Osteoprotegerin Overexpression by Breast Cancer Cells Enhances Orthotopic and Osseous Tumor Growth and Contrasts with That Delivered Therapeutically

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Abstract
Osteoprotegerin (OPG) acts as a decoy receptor for receptor activator of NF-κB ligand (RANKL), which is a pivotal molecule required for osteoclast formation. In vitro OPG inhibits osteoclast formation and in vivo (administered as Fc-OPG) it reduces hypercalcemia and the establishment of osteolytic lesions in mouse models of tumor cell growth in bone. Osteolysis can be induced by parathyroid hormone–related protein (PTHrP) produced by breast cancer cells that results in an increased osteostastic RANKL/OPG ratio. We examined the effect of local tumor production of OPG on the ability of breast cancer cells to establish and grow in bone and mammary fat pad. MCF-7 cells or MCF-7 cells overexpressing PTHrP were transfected with full-length OPG and inoculated into the proximal tibiae of athymic nude mice. Mice injected with cells overexpressing PTHrP and OPG showed enhanced tumor growth, increased osteolysis (2-fold compared with MCF-7 cells overexpressing PTHrP), and altered histology that was reflective of a less differentiated (more aggressive) phenotype compared with MCF-7 cells. In contrast, administration of recombinant Fc-OPG reduced tumor growth and limited osteolysis even in mice inoculated with OPG overexpressing cells. Similarly, OPG overexpression by breast cancer cells enhanced tumor growth following orthotopic inoculation. These results indicate that OPG overexpression by breast cancer cells increases tumor growth in vivo and that there are strikingly different responses between therapeutically administered Fc-OPG and full-length OPG produced by tumor cells. (Cancer Res 2006; 66(7): 3620-8)

Introduction
Breast cancer is the most common malignancy affecting women and is a major cause of death in young middle-aged women in the United States, with 1 in 14 affected in their lifetime. The development of metastatic disease is invariably associated with a fatal outcome. In all series reporting, the most common site of first relapse is bone. When considered with other sites of first metastasis, >50% of patients will develop bone metastases at the time of first recurrence (1). Bone metastases, usually osteolytic, may be expected to complicate the course of up to 80% of patients with disseminated disease, with pathologic fracture and/or hypercalcemia frequently observed. A recently identified member of the tumor necrosis factor (TNF) receptor family, osteoprotegerin (OPG), has the capacity to block osteoclast formation both in vitro and in vivo (2) and is considered a potential therapy to combat cancer-induced bone loss. OPG acts as a decoy receptor blocking the binding of the TNF ligand, receptor activator of NF-κB ligand (RANKL), to its cognate signaling receptor RANK (present on hematopoietic cells), thereby inhibiting osteoclast formation as well as activity (2-5). The requirement of RANKL and RANK for osteoclast formation has been established because mice deficient in either RANKL or RANK are osteopetrotic due to failed osteoclast formation (6-8). Furthermore, the role for the decoy receptor OPG in osteoclast formation was shown by genetic experiments whereby OPG-null mice were shown to be severely osteoporotic (9) whereas transgenic mice overexpressing OPG were osteopetrotic (2). We have shown that human breast cancer cells do not express RANKL, but do express OPG and RANK, and are unable to directly support osteoclast formation in vitro (10). Most importantly, however, we showed that breast cancer cells influence osteolysis by inducing the expression of RANKL by osteoblasts, an effect resulting from parathyroid hormone–related protein (PTHrP) production (10). We also noted that levels of OPG mRNA varied in primary cancers and in breast cancer cell lines (10). The current study was aimed at examining whether overexpressing OPG in breast cancer cells would inhibit tumor-associated osteolysis, using the model of intratibial inoculation of cancer cells in athymic mice, and compared this with recombinant OPG (Fc-OPG) treatment. Surprisingly, it was found that the overexpression of full-length OPG was associated with enhanced tumor growth in vivo, with an accompanying increase in osteolysis, whereas Fc-OPG treatment of mice inhibited osteolysis and, therefore, tumor advance in bone as expected. Furthermore, consistent with this, the overexpression of OPG by breast cancer cells also increased tumor growth following mammary fat pad inoculation.

Materials and Methods
Stable transfection of cell lines. Human breast cancer cell lines (MCF-7) transfected with PTHrP or vector control plasmids were as previously described (10). The coding sequence of human OPG (86-1,310 bp; GenBank accession number NM002546.1) was amplified from RNA extracted from MDA-MB-231 and MCF-7 cells by reverse transcription-PCR (RT-PCR) using the primers OPG-16 [5′-CGCCCTCGAGGGGACCA-CATGAAACAAG-3′] and OPG-17 [5′-CGCGGATCCATTTCCAGTTATAAG-CAG-3′], which included an XhoI and BamHI restriction endonuclease site in primer sequences, respectively. The PCR fragments were agarose gel purified, digested with XhoI and BamHI, and cloned into the XhoI and

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**OPG Enhances Cancer Growth**

BamHI sites of pCEP+; resultant clones (e.g., pSMR969 from MCF-7 RNA and pSMR1141 from MDA-MB-231 RNA) were verified by nucleic acid sequencing. The sequence of pSMR1141 was identical to that of the published sequence of human OPG (GenBank entry NM002546.1) whereas pSMR969 contained a T-to-C transition at position 557 of the human OPG sequence, which resulted in a single amino acid substitution of a serine to a proline at amino acid 155. MCF-7 and MCF-7 + PTHrP cells were trans- 

fected by calcium phosphate precipitation with DNA for either pSMR969, pSMR1141, or pCEP4, and hygromycin-resistant clones (following limiting dilution) and nonclonal (14-day selection and are used for no more than four passages from original derivation) lines were generated. MCF-7 cells and derivatives were maintained in RPMI containing 10% fetal bovine serum (FBS; ref. 10). All other reagents were of analytic grade obtained from standard suppliers. In all assays described herein, cell lines expressing either OPG or Ser155Pro OPG were established and no difference was noted between these cell lines in any of the *in vitro* or *in vivo* assays.

**RT-PCR.** Total RNA extraction from cell lines, cDNA synthesis, and PCR were done as described (11). Oligonucleotides used to amplify human OPG were OPG-4 (5′-GGGACCACATGAAAGTG-3′) and OPG-5 (5′-GCTGGACCATCCTAATCC-3′), and the resultant PCR product was electrophoresed through a 12% wt/vol agarose gel and product verified and quantified by detection with a 35S-labeled internal oligonucleotide [OPG-6 (5′-GCTGCTGTGATGGATTGTCG-3′)] as previously described (10). As a normalizing control, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with oligonucleotides GAPDH-3 and GAPDH-4 and verified with GAPDH-1 as previously described (10). Bound probe was amplified with oligonucleotides GAPDH-3 and GAPDH-4 and verified with GAPDH-1 as previously described (10). Bound probe was detected by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA) and densitometry was done using the software program ImageQuant, version 4.2.

**OPG ELISA.** To establish the level of OPG protein production from the clones, an OPG ELISA of conditioned medium was done by Dr. S. Chandrasekhar (Lilly Research Laboratories, Indianapolis, IN) as previously described (12, 13).

**Animals and animal maintenance.** Congenitally athymic female nude mice (BALB/c, nu/nu, Animal Resource Centre, Perth, WA) were purchased germ-free at 2 to 3 weeks old and housed in the Orthopaedic Department Sterile Facility, St. Vincent’s Hospital.

Before tumor inoculation, radiographic examination, and euthanasia, mice were anesthetized with an intraperitoneal mixture of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (0.75 mg/kg). The St. Vincent’s Hospital Animal Ethics Committee approved all experimental procedures and the animal care was in accordance with the National Health and Medical Research Council animal ethics guidelines.

**Osteoclast formation assays.** Osteoblastic cells were prepared from the calvaria of newborn mice by sequential digestion with 0.1% collagenase (from Worthington Biochemical Co., Freehold, Australia) and 0.2% dispase (Godo Shusei, Tokyo, Japan). Bone marrow was obtained from the femur and tibia of adult C57BL/6j male mice. Osteoblast cells and/or breast cancer cells were cocultured with spleen or marrow cells as previously described (10, 14).

**Intratibial implantation.** Cells in log-phase growth were harvested by trypsinization (0.25% trypsin/0.02% EDTA) and medium containing 10% FBS was added; the cells were then washed thrice by centrifugation in serum-free RPMI and subsequently resuspended in serum-free RPMI (0.5 × 10^6 cells/mL). Cells were kept at 4°C until use (0-2 hours). The cell concentration and viability (trypan blue exclusion, >90% viability) were determined with a hemocytometer.

Four-week-old mice received intratibial inoculation of parental MCF-7, MCF-7 + PTHrP, MCF-7 + OPG, MCF-7 + PTHrP + OPG cells, or control cells for comparison, including MCF-7 + pCEP4, MCF-7 + pcdNANeo, MCF-7 + PTHrP + pCEP4, and MCF-7 + OPG + pcdNANeo (final concentration 0.5 × 10^6 cells/10 μL) as previously described (15). The presence of empty vectors in each of the cell lines did not affect their behavior relative to similar genetically altered cell lines without the empty vector (whether that was pcdNANeo or pCEP4). Right limbs were inoculated with media alone.

Mice were monitored daily for weight loss and at the completion of the experiment for evidence of osteolysis by X-ray analysis. Mice were

anesthetized and radiograms were taken against X-Omat AR film and exposed with X-rays at 35KVP for 6 seconds using a cabinet X-ray system (Faxitron Series, Hewlett-Packard, McMinnville, OR). Long bones were analyzed for evidence of osteolysis, as calculated by the percent area of osteolysis/total area of the tibia, using the M2 Image Analysis System (Imaging Research, Inc., St. Catharines, Ontario, Canada).

Mice were euthanized by anesthetic overdose at 42 days postinoculation. The left and right limbs were harvested and immediately fixed in 4% parformaldehyde in PBS at 4°C for 24 hours, and then transferred into a sterile decalcification solution (15% EDTA/0.5% parformaldehyde in PBS, pH 8.0) for 2 weeks.

**Mammary fat pad inoculation.** Cultured cells in log-phase growth were harvested as described above. The cells were resuspended in serum-free αMEM and with matrigel (5 μg/mL) in a ratio of 1:1 at a final concentration of 0.33 × 10^6 cells/mL before inoculation.

Four-week-old mice received mammary fat pad inoculation of parental MCF-7 cells, MCF-7 cells transfected with vector alone, MCF-7 + PTHrP, MCF-7 + OPG, MCF-7 + PTHrP + OPG cells, MCF-7 + PTHrP + pCEP4, MCF-7 + OPG + pcdNANeo (final concentration, 0.5 × 10^6 cells/10 μL) as described (16). The length and width of the mammary tumors were measured daily with a pair of calipers. At the end of the experiment, the animals were sacrificed, the mammary fat pads containing the tumors were excised, and the tumor size measured and fixed in parformaldehyde or stored frozen for later work.

**Administration of recombinant OPG.** Recombinant OPG was the TNF receptor domain of OPG (amino acids 22-194) fused to the Fc domain of human immunoglobulin G (IgG) as previously described (2, 17) and was kindly provided by Dr. C.R. Dunstan (Amgen, Inc., Thousand Oaks, CA). Recombinant Fc-OPG (2.5 mg/kg/d) or an equivalent volume of PBS (control) was administered daily by s.c. injection to the flank of animals inoculated (intratibial or mammary fat pad) with MCF-7, MCF-7 + PTHrP, or MCF-7 + PTHrP + OPG based on previous work by Caparelli et al. (17).

**Histochemical analysis.** Following fixation, the tissues were paraffin embedded for histologic examination. H&E staining assessed tumor development in these limbs. Tibial sections were also stained for TRAP activity (Sigma Chemical Co., St. Louis, MO) for osteoclasts. Osteoclast number per unit tumor-bone interface surface was measured by standard histomorphometric procedures using the Osteomeasure system (Osteo-Metrics, Inc., Decatur, GA).

**Immunohistochemical analysis.** Immunohistochemistry was done on representative tibial tumors (10-12 tumors per cell line). Serial sections were immunostained to identify proliferating cells by using an antibody to Ki67 (Santa Cruz Biotechnology, Santa Cruz, CA) and apoptotic cells were assayed by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay.

Immunohistochemistry methods for Ki67 employed indirect avidin-biotin–enhanced horseradish peroxidase. Antigen retrieval was done after dewaxing and dehydration of the tissue sections (5 μm) by microwaving for 10 minutes in citrate buffer (pH 6). Sections were cooled to room temperature, treated with 6% hydrogen peroxide in methanol for 30 minutes, and blocked with 10% nonimmune rabbit serum in 5% newborn bovine serum/0.1% Tween 20/PBS (pH 7.4) for 30 minutes. Sections were then incubated with the antibody to Ki67 in newborn bovine serum/ Tween 20/PBS overnight at 4°C. Sections were washed in PBS and incubated with biotinylated rabbit antimouse IgG for 1 hour. After washing, the antibodies were detected with streptavidin-horseradish peroxidase for 30 minutes at room temperature and color was developed with 3,3-diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin. Positive staining for Ki67 was measured using a color image analyzer (MCID-M2 Image Analysis, Imaging Research) and results expressed as the proportion of area with positive staining per field. The measurements were done on four fields per tumor with a minimum of six animals per group.

OPG-specific antisemum was prepared by inoculating rabbits with 1.0 mL of hemocyanin-conjugated peptide (0.5 mg) emulsified with Freund’s adjuvant (Sigma-Aldrich Pty. Ltd., Milwaukee, WI). Complete Freund’s adjuvant was used for the first challenge and incomplete Freund’s adjuvant was used for
the subsequent challenges. The murine OPG peptide CPDHYTDSWHTSDEC (residues 65-80) differs from human OPG in one amino acid (position 69; S, murine and Y, human); however, both peptides and tissues from murine and human sources were detected by the antisera. Samples of preimmune control serum were taken from the rabbits before inoculation. Antisera obtained after the inoculation period were shown to contain OPG-specific antibodies by ELISA and Western blotting.

**Cell death detection ELISA.** MCF-7 cells were plated (5 × 10^4 per well) in 24-well plates and grown for a total of 8 days. On day 8, cells were challenged with 100 ng/mL TRAIL (R&D Systems, Minneapolis, MN) either in fresh medium or in medium in which they had been growing for up to 4 days. Apoptosis was analyzed 24 hours later using the Cell Death Detection ELISA PLUS kit (Roche Diagnostics Corporation, Indianapolis, IN). Cells were also treated with TRAIL in fresh medium supplemented with recombinant OPG-Fc. Each data point is the average absorbance reading of two wells and each cell line was tested at least thrice.

**Serum calcium assay.** Blood was obtained for serum calcium measurement by intracardiac puncture and terminal bleeding under anesthesia at the end of each experiment. The serum calcium concentration was measured with a calcium assay kit (Sigma Diagnostics, Deisenhofen, Germany), on a UV spectrophotometer (Beckman, Buckinghamshire, United Kingdom) according to the instructions of the manufacturer and standardized to serum protein levels.

**Statistical analysis.** Statistics were done using one-way ANOVA, followed by Fisher’s least significant difference test. Values are expressed as mean ± SE and differences were considered to be statistically significant when *P* < 0.05.

**Results**

MCF-7 and MCF-7 + PTHrP cells were transfected with full-length human OPG to determine whether increased OPG production by breast cancers could reverse osteolysis in an in vivo model of breast cancer cell growth in bone: PTHrP-overexpressing cells were used because of their increased ability to induce osteolysis as a result of a PTHrP-induced increase in osteoclastic RANKL production and a corresponding decrease in osteoblastic OPG (10). MCF-7 cells produce very low levels of OPG compared with other cell lines and primary tumor samples (10) and overexpression of OPG in this line enabled us to examine the role of OPG when expressed at levels more comparable to those in tumors from patients.

**OPG overexpression.** RT-PCR analysis showed a 5- to 11-fold increase in OPG mRNA expression in each of the cell lines transfected with OPG compared with the MCF-7 or MCF-7 + PTHrP cells (Fig. 1A and B). Furthermore, ELISA analysis showed that both MCF-7 parental cells and MCF-7 + PTHrP cells produce low levels of OPG, with only 0.1 ng/mL protein produced over a 24-hour period in either cell line. Transfection with full-length OPG resulted in a substantial increase in OPG production, 5.7 ng/mL in MCF-7 + OPG cells (57-fold), and 4.4 ng/mL in MCF-7 + PTHrP + OPG cells (44-fold; Fig. 1C). These nonclonal populations of cells represent cells selected on hygromycin for 14 days, and they were not used for more than four passages from original derivation. Eight clonal lines of MCF-7 + PTHrP + OPG were also generated, which displayed a 1.4- to 3.8-fold increase in OPG mRNA and 12- to 29-fold increase in protein production similar to the nonclonal pools: OPG expression was confirmed by growing the clonally selected cells in selection-free media for 3 weeks and reassaying for OPG expression. The nonclonal pools of cells were used in preference to the clonally selected cell lines to circumvent potential clonal cell line differences that may arise following cell selection by limiting dilution, although the clonal lines phenotypically resembled their counterpart nonclonal pool in the in vitro and in vivo biological assays described below.

**Figure 1.** Overexpression of OPG in MCF-7 cells. A, semiquantitative RT-PCR amplification of OPG mRNA in MCF-7 (lane A), derivative cells MCF-7 + OPG (lane B), MCF-7 + PTHrP (lane C), and MCF-7 + PTHrP + OPG (lane D) compared with amplification of GAPDH mRNA. B, the single amplified PCR products for OPG and GAPDH from three independent experiments such as in (A) were quantified using PhosphorImager analysis and ImageQuant software. Signals corresponding to OPG were normalized with respect to GAPDH mRNA levels and plotted relative to the MCF-7 parental cells. C, levels of OPG in conditioned media produced by the cells in (A and B) over 24 hours. D, effects of OPG on osteoclast formation. Murine primary osteoblast and bone marrow cell cultures were done in the absence (open columns) or presence (black columns) of vitamin D3 and PGE2 in the absence or presence MCF-7, MCF-7 + OPG, or MCF-7 + PTHrP + OPG. After culture for 10 days, TRAP⁺ multinucleated (>3) cells were counted. Each culture was repeated four times in quadruplicate wells; columns, mean; bars, SE. The MCF-7 + PTHrP + OPG cells produced significantly more TRAP⁺ cells (*, *P < 0.05) in both the presence and absence of vitamin D3 and PGE2 compared with the control (without MCF-7 cells) and MCF-7-containing cultures. Cultures with the MCF-7 + PTHrP + OPG cells resulted in significantly less TRAP⁺ cells (**, **P < 0.02) in the presence of D3 and PGE2 compared with the MCF-7 + PTHrP cells.
An in vitro osteoclast formation assay was used to confirm that the OPG produced by the transfected MCF-7 cells was biologically active. Osteoclasts are TRAP⁺ multinucleated cells that exhibit calcitonin receptors and have the ability to resorb bone, and OPG is potent inhibitor of this process (18). The osteoclast formation cultures consisted of mouse bone marrow cells, mouse primary osteoblasts in the presence or absence of vitamin D₃ and prostaglandin E₂ (PGE₂), without and with MCF-7, MCF-7 + PTHrP, or MCF-7 + PTHrP + OPG cells. Osteoclasts (TRAP⁺ cells) formed in the coculture positive control of murine osteoblasts with bone marrow only in the presence of vitamin D₃ and PGE₂ (Fig. 1D), as we have previously described (10, 18). In cultures containing MCF-7 + PTHrP cells, osteoclasts were formed in both the presence and absence of vitamin D₃ and PGE₂, and their numbers were significantly elevated relative to control or MCF-7-containing cultures (Fig. 1D). In cultures containing MCF-7 + PTHrP + OPG cells, osteoclast formation was significantly reduced in the presence of vitamin D₃ and PGE₂ relative to the MCF-7 + PTHrP–containing cultures (Fig. 1D). These results indicated that the OPG expressed by MCF-7 + PTHrP + OPG cells was biologically active, inhibiting osteoclast formation.

**Tumorigenicity of the cells following intratibial inoculation.**
The effect of endogenous OPG on the ability of MCF-7 cells to grow in bone was determined following intratibial inoculation in nude mice. Mice injected with parental MCF-7 cells showed no osteolytic damage compared with control limbs as observed by radiology (Fig. 2B and A, respectively). Furthermore, there was minimal osteolytic damage observed in mice inoculated with MCF-7 + OPG (Fig. 2C).

As expected, the MCF-7 + PTHrP cells promoted bone loss, and osteolysis was readily identified in the tibia of injected mice (Fig. 2D); such a finding is consistent with results reported when these cells were delivered by intracardiac injection, another model that permits tumor cells to establish in bone and induce osteolysis (10, 19). Most striking was the effect of OPG overexpression in the MCF-7 + PTHrP cells (Fig. 2E) where enhanced osteolysis and increased tumor size were observed radiologically when compared with mice injected with MCF-7 + PTHrP cells (Fig. 2D).

OPG, administered as a recombinant protein (Fc-OPG), has been shown to inhibit osteoclast formation in vitro and in vivo and prevents osteolysis in models of cancer-induced bone loss (17, 20–22). Fc-OPG was administered from the time of intratibial inoculation with MCF-7, MCF-7 + PTHrP, or MCF-7 + PTHrP + OPG cells. Because parental MCF-7 cells do not establish well in bone, no obvious effect on osteolysis was noted in mice treated with Fc-OPG, although it should be noted that the femora of these mice (Fig. 2G) as well as those of the sham-treated animals (Fig. 2F) appeared more radiologically dense than the femora of animals that had not received Fc-OPG (Fig. 2, F relative to A and G relative to B), indicating that Fc-OPG was active (17). However, Fc-OPG dramatically reduced osteolytic lesions in mice injected...
Figure 3. Histology of hind limbs. Mice were treated as in Fig. 2. A to G, H&E sections of mice hind limbs at 42 days following intratibial inoculation into the left hind limb of MCF-7 (A and B), MCF-7 + OPG (C), MCF-7 + PTHrP (D and E), and MCF-7 + PTHrP + OPG (F and G). Arrows, tumor masses in the bone. H to K, TRAP staining of tumor-bone interface in mice injected with MCF-7 + PTHrP (H and I), MCF-7 + PTHrP + OPG (J and K); I and K, arrows, flattened osteoclasts at the tumor-bone interface. Mice represented in (B, E, G, I, and K) received s.c. injections of Fc-OPG (2.5 mg/kg/d). Bar, 100 μm. L and M, osteoclast numbers at the tumor-bone interface surface (OcS/TBIS) and serum calcium levels. Columns, mean; bars, SE. *, P < 0.01; **, P < 0.001.
with MCF-7 + PTHrP or MCF-7 + PTHrP + OPG cells (Fig. 2, H and I compared with D and E, respectively).

When the percentage area of osteolysis was quantified, OPG overexpression resulted in a doubling of the area of osteolysis for the MCF-7 + PTHrP cells (Fig. 2J). As mentioned earlier, no osteolysis was observed radiologically as a result of OPG overexpression in MCF-7 parental cells. Furthermore, quantification showed that Fc-OPG significantly reduced osteolysis in the MCF-7 + PTHrP and MCF-7 + PTHrP + OPG cells compared with untreated controls (Fig. 2J). This implies that full-length OPG, when expressed by breast cancer cells, does not inhibit osteolysis whereas OPG, when administered as a recombinant protein (Fc-OPG), is able to reduce cancer-induced osteolysis.

Histologic examination of the tibiae of these mice confirmed the changes in bone destruction evident from the Faxitron analyses (Fig. 2) along with accompanying cancer growth (Fig. 3). Small tumor nests were evident in mice inoculated with MCF-7 cells, either without or with Fc-OPG administration (Fig. 3A and B), compared with control limbs where no tumor cells were evident (data not shown). Moderately larger tumors developed in mice inoculated with MCF-7 + OPG cells although these showed little bone involvement (Fig. 3C). Inoculation of MCF-7 + PTHrP and MCF-7 + PTHrP + OPG cells resulted in the formation of large tumors that invaded the marrow cavity and the surrounding bone (Fig. 3D and F).

Similar levels of nuclear pleomorphism and mitoses in all of the tumors examined were revealed by histologic analysis. Interestingly, less tubule formation was apparent in the tumors overexpressing both PTHrP and OPG compared with tumors overexpressing PTHrP alone, indicating that these tumors represent a higher tumor grade (grade 9 versus grade 8, respectively) according to the modified Scarff-Bloom-Richardson grading system (23). This suggests that OPG expressed by cancer cells may affect tumor growth and differentiation, resulting in a less differentiated, more aggressive tumor. Furthermore, these effects were not restricted to MCF-7 cells because similarly engineered MDA-MB-231 cells (e.g., MDA-MB-231, MDA-MB-231 + OPG) showed similar enhanced growth in bone and osteolysis in a series of corresponding experiments (data not shown).

Fc-OPG treatment of mice resulted in reduced tumor burden in bone in mice injected with MCF-7 + PTHrP or MCF-7 + PTHrP + OPG (Fig. 3, E and G compared with D and F, respectively). Compared with the MCF-7 and MCF-7 + OPG cells, a large number of osteoclasts were present at the bone-tumor interface in the tumors produced by MCF-7 + PTHrP and the MCF-7 + PTHrP + OPG cells, as observed by H&E and TRAP staining (e.g., Fig. 3H and J). Fc-OPG treatment dramatically reduced osteoclast numbers at the tumor-bone interface in mice with MCF-7 + PTHrP tumors, but osteoclast numbers were unaltered by Fc-OPG treatment in mice with tumors produced by MCF-7 + PTHrP + OPG cells (Fig. 3L). Fc-OPG treatment of mice injected with either cell type resulted in a change of osteoclast morphology, with a flattened appearance adjacent to bone and reminiscent of a quiescent osteoclast (Fig. 3I and K).

Concomitant with the presence of large osteolytic lesions was a rise in blood ionized calcium in mice inoculated with MCF-7 + PTHrP + OPG cells (Fig. 3M). Fc-OPG administration was able to correct this rise in blood ionized calcium evident in these mice (Fig. 3M). This finding, together with the appearance of the flattened osteoclasts following Fc-OPG treatment (Fig. 3K), suggests that these osteoclasts have reduced resorptive activity.

TRAIL-induced apoptosis. Because OPG has been reported to influence cellular apoptosis (24, 25), we examined the effect of OPG overexpression to modulate TRAIL-induced apoptosis of MCF-7 cells; cultures were treated with 100 ng/mL TRAIL in fresh media and in conditioned media. MCF-7 parental and MCF-7 + PTHrP cells treated with TRAIL showed a 2.1- to 2.8-fold increase in apoptosis versus the control cultures (Fig. 4). However, there was no significant difference in levels of apoptosis in MCF-7 + OPG or MCF-7 + PTHrP + OPG cell lines versus the MCF-7 or MCF-7 + PTHrP cells, respectively. Histologic sections of tumors were also examined for the presence of apoptotic cells, which were dispersed uniformly through out the tumors, as observed by apoptotic bodies and by TUNEL staining. Similar to the in vitro findings, when cells were challenged with TRAIL to induce apoptosis (Fig. 4), there was no difference in the number of apoptotic cells in the intratibial tumors overexpressing OPG, compared with their counterparts, or following treatment with Fc-OPG (Fig. 5A-C).

Cell proliferation within the intratibial tumors was examined using Ki67 staining. Whereas there was some nuclear staining for Ki67 in the intratibial tumors produced by MCF-7 + PTHrP cells (Fig. 5D), significantly more cells stained positive for Ki67 in the
MCF-7 + PTHrP + OPG tumors (Fig. 5E), which was independent of Fc-OPG treatment (Fig. 5F).

To determine if OPG expression was maintained by tumor cells after sustained growth in vivo, sections were stained for OPG expression. OPG protein levels were clearly elevated in tumors that were derived from cells overexpressing OPG relative to their controls (e.g., MCF-7 + PTHrP compared with MCF-7 + PTHrP + OPG tumors; Fig. 5G and H).

**Tumorigenicity of the cells following mammary fat pad inoculation.** We examined the effect of OPG overexpression in breast cancer cells following mammary fat pad inoculation to determine whether the effect of OPG that we had observed was specific to cells growing in the bone microenvironment. MCF-7, MCF-7 + OPG, MCF-7 + PTHrP, and MCF-7 + PTHrP + OPG cells (along with appropriate vector control cells) were injected into the mammary fat pads of 4-week-old female nude mice. Tumors were measured from 21 days postinoculation once the tumors became palpable. MCF-7 parental cells exhibited an initial latent period following inoculation into the mammary fat pad and grew slowly until the end of the experiment (Fig. 6); similar results were obtained for the MCF-7 transfected with the empty expression vector (data not shown). At day 21, there was a significant difference in tumor size in mice inoculated with MCF-7 + OPG, MCF-7 + PTHrP, and MCF-7 + PTHrP + OPG versus animals inoculated with MCF-7 cells (P = 0.01, ANOVA). By day 28, not only were all these tumors (MCF-7 + OPG, MCF-7 + PTHrP, or MCF-7 + PTHrP + OPG derived) larger than the MCF-7 cell line tumors (P = 0.007, ANOVA) but tumors from MCF-7 + PTHrP + OPG cells were also significantly larger than those from MCF-7 + PTHrP cells (P = 0.03). These differences in growth rates continued for the remainder of the experiment. We found no effect of PTHrP overexpression on MCF-7 proliferation in vitro (data not shown); however, another report of PTHrP overexpression in MCF-7 cells resulted in increased cell adhesion, migration, and invasion (26), and these actions may be responsible for enhanced tumor growth.

Mammary fat pad tumors obtained from animals 49 days postinoculation were examined for expression of Ki67 and apoptosis (TUNEL) to determine whether the enhanced mammary tumor growth was the result of increased breast cancer cell proliferation, decreased apoptosis, or a combination of the two. As was the case with the intratibial-established tumors, OPG had no effects on cell apoptosis in tumors at the orthotopic site (data not shown). MCF-7 + PTHrP + OPG tumors (Fig. 5E), which was independent of Fc-OPG treatment (Fig. 5F).

**Figure 5.** Immunohistochemical analysis of hind limbs. Mice were treated as in Fig. 2. TUNEL staining of sections of mice hind limbs at 42 days following intratibial inoculation into the left hind limb of MCF-7 + PTHrP (A) and MCF-7 + PTHrP + OPG (B). Arrows, apoptotic cells. C, number of cells that have a positive TUNEL stain. Ki67 staining in intratibial tumors from mice inoculated with MCF-7 + PTHrP (D) and MCF-7 + PTHrP + OPG (E). F, proportional area (%) of Ki67 staining in intratibial tumors, where positive staining for Ki67 was measured using a color image analyzer and results expressed as the proportion of area with positive staining per field as described in Materials and Methods. OPG staining in intratibial tumors from mice inoculated with MCF-7 + PTHrP (G) and MCF-7 + PTHrP + OPG (H). Bar, 100 μm. Columns, mean; bars, SE. *, P < 0.05.
Many breast cancers possess properties that enable them to establish and grow in bone. One of the primary mechanisms used by breast cancers to achieve this is their ability to induce osteolysis as a result of enhanced osteoclast formation and activity. An important mediator of this process is PTHrP, which is produced by breast cancer cells and promotes the expression of osteoblast-derived RANKL, enabling osteoclast formation. We have previously reported that concomitant with the induction of RANKL in the osteoblast is a reduction in osteoblast-derived PTHrP production (10). The combination of these two outcomes was considered favorable for the establishment of the cancer in bone because of the enhanced osteolysis (10). However, in light of the results presented here, we must reconsider the pathophysiologic role of OPG in tumor growth at the primary site as well as during cancer-induced osteolysis. Breast, lung, and prostate cancers, multiple myeloma, and osteosarcomas express OPG (10, 20–22, 27), and increased OPG expression has been associated with poor outcomes in gastrointestinal carcinoma and pancreatic cancer (28, 29).

Delivery of Fc-OPG as a recombinant protein has shown promise as a potential therapy through experiments in animal models, in that OPG limits hypercalcemia and osteolysis induced by myeloma, breast, lung, or prostate cancer and reduces tumor establishment in bone (17, 20–22). Indeed, we reaffirm the ability of Fc-OPG to limit breast cancer cell growth in bone and accompanying osteolysis, even in cells that avidly promote osteolysis due to high level PTHrP production. In contrast to these actions, OPG expression by breast cancers seems to be advantageous to the growth of MCF-7 cells in bone.

We assessed whether the OPG-mediated enhanced growth of MCF-7 cells was a universal property, rather than restricted to the growth of these cells in bone, by examining whether this effect was mimicked in soft tissue tumors. As was the case with the intratibial inoculation, OPG overexpression by MCF-7 cells was associated with an enhanced growth rate for both MCF-7 and MCF-7 + PTHrP cells following mammary fat pad inoculation. The enhanced growth seemed to result from increased proliferation because OPG expression did not affect apoptosis. Whereas OPG overexpression significantly increased tumor mass following mammary fat pad inoculation, the increased Ki67 staining, indicating cellular proliferation provided by the OPG overexpressing tumors, was not as significant at this site as bone. The increased tumor mass may have resulted from altered matrix deposition in the breast or effects on breast stromal cells: the Ki67 antibody only detects positive staining per field as described in Materials and Methods. * P < 0.05.

Discussion

Many breast cancers possess properties that enable them to establish and grow in bone. One of the primary mechanisms used by breast cancers to achieve this is their ability to induce osteolysis as a result of enhanced osteoclast formation and activity. An important mediator of this process is PTHrP, which is produced by breast cancer cells and promotes the expression of osteoblast-derived RANKL, enabling osteoclast formation. We have previously reported that concomitant with the induction of RANKL in the osteoblast is a reduction in osteoblast-derived PTHrP production (10). The combination of these two outcomes was considered favorable for the establishment of the cancer in bone because of the enhanced osteolysis (10). However, in light of the results presented here, we must reconsider the pathophysiologic role of OPG in tumor growth at the primary site as well as during cancer-induced osteolysis. Breast, lung, and prostate cancers, multiple myeloma, and osteosarcomas express OPG (10, 20–22, 27), and increased OPG expression has been associated with poor outcomes in gastrointestinal carcinoma and pancreatic cancer (28, 29).

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Whereas OPG expressed by breast cancer cells could inhibit osteoclast formation in vitro, it did not suppress osteoclast numbers at the tumor-bone interface when tumor cells were delivered directly into the tibiae. This finding might suggest either that the OPG made by tumor cells was insufficient to inhibit osteoclast formation and activity or that the enhancement of cancer cell growth conferred by OPG expression was the predominant action and thus permitted tumor growth. Furthermore, Fc-OPG administration did not limit osteoclast numbers in areas proximal to highly proliferative tumor cells although osteoclasts appeared flattened on the bone surface, suggesting that Fc-OPG treatment inhibited osteoclast activity, but not osteoclast generation, in these tumor-bearing mice. The enhanced proliferation of tumor cells seems to favor increased bone loss, with Fc-OPG limited in its action in ablating RANKL. Comparison of this response with an anti-RANKL antibody would be worthwhile to pursue. Unfortunately AMG162, an anti-RANKL antibody, is human specific and does not target murine RANKL (30), which is essential for osteoclast formation in the nude mouse models that are routinely used.

OPG is a receptor for the cytotoxic TNF-related apoptosis inducing ligand (TRAIL) that induces apoptosis, both in vitro and in vivo, in a wide range of tumor cells with little effect on normal
tissues (24). TRAIL binds to its transmembrane receptors, DR4 and DR5, which are expressed on many normal and malignant cell types, including MCF-7 human breast cancer cells, inducing apoptosis through the caspase-8 cascade. Two decay receptors, DcR1 and DcR2, which lack functional death domains and are therefore unable to activate the caspase-8 cascade, also bind to TRAIL. DcR1 and DcR2 are expressed at high levels by normal cells but are either not expressed or only expressed at low levels by tumor cells, thereby restricting the cytotoxic activity of TRAIL to malignant tissue. OPG represents the third decay receptor for TRAIL (24) and Fc-OPG has been shown to inhibit TRAIL-induced apoptosis in Jurkat cells and prostate cancer cells in vitro (24, 25).

The effect of native OPG produced by MCF-7 cells seems to be TRAIL independent because we observed (a) no difference in apoptotic cell number between tumors from OPG-overexpressing cells and those from their parental derivatives, and (b) no difference in apoptosis induction by TRAIL between MCF-7 or MCF-7 + PTHrP cells with or without OPG overexpression. The enhanced growth of MCF-7 cells in vivo in the absence of altered apoptosis would argue that full-length OPG acts primarily to increase cellular proliferation, and this has been proposed for other members of the TNF receptor superfamily (31). Consistent with this, we noted increased Ki67 staining, suggesting increased cell proliferation in tumors that were overexpressing OPG relative to cell lines that were not. The addition of recombinant full-length OPG to MCF-7 cells did not result in increased proliferation in vitro (data not shown), suggesting the actions that we observed may be a consequence of intracellular actions of OPG.

Whereas the TNF receptor domains of OPG fused to the IgG Fc has osteoclast inhibitory activity both in vitro and in vivo, we have established that overexpression of native OPG results in enhanced growth of breast cancer cells in both soft tissue and bone. The actions of native OPG to promote cell growth relative to Fc-OPG may result from the differences between these molecules because Fc-OPG lacks the potential death domains, the heparin binding region, and the dimerization sequences, and, unlike native OPG, cannot participate in intracellular actions. These differences, either alone or in combination, may contribute to the detrimental outcome of OPG overexpression by breast cancer cells.

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References


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