01; Fig. 4).

**Effect on Cell Proliferation in Vitro and Tumor Growth in Vivo.** To further understand the mechanism of this effect, we performed *in vitro* studies for cell proliferation and survival assays (to assess apoptosis). These results are summarized in Table 2. In serum-treated cells, EB1089 significantly inhibited cellular growth in a dose-dependent fashion achieving a maximal inhibition at 10⁻⁷ M. However, no effect on cell survival was detected in serum-deprived experiments (data not shown).

Tumor growth velocity was assessed before and during infusion of EB1089 or vehicle alone (Table 2). Tumors continued to grow in both treated and control group. However, in EB1089-treated animals, tumor growth velocity decreased significantly as compared with control animals (16.8% ± 5% versus 95% ± 32%; P < 0.05).

**Discussion**

1,25(OH)₂D₃ analogues with low calcemic activities are of potential value as anticancer agents (13, 18, 25–28). These analogues retain strong antiproliferative effects, although less calcemic than 1,25(OH)₂D₃. In previous studies, we have used one such analogue, EB1089, and demonstrated its strong capacity to inhibit PTHRP production *in vitro* (13) and *in vivo* (18). Our present data indicate that this analogue can also reverse established hypercalcemia in nude mice that have been implanted with a human squamous cancer. This tumor produces high levels of PTHRP (11, 13), a mediator linked to MAH of the majority of solid tumors in humans (9). Our previous demonstration (18) that EB1089 could be used as a preventative agent in the treatment of hypercalcemia in the rat Leydig cell tumor suggested that such an analogue could also be effective in reversing established hypercalcemia, a clinical situation frequently encountered in advanced cancer. Furthermore, a human model of epithelial carcinogenesis was chosen to closely mimic the human

**Table 2** Effect of EB1089 on FBS-stimulated cell growth in HPK1A-ras cells in *vitro* and on tumor growth in vivo

<table>
<thead>
<tr>
<th>EB1089 concentration (M)</th>
<th>Cell proliferation</th>
<th>Tumor growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell number</td>
<td>Formazan production</td>
</tr>
<tr>
<td>Control (100%)</td>
<td>vehicle 95 ± 32</td>
<td>EB1089 16.8 ± 5°</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>86.4 ± 3.5°</td>
<td>92.3 ± 3.2°</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>82.4 ± 0.84°</td>
<td>78.3 ± 5.0°</td>
</tr>
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*Significant difference from control values or vehicle-treated animals (P < 0.05).
clinical syndrome of MAH. Our strategy was to use a continuous infusion of EB1089, which does not produce calcium elevation in control non-tumor-bearing animals. A dosage of 18 pmol/24 h was determined, and the pump was implanted adjacent to the tumor to deliver a high concentration of the analogue to the tumor site. This experimental design was favored to achieve maximal local inhibition of PTHRP production by tumor cells. Although we cannot exclude a strong systemic effect of EB1089, it is likely that this experimental design favors a strong local effect of EB1089 on the tumor. To be useful clinically, such agents will require adequate adverse effects in treatment of cancer patients to mimic the experimental design presented here.

The administration of EB1089 was effective in reversing hypercalcaemia in tumor-bearing animals in which calcium levels were ≥2.85 mmol/liter. This cutoff value was used because it represents the clinical situation in cancer patients in which hypercalcaemia often requires treatment with antiresorptive agents such as bisphosphonates. Bisphosphonates are highly effective in reversing hypercalcaemia but their effect are short-lived (29), and patients with elevated PTHRP levels are often nates are highly effective in reversing hypercalcaemia but their effect are short-lived (29), and patients with elevated PTHRP levels are often nates are highly effective in reversing hypercalcaemia but their effect are short-lived (29), and patients with elevated PTHRP levels are often nates are highly effective in reversing hypercalcaemia but their effect are short-lived (29), and patients with elevated...
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