Expression and regulation of parathyroid hormone-related peptide in normal and malignant melanocytes

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El Abdaimi, Khadija, Vasilios Papavasiliou, David Goltzman, and Richard Kremer. Expression and regulation of parathyroid hormone-related peptide in normal and malignant melanocytes. Am J Physiol Cell Physiol 279: C1230–C1238, 2000.—We examined parathyroid hormone-related peptide (PTHrP) production and regulation in both normal human melanocytes and in a human amelanotic melanoma cell line (A375). Northern blot and immunocytochemical analysis demonstrated that both cultured A375 cells and normal human melanocytes express PTHrP, but A375 cells expressed much higher levels of the peptide. PTHrP secretory rate increased at least 10-fold after treatment with 10% fetal bovine serum (100.2 ± 2.8 pmol/10⁶ cells vs. basal <15 pmol/10⁶ cells) in proliferating A375 cells but only twofold in confluent cells. Treatment of A375 cells with increasing concentrations of 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃] or its low-calcemic analog EB-1089 revealed that EB-1089 was 10-fold more potent than 1,25-(OH)₂D₃ on inhibition of both cell proliferation and PTHrP expression. Furthermore, inoculation of A375 cells into the mammary fat pad of female severe combined immune deficiency mice resulted in the development of hypercalcemia and elevated concentrations of plasma immunoreactive PTHrP in the absence of detectable skeletal metastases. Our study, therefore, demonstrates a stepwise increase in PTHrP expression when cells progress from normal to malignant phenotype and suggests that EB-1089 should be further evaluated as a therapeutic agent in human melanoma.

Malignant melanoma can metastasize to almost every major organ and tissue. In clinical series, osteolytic bone metastases have been recognized with a frequency ranging from 23 to 49% (4). Hypercalcemia is uncommon in this condition, and its pathogenesis remains uncertain (5, 31). As in other malignancies, it may be caused by local osteolytic mechanisms and/or by production of the tumor’s osteoclast-stimulating factors. In breast cancer, PTHrP is known to induce osteolysis in malignant melanoma; vitamin D analog EB-1089.

MATERIALS AND METHODS

Culture conditions. The human amelanotic malignant melanoma cell line A375 was established from a metastatic lesion in the lung (27). These cells metastasize to the lung at a high frequency when injected subcutaneously or intravenously into athymic nude mice or form skeletal metastases when injected into the left cardiac ventricle (17, 35). A375 cells were obtained from American Type Culture Collection (Rockville, MD), maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Grand Island, NY) that contained 10% heat-inactivated fetal bovine serum (FBS; Wisent, Montreal, Quebec, Canada) and 1× antibiotic-antimycotic solution (GIBCO) at 37°C in an atmosphere of humidified air and 5% CO₂, and passaged once weekly.

Normal human epidermal melanocytes (NHEM) were obtained from Clonetics (San Diego, CA) and grown in a complete medium, melanocyte growth medium (MMG), consisting of melanocyte basal medium (MM-2; Clonetics) supplemented with 1 ng/ml human basic fibroblast growth factor (bFGF; Clonetics), 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10 ng/ml phorbol 12-myristate 13-acetate (Clonetics), 0.5% FBS, and 15 μg/ml bovine pituitary extract. For studies involving mRNA detection, cells were grown to 60% confluence in MMG. After a 24-h incubation in MMG-2 (basal conditions), the medium was changed and replaced with

osteolytic bone lesions and the growth of metastatic deposits in a nude mouse model of bone metastases injected with a PTHrP-producing human breast cancer cell line (15), suggesting that PTHrP expression by breast cancer cells enhances their metastatic potential to bone. To gain further insight into the mechanism of tumor-induced osteolysis in malignant melanoma, we examined the expression and regulation of PTHrP production in normal and malignant melanocytes. In addition, we examined the in vivo production of PTHrP by the tumor and its calcemic effect after implantation of melanoma A375 cells into the mammary fat pad of athymic severe combined immunodeficiency (SCID) mice. Here, we demonstrated a stepwise increase in PTHrP expression when cells progress from normal to malignant melanocytes in vitro and its regulation by the vitamin D₃ analog EB-1089. Furthermore, we show in vivo induction of hypercalcemia by the tumor in the absence of skeletal metastases.

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MG5M for 4 h. Cells were then trypsinized and lysed with TRIzol solution (GIBCO) for subsequent analysis by Northern blot hybridization as described in Northern blot analysis.

Cell proliferation assays. A375 cells were seeded at a density of 1 × 10^4 cells/well in 24-well cluster plates and grown to 30% confluence. After 24 h in serum-deprived DMEM, fresh medium that contained 1% FBS with or without increasing concentrations (10^-10 to 10^-7 M) of EB-1089 or 1,25-dihydroxyvitamin D_3 (1,25-(OH)_2D_3) was added to cultured cells, and incubations continued for 48–72 h. Ethanol concentration in all cultures was 0.1%. Cells were trypsinized at timed intervals, an aliquot was counted (Coulter Electronics, Beds, UK), and the remaining cell suspension was spun and processed for Northern blot analysis.

DNA synthesis was assessed by measuring incorporation of [3H]thymidine into cellular DNA. [3H]thymidine (1 μCi/ml, DuPont-NEN) was added to the cells during the last 2 h of incubation. The medium was aspirated, and the cells were then washed twice with cold Hanks' balanced salt solution and incubated in 5% cold trichloroacetic acid (TCA) for 15 min. TCA was removed, and the cells were dissolved in 0.5 ml of 0.6 N NaOH, and an aliquot was counted by liquid scintillation. Results were calculated as counts per minute (cpm)/10^6 cells and then were expressed as a percentage of [3H]thymidine incorporation measured in the absence of 1,25-(OH)_2D_3 or EB-1089.

The MTS tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay assesses cellular growth on the basis of the intensity of a colorimetric reaction that results from the reduction of a tetrazolium reagent (MTS; Promega, Madison, WI) to a soluble formazan salt by growing cells, with absorbance being directly proportional to cell density. Briefly, NHEM cells were seeded at 4 × 10^3 cells/100 μl into 96-well microtiter plates in MGM until 50% confluence. After 24 h in basal conditions (MBM-2), medium was removed and replaced with fresh MGM that contained increased concentrations of 1,25-(OH)_2D_3 or EB-1089 (10^-7 to 10^-2 M). MTS solution, 20 μl to each well, was added during the last 2 h of incubation at 37°C. Absorbance at 490 nm was measured using a Bio-Rad microplate reader. The background 490-nm absorbance from the control wells that contained MGM alone (without cells) was subtracted at each point.

Assay for immunoreactive PTHrP in conditioned medium. An immunoradiometric assay (INCSTAR, Stillwater, MN) that employs two polyclonal antibodies, one labeled with 125I that binds to the COOH-terminal 57–80 region, and a second constant, was used. The detection limit of the assay is 1.5 pmol/l. A375 cells were plated at a density of 1 × 10^4 cells/well in 24-well cluster plates and grown to 30% confluence. After 24 h in serum-deprived DMEM, cells were treated with increasing concentrations of EB-1089 or 1,25-(OH)_2D_3 (10^-7 to 10^-2 M), insulin (1–10 μg/ml), or hFGF (0.5–50 ng/ml). Conditioned medium was collected, centrifuged to remove debris, and stored at −80°C until assayed. Cells were trypsinized and an aliquot was counted. Results were corrected for cell number and expressed as percentage of human PTHrP (rPTHrP(1–84))/10^6 cells in the absence of 1,25-(OH)_2D_3 or its analog (% of control).

Northern blot analysis. Total cellular RNA from cells or tumor tissues was isolated with TRIzol solution according to the manufacturer's specifications (GIBCO), dissolved in water, and stored at −80°C for future use. Twenty micrograms of total RNA were electrophoresed in a 1.2% formaldehyde-agarose gel and transferred by blotting to a nylon membrane. Filters were air dried, baked at 80°C under vacuum for 2 h, and then prehybridized for at least 2 h at 65°C in a solution of 5× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate), 5× Denhardt’s solution, 0.5% sodium dodecyl sulfate (SDS), and denatured salmon sperm DNA. Hybridization was carried out overnight at 65°C in the same buffer using a 537-bp Sac I-Hind III restriction fragment encoding exon III (coding region) of the hPTHrP gene labeled with [32P]dCTP (ICN Biomedicals, Mississauga, Ontario, Canada) using a random primer kit (Pharmacia Biotech, Baie d’Urfé, Quebec, Canada). Filters were washed twice at room temperature in 2× SSC and 0.1% SDS for 10 min and once for 15 min at 65°C in 1× SSC and 0.1% SDS. Autoradiography of filters was carried out at −80°C using Kodak XAR film and two intensifying screens. Filters were also probed with an 800-bp BamH I restriction fragment of rat cyclophilin as a control for PTHrP mRNA changes.

RNA analysis by RT-PCR. Vitamin D receptor (VDR) was amplified using the following primers: VDR upstream primer, 5′-ATGGCGGCGACACTTCCGTCGTCGAC-3′; VDR downstream primer, 5′-CTCTCCTTCGCTTCCAGGATCTC-3′. Briefly, 5 μg of total RNA were reverse transcribed using an RT mix consisting of 4 μl of 5× first-strand buffer (GIBCO BRL, Montreal, Quebec, Canada), 2 μl of deoxynucleotide triphosphate (10 mM), 1 μl of random hexamer primers, 0.25 μl of RNase inhibitor, 0.5 μl of RT, 0.4 μl of dithiothreitol (0.1 M), and 5.55 μl of water. Tubes were then placed in a thermocycler (Perkin-Elmer) and treated at the following temperatures: 10 min at 23°C, 45 min at 42°C, and 5 min at 95°C. A PCR mix consisting of 5 μl of 10× PCR buffer (GIBCO BRL), 2.5 μl (100 pmol) of each primer, 0.5 μl of Taq polymerase, and 34.5 μl of water was then added to 5 μl of RT product, and the DNA was amplified for 30 cycles. Analysis was performed on a 2% agarose gel stained with ethidium bromide.

Immunocytochemical analysis. Cellular content of PTHrP was determined by immunocytochemistry. Cells were seeded in four chamber glass slides (Nalge Nunc, Naperville, IL) at a density of 10^4 cells per chamber in either DMEM/10% FBS (A375 cells) or MGM (NHEM). Medium was changed 24 h before staining. Cells were then fixed in 1% Formalin in phosphate-buffered saline (PBS) for 15 min at 4°C, rinsed with PBS, and stained with a rabbit polyclonal antibody raised against hPTHrP(1–34) using a modification of the three-layer peroxidase antiperoxidase technique (28).

Animal protocols. SCID female mice (Charles River, St-Constant, Quebec, Canada), weighing 13–16 g (3–4 wk old), were used in all studies. Approximately 1 × 10^6 A375 viable cells in 0.1 ml of FBS-free DMEM were injected subcutaneously on the backs of animals with a 27-gauge needle. To examine the effect of EB-1089 on tumor growth, osmotic minipumps (model 2ML4; Alza, Palo Alto, CA) were implanted subcutaneously, immediately adjacent to the tumor site, the same day as the injection of A375 cells. Each minipump contained EB-1089 or vehicle alone, dissolved in 50% propylene glycol, 10% ethanol, and 40% saline, to deliver a continuous dose of the compound for up to 4 wk at a delivery rate of 16 μM/24 h. This dose of EB-1089 given to SCID mice was the maximal dose that did not induce hypercalcemia and was tolerated without weight loss (data not shown). Each group consisted of five mice. All animals were examined once a week for the development of a palpable tumor at the site of injection. Three-dimensional tumor measurements were done using calipers. Tumor diameters, long axis (L), and mean midaxis width (W), were measured to estimate the volume using the following formula: 4/3π(L/2)(W/2)^2. A growth curve was generated by plotting the mean tumor volume over time. Five weeks after tumor implantation.
tion, animals were killed, and tumors were removed and weighed.

**Plasma calcium and PTHrP measurements.** To examine the ability of A375 cells to develop hypercalcaemia and elevated serum immunoreactive PTHrP (iPTHrP), $1 \times 10^6$ viable semiconfluent A375 cells were injected through a 27-gauge needle into the mammary fat pad (a single injection per mouse) of a SCID female. Blood was collected by orbital bleeding for measurement of total plasma calcium and albumin. Plasma calcium and albumin levels were determined by microchemistry (Kodak Ektachrome, Mississauga, Ontario, Canada). Corrected plasma calcium was calculated using the following formula: plasma total calcium + (40 – plasma albumin) × 0.02. Seven weeks after tumor cell inoculation, mice were killed by cardiac puncture, and tumors were excised and snap frozen for further RNA analysis. Plasma samples were stored at $-80^\circ$C until PTHrP analysis using an immunoradiometric assay specific for PTHrP(1–86) (Diagnostic Systems Laboratories, Webster, TX) as described previously (29). Results were expressed as picogram equivalents of PTHrP(1–86) per milliliter. The detection limit of the assay was 2 pg equivalent of PTHrP(1–86)/ml plasma.

**X-ray analysis of nude mice.** Mice were anesthetized, placed in a prone position against the films (18 × 24 cm; AGFA, Mortsel, Belgium), and exposed to an X-ray at 25 kV for 5 s using a Mammo Diagnost UC (Philips, Hamburg, Germany). Films were developed using a Curix compact processor (AGFA). Radiographs were analyzed by three investigators, including one radiologist who had no knowledge of the experimental protocol.

**Statistical analysis.** All results are expressed as means ± SE of triplicate determinations, and statistical comparisons are based on one-way analysis of variance or Student’s $t$-test. A probability value of $P < 0.05$ was considered significant.

**RESULTS**

**Expression of PTHrP mRNA in human melanoma A375 cells, melanoma tumor tissue, and NHEM.** PTHrP was seen as a single 1.6-kb transcript in both FBS-treated melanoma A375 cells or growth factor-treated NHEM (Fig. 1). PTHrP expression in melanoma A375 cells was at least 10 times higher than in normal melanocytes. The data obtained in NHEM were done using melanocytes obtained from different donors and were found similar. PTHrP was also detected in melanoma tumor tissue produced by inoculation of A375 cells into athymic mice.

**Immunocytochemical staining of normal and malignant human melanocytes.** Immunocytochemical staining using a PTHrP antibody directed against PTHrP(1–34) showed intense and widespread staining in human melanoma A375 cells, compared with control cells treated with nonimmune serum (Fig. 2, A and B). In contrast, the same PTHrP antibody stained only a small number of normal human melanocytes (Fig. 2, C and D), in keeping with the difference observed with PTHrP mRNA expression.

**PTHrP secretion in proliferating and confluent melanoma A375 cells and in NHEM.** We then assessed iPTHrP release into the conditioned medium of A375 cells (Fig. 3). In proliferating subconfluent A375 cells incubated with 10% FBS, there was a marked (10-fold) increase of PTHrP secretory rate, compared with cells incubated in the absence of FBS (basal secretory rate).

In contrast, when confluent cultures of A375 were treated in the same conditions, FBS produced only a twofold increase in the secretory rate of PTHrP, indicating that PTHrP secretion by A375 cells is much more pronounced during the proliferative phase.

In contrast to those high levels of PTHrP production in A375 cells, PTHrP was undetectable in the conditioned medium of NHEM treated with growth factors (data not shown).

**Effect of insulin and bFGF on PTHrP secretion in human melanoma cells.** We subsequently analyzed the effect on PTHrP production of growth factors previously shown to promote melanocyte cell proliferation. In these experiments, cells were grown in DMEM in the absence (basal) or presence (control) of a low-FBS (0.5%) concentration. The potential additive effect of insulin (10 μg/ml) or bFGF (50 ng/ml) was analyzed in the presence of 0.5% FBS. A small increase above basal level was observed with 0.5% FBS (24 ± 0.5 vs. 20.1 ± 0.4, $P < 0.05$) but no further increase was seen with either insulin or bFGF (Table 1).

**Effects of 1,25-(OH)$_2$D$_3$ and EB-1089 on the proliferation of normal and malignant melanocytes.** We examined the effects of 1,25-(OH)$_2$D$_3$ and its low-calcemic analog EB-1089 on proliferation of NHEM and melanoma A375 cells. These cells express VDR mRNA, as determined by RT-PCR of total RNA (data not shown). Addition of increasing concentrations of 1,25-(OH)$_2$D$_3$ or EB-1089 to the culture medium of A375 cells caused a significant dose-dependent inhibition of cell growth.
EB-1089 was, on average, 10-fold more potent than 1,25-(OH)2D3, as determined by the concentration producing a 30% decrease of maximal inhibition of cell growth (10⁻¹⁰ M vs. 10⁻⁹ M) (Fig. 4A). Similar results were observed by assessing inhibition of [³H]thymidine incorporation in EB-1089 or 1,25-(OH)2D₃-treated cells (Fig. 4B). In addition, both 1,25-(OH)₂D₃ and EB-1089 produced a significant inhibition of normal melanocyte growth, as assessed by formazan production (Fig. 4C).

Effects of 1,25-(OH)₂D₃ and EB-1089 on PTHrP secretion. Effects of increasing concentrations of 1,25-(OH)₂D₃ and EB-1089 (10⁻¹⁰ to 10⁻⁷ M) on PTHrP secretion were next examined. Untreated cells produced 75.5 ± 3.81 pmol/10⁶ cells/24 h, whereas both EB-1089 and 1,25-(OH)₂D₃ produced a significant and dose-dependent inhibition of PTHrP secretion, similar to the effects observed on cell growth (Fig. 5). EB-1089 was again, on average, 10-fold more potent than 1,25-(OH)₂D₃, as assessed by the concentration of the hormone inducing a 30% inhibition of PTHrP secretion. Results were corrected for cell number, indicating that the observed effects were independent of the antiproliferative action of EB-1089 and 1,25-(OH)₂D₃.

Effect of 1,25-(OH)₂D₃ and EB-1089 on PTHrP mRNA expression in melanoma A375 cells. EB-1089 and 1,25-(OH)₂D₃ effects on PTHrP mRNA was examined by Northern blot analysis. Treatment of A375 cells with increasing concentrations of 1,25-(OH)₂D₃ or EB-1089 (10⁻⁹ to 10⁻⁷ M) for 3 h produced a dose-dependent decrease in PTHrP mRNA levels (Fig. 6).

Fig. 2. Immunocytochemical stain of cultured normal and malignant human melanocytes for PTHrP. Cells were fixed, permeabilized, and stained with a polyclonal antibody directed against hPTHrP(1–34), using a modification of the 3-layer peroxidase antiperoxidase technique. Positive PTHrP-containing cells are stained dark brown. Mela-
noma A375 cells showed intense staining (A). Normal melanocytes that expressed the highest levels of PTHrP are indicated by arrows (C). Original magnifications, ×10 (NHEM) and ×40 (A375 cells). Control incubations (B and D) with nonimmune serum did not stain.

Table 1. Effect of insulin and bFGF on PTHrP secretion

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<th>PTHrP, pmol/10⁶ cells</th>
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<tr>
<td>Basal</td>
<td>20.1 ± 0.4</td>
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<tr>
<td>Control</td>
<td>24 ± 0.5*</td>
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<tr>
<td>bFGF (50 ng/ml)</td>
<td>24.2 ± 2.1*</td>
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<tr>
<td>Insulin (10 µg/ml)</td>
<td>23.9 ± 0.2*</td>
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Values are means ± SE of 3 different experiments done in quadruplicate. A375 cells were grown as described in MATERIALS AND METHODS, serum deprived for 24 h (basal), and then treated with either 0.5% fetal bovine serum (FBS; control), insulin (10 µg/ml), or basic fibroblast growth factor (bFGF, 50 ng/ml) in DMEM containing 0.5% FBS. Medium was collected at 48 h for measurements of parathyroid hormone-related peptide (PTHrP). Cells were trypsinized and counted. PTHrP levels were expressed as picomole equivalents of human PTHrP(1–84) per 10⁶ cells. *Significant difference from PTHrP measured in basal medium (P < 0.05).

Fig. 3. PTHrP secretion in proliferating and confluent human melanoma cells. After 24-h FBS deprivation (basal), A375 cells were incubated with 10% FBS for 24 h. Medium was collected for measurements of PTHrP. Cells were trypsinized and counted. Results are expressed as picomole equivalents of hPTHrP(1–84) per 10⁶ cells. The horizontal dashed line represents the detection limit of the assay. Bars represent the means ± SE of 3 replicates assayed in duplicate. *Significant difference from PTHrP basal values (serum-deprived cells) at P < 0.05.
with EB-1089 being more potent than 1,25-(OH)2D3 at 10\textsuperscript{-9} M and 10\textsuperscript{-8} M but not at 10\textsuperscript{-7} M.

In vivo analysis of calcium and PTHrP levels in SCID mice inoculated into the mammary fat pad with A375 cells. Palpable tumors developed within 10 days postinoculation, and animals killed at 7 wk had metastases, primarily in the lymph nodes and lungs, as determined by histological examination. However, no visible osteolytic bone lesions were observed by X-ray analysis (data not shown). Blood was collected in tumor-bearing animals at 7 wk and demonstrated an increase both in plasma calcium and iPTHrP (Table 2).

Fig. 4. Effects of 1,25-dihydroxyvitamin D\textsubscript{3}, [1,25-(OH)\textsubscript{2}D\textsubscript{3}] and EB-1089 on cell growth. A375 cells were grown in DME supplemented with 10\% FBS to \sim 30\% confluency. The medium was changed to serum-free DME for 24 h, and the cells were then treated for 48–72 h with increasing concentrations of 1,25-(OH)\textsubscript{2}D\textsubscript{3} or EB-1089 (10\textsuperscript{-10} to 10\textsuperscript{-7} M). NHEM were grown in MGM until 50\% confluence, starved for 24 h, and then incubated in MGM that contained increasing concentrations of each compound for 6 days. A: cell number assessed at 72 h in A375 cells. B: \textsuperscript{3}H]thymidine incorporation expressed as a percentage of control per 10\textsuperscript{6} cells (100\%) in A375 cells. C: formazan production assessed at 6 days using the MTS assay as described in MATERIALS AND METHODS in NHEM. C (control) represents cell number, \textsuperscript{3}H]thymidine incorporation, or optical density in the presence of vehicle alone (0.1\% ethanol). Each value represents the mean \pm SE of 3 different experiments done in quadruplicate. *Significant difference from control cells; \(,\) significant difference between 1,25(OH)\textsubscript{2}D\textsubscript{3} and EB-1089 at their respective concentrations (\(P < 0.05\))

Fig. 5. Effects of 1,25-(OH)\textsubscript{2}D\textsubscript{3} and EB-1089 on PTHrP secretion by A375 cells. A375 cells were grown as described in MATERIALS AND METHODS, serum deprived for 24 h, and then treated with increasing concentrations of 1,25-(OH)\textsubscript{2}D\textsubscript{3} or EB-1089 for 24 h. Control (C) represents PTHrP concentrations in conditioned medium from cells treated with vehicle alone (0.1\% ethanol). Conditioned medium was collected and immunoreactive PTHrP concentrations were determined by radioimmunoassay. The control value was 75.5 \pm 3.81 pmol of PTHrP(1–84)/10\textsuperscript{6} cells. Each point represents the mean \pm SE of 3 independent experiments done in quadruplicate. Values were calculated as a percentage of the control (vehicle-treated) cells after correction for cell number. *Significant difference from control cells; \(,\) significant difference between 1,25(OH)\textsubscript{2}D\textsubscript{3} and EB-1089 at their respective concentrations (\(P < 0.05\)).

Fig. 6. Effects of 1,25-(OH)\textsubscript{2}D\textsubscript{3} and EB-1089 on PTHrP mRNA expression in A375 cells. A375 cells were exposed to vehicle (0.1\% ethanol) or increasing concentrations of 1,25-(OH)\textsubscript{2}D\textsubscript{3} or EB-1089 for 3 h. Basal represents PTHrP mRNA expression in cells after 24 h of serum (FBS) deprivation. Total cellular RNA (20 \mu g/lane) was electrophoresed on 1.2\% agarose gel, and filters hybridized with a \textsuperscript{32}P-labeled hPTHrP cDNA probe as described in MATERIALS AND METHODS. As a control for RNA loading, filters were hybridized with a \textsuperscript{32}P-labeled cyclophilin probe. These results are representative of 3 different experiments.
In contrast, PTHrP was undetectable in nontumor-bearing mice.

**Effect of EB-1089 on tumor growth and body weight.**

After A375 cell implantation, palpable tumors occurred after the second week and showed a rapid increase in volume until death of the SCID mice at 5 wk (Fig. 7A). Three weeks after tumor implantation, there was a significant tumor growth inhibition in EB-1089-treated animals compared with the control group (0.249 ± 0.098 vs. 0.411 ± 0.107 cm³; n = 5, P < 0.05). At 5 wk, the mean tumor volume in control mice was 2.5 ± 0.43 cm³, compared with 1.43 ± 0.11 cm³ in EB-1089-treated animals (P < 0.03). In addition, tumor weight, measured at death (5 wk), was significantly lower in EB-1089-treated mice (0.86 ± 0.12 g) than in control mice (1.4 ± 0.2 g; P < 0.04; Fig. 7B). In contrast to the significant differences in tumor volume and weight, animal weight was similar between the two groups for the duration of the experiments (Fig. 7C).

**DISCUSSION**

A number of normal and cancer cells have previously been shown to produce PTHrP (9, 12, 19, 20, 28, 30, 36, 38, 39). Studies in normal cells (12, 18, 28) and knock-out animals (3, 26) indicate that PTHrP modulates cellular growth and differentiation of numerous tissues, including skin (24, 25, 28), cartilage (3), and breast (39, 40). In the present study, we demonstrated that PTHrP is also expressed at low levels in normal human melanocytes. One dominant transcript was observed similar to what has been reported in other cultured normal and cancer cell lines (10, 20, 28, 32). Immunocytochemical analysis indicates that few normal human melanocytes indeed express the peptide. However, PTHrP was undetectable in the conditioned media of NHEM treated with growth factors, suggesting that this peptide is unlikely to play an autocrine/paracrine function in this model. This low expression contrasts with the high expression observed in the human malignant melanoma A375 cells. In these cells, PTHrP mRNA was at least 10-fold higher than in normal melanocytes. Furthermore, immunocytochemical analysis revealed that all cells expressed the peptide. The expressed PTHrP was also released in high levels in the culture medium in vitro. These properties were also observed in vivo after implantation of tumor cells into the mammary fat pad. Animals grew tumors

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<th>Corrected Calcium, mmol/l</th>
<th>iPTHrP, pg/ml</th>
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<tr>
<td>Control mice (n = 6)</td>
<td>2.25 ± 0.04</td>
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<tr>
<td>A375-bearing mice (n = 6)</td>
<td>2.74 ± 0.26</td>
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Values are means ± SE (n = 6 per group). Mice were bled 7 wk after tumor cell inoculation, and plasma calcium and immunoreactive PTHrP (iPTHrP) were determined as described in MATERIALS AND METHODS. The detection limit of the assay was 2 pg PTHrP(1–86)/ml plasma. *Significant difference in plasma calcium and iPTHrP from nontumor-bearing animals, (P < 0.05).

Fig. 7. Effects of EB-1089 on tumor growth and body weight in A375 tumor-bearing mice. Tumor-bearing severe combined immunodeficiency mice were treated continuously with EB-1089 (16 pM/24 h) or with vehicle alone. Animal weight (C) and tumor volume (A) were determined once a week, and the tumor volume was calculated by the formula described in MATERIALS AND METHODS. Five weeks after tumor cell inoculation and treatment with EB-1089 or vehicle, mice were killed and the weight (B) of each tumor was measured. Data are expressed as means ± SE for 5 mice in each group. **Significant difference from control tumor-bearing animals at each time point.
that rapidly metastasized to several organs. mRNA extracted from tumors expressed high levels of PTHrP, and the peptide was detected in the blood of animals that rapidly developed hypercalcemia, indicating the endocrine effect of PTHrP. A careful analysis of the metastatic properties of these cells indicated that several major tissues were targeted. However, no obvious osteolytic skeletal metastases were detected. It remains possible, however, that small metastatic foci remain undetected on X-rays. In previous studies (17, 35), the same cell line was injected into the left ventricle of nude mice, inducing the rapid development of skeletal metastases with concomitant hypercalcemia. The authors postulated that transforming growth factor-α overexpression by A375 tumor cells could be responsible for tumor-induced osteolysis and subsequent hypercalcemia. However, PTHrP was not measured in the blood of the animals or in the tumor cells within bone.

Although uncommon, hypercalcemia has been reported in patients with human melanoma with or without bone metastases, but its pathogenesis remains uncertain. In some studies, hypercalcemic patients with melanoma were reported to have suppressed levels of parathyroid hormone but elevated levels of neoprogenous cAMP excretion (5, 13, 31), which suggests a possible link with PTHrP production by melanoma cells. Recently, several studies have reported the occurrence of hypercalcemia in patients with malignant melanoma and identified PTHrP as one of the causative factors of hypercalcemia (23, 34, 41). Osteolytic bone metastases are also a manifestation of malignant melanoma. However, as postulated for breast cancer (14, 15, 42), PTHrP may contribute to the pathogenesis of osteolytic bone destruction in this condition. Further studies will be required to determine whether PTHrP contributes to the development of bone metastases in this melanoma model.

The biologically active metabolite of vitamin D₃, 1,25-(OH)₂D₃, has properties that extend beyond those of regulating bone mineralization and calcium homeostasis. Previous in vitro and in vivo studies have clearly demonstrated that 1,25-(OH)₂D₃ is a potent antiproliferative agent and suggest a possible clinical use of this hormone in the treatment of hyperproliferative disorders. However, its clinical usefulness is restricted by its strong calcemic activity. Consequently, the search for new vitamin D analogs with more potent growth inhibitory properties but reduced calcemic activity has intensified in the past several years. Among these analogs, EB-1089, characterized by an ethyl group and double bonds in the side chain and a half-life similar to 1,25-(OH)₂D₃ in vivo (33), has been studied extensively. This compound has more potent antiproliferative activity than 1,25-(OH)₂D₃ while being 50% as calcemic as its parent compound (2, 7, 8, 16, 22, 37, 44). In the present study, we show that the vitamin D analog EB-1089, as well as 1,25-(OH)₂D₃, inhibits melanoma A375 cell growth and PTHrP expression and secretion. Furthermore, EB-1089 is more potent than the native hormone by at least one order of magnitude. These results are consistent with the reported observations that PTHrP is negatively regulated by 1,25-(OH)₂D₃ and its synthetic analogs in several normal and cancer cells, including cultured normal human mammary cells (36), cultured human keratinocytes (28, 44), human lung squamous cancer cells (11), MT-2 cells (a cell line derived from human T cell leukemia virus I-infected T cells) (21), and oral cancer cells (1). In addition to these effects in vitro, our data also indicate that EB-1089 significantly reduces the growth of melanoma A375 cells in vivo. This effect occurs without significant calcium elevation and body weight loss. Several in vivo studies have previously demonstrated the efficacy of EB-1089 in reducing the growth of a variety of malignancies without affecting serum calcium levels (15, 18, 28–30), indicating that EB-1089 has selective properties on target tissues and, particularly, cancer cells at a dose that has no apparent effect on calcium homeostasis. In view of the fact that the VDR is expressed in a wide variety of cancer cells, including human malignant melanoma cells (6), EB-1089 may prove to be an interesting adjuvant in the chemotherapeutic arsenal against human melanoma.

Because normal human melanocytes require specific growth factors, such as bFGF, to proliferate in culture, and because bFGF is one of the most abundant growth factors in the bone matrix (43), we assessed the influence of bFGF on PTHrP release from melanoma A375 cells. The bFGF effect was analyzed in various conditions both in the absence and in the presence of FBS over several days and did not significantly modulate either cellular proliferation or the secretion of PTHrP into the conditioned medium. The absence of a bFGF effect is likely due to an absent or inactive bFGF receptor in this model. Finally, we examined the effect of insulin on both cellular proliferation and PTHrP secretion. Although insulin was a potent mitogen in this system, we could not demonstrate any effect of insulin on PTHrP secretion. The absence of an insulin effect in this study is similar to our previous report on normal human keratinocytes (28) but contrasts with the well-described influence of insulin on modulating PTHrP secretion reported in normal human mammary epithelial cells (36), suggesting that PTHrP stimulation by mitogens is regulated in a tissue-specific fashion.

In conclusion, our results indicate that PTHrP is expressed in both melanocytes and the human melanoma cell line A375 and that its high level of expression in the latter model is closely associated with the development of hypercalcemia in the absence of osteolytic bone lesions. The potent action of EB-1089 on inhibition of both cell proliferation and PTHrP expression may provide a new strategy for the treatment of this common malignancy.

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