Osteoprotegerin Prevents and Reverses Hypercalcemia in a Murine Model of Humoral Hypercalcemia of Malignancy

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Abstract

Osteoprotegerin (OPG), a novel, secreted tumor necrosis factor receptor family member that inhibits osteoclast formation and activity was examined for its activity in a syngeneic tumor model of humoral hypercalcemia of malignancy. Normal mice bearing Colon-26 tumors develop increases in both parathyroid hormone-related protein (PTHrP) expression and plasma PTHrP, marked hypercalcemia, and increased bone resorption. OPG, given either at the onset of hypercalcemia or after it had occurred, blocked tumor-induced increases in bone resorption and hypercalcemia and rapidly normalized blood ionized calcium. In tumor-bearing mice, OPG treatments reduced osteoclast activity from approximately 2-fold above normal into the subphysiological range but had no effects on tumor size, tumor-induced cachexia, or PTHrP levels. The potent effects of OPG in this humoral hypercalcemia of malignancy model suggest a potential therapeutic role for OPG in the prevention and treatment of this disorder.

Introduction

HHM results from the systemic actions of tumor generated factors that alter calcium metabolism (1) and accounts for between 75 and 80% of all of the malignancy-associated cases of hypercalcemia (2). Tumor PTHrP production is the etiology of most cases of HHM (3–5). Pathophysiologically, HHM is characterized by increases in osteoclastic bone resorption and renal calcium reabsorption, with increased bone resorption being the principal source of the increased systemic calcium burden (6). Recently, a novel member of the TNF receptor family termed OPG (7) or osteoclastogenesis-inhibitory factor (OCIF; Ref. 8) was discovered that inhibits osteoclast formation and activity in vitro and in vivo (7). OPG-overexpressing mice are osteopetrotic (7), whereas OPG-deficient mice are osteoporotic (9) underscoring the pivotal role of this protein in regulating bone mass. OPG binds and neutralizes OPGL (10), a potent TNF family member that is pivotal, if not essential, for the differentiation and activation of osteoclasts both in vivo and in vitro (10, 11). OPGL is expressed by osteoblasts and bone marrow stromal cells (10, 12). OPGL expression is up-regulated by factors known to increase bone resorption and potentially cause hypercalcemia, including PTH (12, 13). Previous studies have shown that OPG can inhibit bone resorption caused by PTHrP, IL-1, 1,25-dihydroxyvitamin D3, PTH, TNF, or OPGL (10, 14), as well as inhibiting the bone loss caused by ovariectomy (7). In the present study, we show that OPG can both prevent and reverse HHM in immune-competent mice inoculated with syngeneic adenocarcinoma (colon) cells.

Materials and Methods

C-26 Tumor Model. Male BALB/c × DBA/2 (CDF1) mice at 10–12 weeks of age were purchased from either Harlan Sprague Dawley (San Diego, CA) or Charles River (Wilmington, MA) and were maintained in an AALAC-approved facility under the guidance of Amgen’s Institutional Animal Care and Use Committee. C-26 cells were originally isolated from a chemically induced mouse adenocarcinoma (15) and have been reported to induce hypercalcemia and cachexia (16–18). C-26 cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) plus 10% FCS (Life Technologies, Inc.), 1× penicillin-streptomycin/glutamine (Life Technologies, Inc.), and 1× nonessential amino acids (Life Technologies, Inc.). Cells were harvested by trypsinization (Life Technologies, Inc.) and resuspended in unsupplemented DMEM (Life Technologies, Inc.). Aliquots (200-μL, 0.5 × 106 cells) of these suspensions were implanted s.c. on the right flank of mice by injection.

Administration of Recombinant OPG. The recombinant OPG used for these studies comprised the ligand binding domain of human OPG fused to the Fc domain of human IgG (7, 14). In the prevention model, recombinant OPG or an equivalent volume of PBS control was administered daily by s.c. injection for 7 days beginning on day 9 after tumor injection. In the model of established hypercalcemia, OPG was administered daily by s.c. injection for 4 days beginning when an individual mouse attained an ionized calcium level greater than 1.60 mmol/L. Body weight and blood ionized calcium levels from retro-orbital samples (blood ionized calcium/pH analyzer, Chiron Diagnostics, Norwood, MA) were periodically measured throughout each study. At the conclusion of the prevention study, the tumors were excised, dissected free of skin and soft tissue, and weighed.

PTHrP RPA Analysis. Total RNA was isolated from freshly dissected tumors using the RNA STAT-60 reagent (Tel-Test “B”, Inc., Friendswood, TX). A 278-bp portion of the mouse PTHrP sequence (nucleotide 152–428 of Gb:M60056) was prepared using a reverse transcription PCR (mouse brain)-generated fragment that was cloned into pGEM-T (Promega, Madison, WI). A 103-bp mouse cyclophilin probe (Ambion, Inc., Austin, TX) was used as an internal control. The probes were generated, and the RPA performed as described previously (10). The gels were analyzed using a phosphor imager (Molecular Dynamics, Sunnyvale, CA) and cyclophilin as an internal reference.

PTHrP Assay. PTHrP levels were measured in mouse plasma treated with protease inhibitors (Nichols Institute, San Juan Capistrano, CA). PTHrP levels were quantitated using a sandwich immunoradiometric assay kit (Nichols Institute) designed for the detection of human PTHrP with a detection limit of 0.3 pmol/L human PTHrP. Although studies using this assay on mouse PTHrP have not been reported, the similarities of the mouse and human PTHrPs in the two regions used are 88%and 100%, respectively. Also, the antibodies used in this assay have been reported to cross-react with rabbit PTHrP.

Histological Analysis. The femurs and tibiae were processed as described previously (7). Sections of the femur and tibia were stained for TRAP activity (leukocyte acid phosphatase kit, Sigma, St. Louis, MO) and counterstained with hematoxylin. In this staining procedure, osteoclasts are red, and the remaining tissue is stained blue. Measurements were made in a 1 mm × 1 mm...
square area both in the primary spongiosa immediately distal to the proximal tibial growth plate and in an area of the tibial cortical shaft 2 mm distal to the first measurement area using an Osteomeasure bone analysis program (Osteometrics Inc., Decatur, GA). Osteoclasts were scored based on contact with bone surface and TRAP-positive staining and were recorded as osteoclast number/mm² tissue area (OcS/TA) and percent osteoclast surface/bone surface (OcS%BS).

For cathepsin K immunohistochemistry, deparaffinized 4-um sections were pretreated with 0.1% trypsin and blocked with CAS Block (Zymed Lab., San Francisco, CA) containing avidin (Vector Lab., Burlingame CA) according to the manufacturer’s instructions. Sections were then incubated with anti-cathepsin K polyclonal antibodies (19) at 10 μg/ml and biotin (Vector Lab.). A biotinylated goat antirabbit IgG (Vector Lab.) was used as the secondary antibody. The tertiary was Alkaline Phosphatase ABC complex (Vector Lab.). Vector Red AP Substrate (Vector Lab.) was used to visualize all of the staining reactions. All of the sections were lightly counterstained with hematoxylin.

Statistics. Statistical analysis was performed using JMP Statistical Software, (SAS Institute, Inc., Cary, NC). Comparisons were made using Dunnett’s test for comparison of multiple treatment groups with a control or the manufacturer’s instructions. Sections were then incubated with anti-cathepsin K polyclonal antibodies (19) at 10 μg/ml and biotin (Vector Lab.). A biotinylated goat antirabbit IgG (Vector Lab.) was used as the secondary antibody. The tertiary was Alkaline Phosphatase ABC complex (Vector Lab.). Vector Red AP Substrate (Vector Lab.) was used to visualize all of the staining reactions. All of the sections were lightly counterstained with hematoxylin.

Results

Treatment of C-26 tumor-bearing mice with OPG prior to the onset of hypercalcemia resulted in a dose-dependent inhibition of tumor-induced hypercalcemia. As shown in Fig. 1A, tumor-bearing mice had slightly increased whole-blood ionized calcium levels (1.34 ± 0.06 mmol/L) versus 1.25 ± 0.02 mmol/L in normal controls) just before commencing OPG or PBS treatments. In the PBS group, blood ionized calcium reached a maximum of 1.84 ± 0.12 mmol/L on days 13 and 15 and decreased slightly by day 16. OPG dose-dependently inhibited this increase in whole blood ionized calcium throughout the treatment period, with ionized calcium levels reaching a maximum of 1.38 ± 0.06 mmol/L on day 15 in the 2.5 mg/kg group. This level of ionized calcium was modestly higher than the ionized calcium level in non-tumor-bearing control mice but significantly lower than the ionized calcium levels in vehicle-treated tumor-bearing mice. OPG treatment had no effect on ionized calcium levels in non-tumor-bearing mice (data not shown).

In mice with established hypercalcemia (1.79 ± 0.04 mmol/L versus normal levels of 1.14 ± 0.03 mmol/L), OPG treatments resulted in complete normalization of ionized calcium (Fig. 1B). Within 24 h of treatment initiation, ionized calcium decreased to 1.43 ± 0.03 mmol/L, and by 48 h, ionized calcium levels were within the normal range (1.22 ± 0.03 mmol/L). Continued daily OPG treatments maintained lowered ionized calcium levels for the final 2 days of the study.

The use of OPG (2.5 mg/kg for 7 days) to prevent hypercalcemia had no effect on tumor size (PBS, 0.75 ± 0.05 g; OPG, 0.78 ± 0.05 g), nor did it affect tumor-associated weight loss (PBS, 22.52 ± 5.00%; OPG, 18.64 ± 3.58%). The effects of OPG on these parameters were similar in tumor-bearing mice with established hypercalcemia that were treated with OPG (data not shown).

Plasma PTHrP levels were elevated in C-26 tumor-bearing mice (5.12 ± 0.75 pmol/L) compared with normal mice (PTHrP levels less than the 0.3 pmol/L detection limit of the immunoradiometric assay). OPG treatment had no significant effect on plasma PTHrP levels (5.84 ± 0.78 pmol/L average for all of the prophylactic OPG doses) in tumor-bearing animals. The effects of OPG on plasma PTHrP levels were similar in tumor-bearing mice with established hypercalcemia that were treated with OPG (data not shown). Consistent with the increase in circulating PTHrP levels, C-26 tumors excised from tumor-bearing mice expressed PTHrP at a level approximately 10-fold higher than the level found in skin adjacent to the tumor as quantitated by RPA analysis (data not shown). The increase in PTHrP expression was equivalent in both vehicle- and OPG-treated C-26 tumor-bearing mice. OPG also had no effect on C-26 tumor cell proliferation in vitro (data not shown), further indicating a lack of direct antitumor effects. The lack of an OPG effect on tumor size, plasma PTHrP levels, tumor PTHrP mRNA expression, and tumor-induced cachexia strongly suggest that OPG is not directly acting on the C-26 tumor.

The marked reduction in blood ionized calcium suggested that OPG treatments were likely blocking increased bone resorption caused by PTHrP. In fact, osteoclast-lined surfaces and osteoclast numbers were markedly increased in hypercalcemic mice bearing C-26 tumors (Fig. 2). Prophylactic OPG treatment (2.5 mg/kg) prior to the development of hypercalcemia resulted in the virtual complete disappearance of osteoclasts (Fig. 2A). Quantitatively, the percent osteoclast surface per bone surface (OcS%BS) was about 2-fold greater in the untreated tumor-bearing mice (8.95 ± 2.10%, Fig. 2B), compared with untreated non-tumor-bearing controls (3.91 ± 1.10%, OPG (2.5 mg/kg) reduced osteoclast surface to 0.13 ± 0.07%, which is significantly lower than the levels found in non-tumor-bearing control mice. A 0.5-mg/kg dose of OPG also had a significant but less marked effect on osteoclast surface (2.72 ± 1.47%). A similar pattern of findings emerged when the number of osteoclasts per mm² tissue were assessed (Fig. 2C).
OPG had similar effects when used to treat established hypercalcemia. The C-26 tumor-bearing mice developed significant increases in osteoclast-lined bone surfaces as well as in osteoclast number when compared with non-tumor-bearing mice (Fig. 3). OPG treatment for 4 days after the establishment of hypercalcemia resulted in a significant reduction in percent osteoclast surface per bone surface from $8.95 \pm 0.67\%$ (Fig. 3B) in vehicle-treated tumor-bearing mice to $1.26 \pm 0.46\%$, which is significantly below the levels found in normal non-tumor-bearing mice ($3.66 \pm 0.41\%$). The number of osteoclasts per mm$^2$ of tissue area was also significantly reduced with OPG (2.5 mg/kg) treatment from $32.39 \pm 4.85$/mm (Fig. 3C) in vehicle-treated tumor-bearing mice to $5.56 \pm 4.19$/mm with 4 days of OPG treatment. This level is significantly below the normal physiological value of $17.51 \pm 3.42$/mm found in vehicle-treated non-tumor-bearing mice.

Discussion

The etiology of HHM involves tumor-produced factors that stimulate osteoclast recruitment and bone resorption as well as increasing renal calcium reabsorption. In the C-26 murine model of HHM, we demonstrate that OPG is able to both prevent and reverse the profound hypercalcemia that occurs in tumor-bearing mice. OPG treatment given before or after the establishment of hypercalcemia was associated with the almost complete disappearance of osteoclasts, which indicated that the inhibition of bone resorption was the primary tissue...
effect of OPG that led to normalization of blood calcium levels. The effects of OPG were rapid. Treatment of mice with established hypercalcemia with a maximal dose of OPG (2.5 mg/kg) resulted in normalization of blood ionized calcium within 48 h.

OPG acts by binding and inactivating OPGL, which is essential for osteoclast differentiation in normal mice (11) and is a survival factor for osteoclasts in vitro (20, 21). The lack of osteoclasts in C-26 tumor-bearing OPG-treated mice may be due to the inhibition of osteoclastogenesis or by the induction of apoptosis of mature osteoclasts. Low osteoclast numbers are also observed in normal OPG-treated mice (14) and in transgenic mice overexpressing OPG at high levels (7). In vitro, OPG inhibits osteoclastogenesis (7, 22), blocks bone resorption by mature osteoclasts (10, 23), and promotes apoptosis of osteoclasts (20, 21, 24). In vivo, OPG also rapidly leads to osteoclast apoptosis. The mechanism of the hypercalcemic effects of C-26 tumor cells in this model is not completely established. Previous studies have shown that hypercalcemia in the C-26 model of HHM is partially mediated by IL-6, but failure to completely normalize calcium levels with an IL-6 antibody indicated that other factors might be involved (16, 18). Our results strongly suggest a potential role for PTHrP in the hypercalcemic response to this tumor. We have found that C-26 tumors express PTHrP mRNA and that plasma PTHrP

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Fig. 3. Effects of OPG treatments on osteoclasts in hypercalcemic C-26 tumor-bearing mice. Mice were treated as in Fig. 1B and killed, and the femur and tibia were collected. Sections were prepared as in Fig. 2A. In A, fluorescent images of the proximal tibial metaphysis adjacent to the growth plate are shown. Osteoclasts appear orange (yellow arrows), whereas bone (B) and marrow (M) appear green. Scale bar, 100 μm. In B, the percent osteoclast surface per bone surface (OcS%BS) measurements taken of the primary spongiosa and the cortical shaft are shown. In C, osteoclast number/mm² tissue area (NOc/TAr) measurements made in the same area as in B are shown. * and #, different (P < 0.05) from vehicle-treated tumor-bearing mice or normal mice, respectively. Values are mean ± SE, n = 4–6.

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levels are markedly elevated in hypercalcemic C-26 tumor-bearing mice. Furthermore, blood ionized calcium levels remained slightly elevated in tumor-bearing mice treated prophylactically with OPG, although high doses of OPG had virtually eradicated the osteoclast population. This observation is consistent with a renal calcemic response to PTHrP (25), because PTHrP expression and plasma levels are not influenced by OPG treatment. Additionally, PTHrP and IL-6 have been shown to synergistically induce hypercalcemia in mice (26), and the action of both of these factors may explain the rapid and severe hypercalcemia that these mice develop.

The activity of OPG in this model indicates that blocking OPGL is an effective strategy to prevent bone resorption induced by tumor derived factors. The results of this study suggest that OPG may have clinical utility in treating hypercalcemia caused by malignant tumors and other disorders of excessive bone resorption. OPG seems to have low toxicity because transgenic mice overexpressing OPG develop normally (7), and have a normal life expectancy. OPG was also well tolerated in a Phase 1 clinical trial (27). Bisphosphonates can effectively reverse hypercalcemia over 3–5 days in most patients (28). In a murine model of HHM, OPG reduced hypercalcemia more rapidly than did pamidronate (29). If clinical trials demonstrate safety and efficacy in human HHM, OPG could provide an alternative to bisphosphonate therapy for patients with HHM.

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