Osteoprotegerin in Prostate Cancer Bone Metastasis

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Abstract

Osteoprotegerin (OPG), a critical regulator of osteoclastogenesis, is expressed by prostate cancer cells, and OPG levels are increased in patients with prostate cancer bone metastases. The objective of this study was to investigate the effects of OPG overexpression on prostate cancer cells and prostate cancer/bone cell interactions in vitro and in vivo. OPG-transfected C4-2 cells expressed 8.0 ng OPG per mL per 10⁶ cells, whereas no OPG was detected in the media of C4-2 cells transfected with a control plasmid. OPG overexpressed by C4-2 cells protected these cells from tumor necrosis factor-related apoptosis-inducing ligand–induced apoptosis and decreased osteoclast formation. Subcutaneous OPG-C4-2 and pcDNA-C4-2 tumors exhibited similar growth and take-rate characteristics. However, when grown in bone, tumor volume was decreased in OPG-C4-2 versus pcDNA-C4-2 (P = 0.0017). OPG expressed by C4-2 cells caused increases in bone mineral density (P = 0.0074) and percentage of trabecular bone volume (P = 0.007), and decreases in numbers of osteoblasts and osteoclasts when compared with intratibial pcDNA-C4-2 tumors (P = 0.003 and P = 0.019, respectively). In summary, our data show that increased expression of OPG in C4-2 cells does not directly affect proliferation of prostate cancer cells but indirectly decreases growth of C4-2 tumors in the bone environment. Our data also show that OPG expressed by C4-2 cells inhibits bone lysis associated with C4-2 bone metastasis, which results in net increases in bone volume. We therefore hypothesize that OPG expressed in prostate cancer patient bone metastases may be at least partially responsible for the osteoblastic character of most prostate cancer bone lesions.

Introduction

Bone metastases of prostate cancer are the most critical complication of advanced prostate cancer, often resulting in severe pain, morbidity, and mortality (1, 2). Prostate cancer bone metastases stimulate an overall increase in both bone remodeling and bone volume (3), but the mechanisms underlying this process are not fully understood. Characterization of the factors involved at the molecular level and elucidation of their interactions are current objectives of prostate cancer/bone biology research.

A key molecular system that influences bone remodeling consists of osteoprotegerin (OPG), receptor activator of NFκB ligand (RANKL), and RANK. OPG is a soluble member of the tumor necrosis factor receptor superfamily that prevents association of RANKL with RANK by acting as a decoy receptor. Interactions between RANKL and RANK are critical for the recruitment, activity, and survival of osteoclasts. An increase in the ratio of OPG to RANKL inhibits these processes and results in increased bone mass (4). OPG inhibits osteoclastogenesis in vitro and blocks bone loss following ovariectomy in vivo (5). Transgenic mice overexpressing OPG exhibit an osteopetrotic phenotype, whereas OPG-knockout mice have severe osteoporosis (6, 7).

The OPG/RANKL/RANK system is also involved in various pathologies associated with metastatic disease in bone (8). Increased expression of RANKL has been observed in osteolytic malignancies, such as breast cancer and multiple myeloma, and inhibition of osteoclastogenesis has been considered as an intervention strategy (9–12). Treatment of osteolytic bone metastases with OPG inhibits osteolysis and decreases skeletal tumor burden in myeloma and breast cancer (13, 14).

Although prostate cancer bone metastases are usually osteoblastic in character, the complex nature of bone remodeling and its interactions with prostate cancer seem to involve effects on osteolysis as well; increased osteolysis has been reported in patients with prostate cancer metastases (3). Based on previous findings, prostate cancer growth in bone is thought to be influenced by the osteolytic events that accompany the overall osteoblastic response. Exogenous administration of Fc-OPG and zoledronic acid, potent inhibitors of osteoclastogenesis and therefore osteolysis, were reported to inhibit intraosseous growth of C4-2B prostate cancer cells (15), LNCaP cells (16), and osteoblastic LuCaP 23.1 (17, 18), and it has also been reported that administration of Fc-RANK inhibited prostate cancer tumor growth in bone (19). Moreover, we have recently reported increased expression of OPG and RANKL in prostate cancer bone metastases (20), and increased levels of OPG in the serum of patients with prostate cancer bone metastases were reported by us and others (21–23). Taken together, these findings indicate significant involvement of the OPG/RANKL/RANK system in prostate cancer bone metastases.

This study was undertaken to evaluate the biological effects of OPG expressed by prostate cancer cells on these cells and their interactions with the bone environment. We examined whether OPG expression results in selective inhibition of growth in the bone when compared with growth at a subcutaneous site and in decreased osteolysis upon implantation in bone. Furthermore, because there is a delicate balance between bone degradation and new bone deposition, we examined whether suppression of osteolytic events may result in an increased osteoblastic response.

Materials and Methods

Cell Lines

C4-2 cells, a subline of LNCaP prostate cancer cells, were purchased from Urocor, Inc. (Oklahoma City, OK) and maintained under standard tissue
culture conditions in RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) and 2 mmol/L L-glutamine. RAW 264.7 mouse monocyte-macrophage cells (American Type Culture Collection, Manassas, VA) were maintained under standard tissue culture conditions in DMEM (BioWhittaker) with 10% fetal bovine serum.

Preparation and Characterization of Transfected Cells

In vitro Plasmid Preparation and Transfection

The coding region of human OPG was amplified by cDNA derived from MG-63 cells, an osteosarcoma cell line, using sense primer ACCCAAT-GAACAAGTTGGTGTGCCTGCGGT and antisense primer TTTAAG-CAGCTTATTTTTATGTT and ligated into pcDNA3.1 V5-His-TOPO (Invitrogen, Inc., Carlsbad, CA). Clones containing the OPG insert were sequenced and a 100% matching clone was transfected into C4-2 cells using Lipofectamine 2000, according to the manufacturer’s recommendations (Life Technologies, Rockville, MD). Selection of transfected cells was done with 600 μg/mL G418 and cells were maintained on 300 μg/mL G418.

Quantification of Osteoprotegerin mRNA and Protein Levels

Real-time PCR. OPG and pcDNA3.1V5-His-transfected C4-2 cells were harvested and total RNA was isolated using STAT-60 (Tel-Test, Friendswood, TX). One microgram of total RNA was transcribed as described previously (24). Expression of OPG was evaluated by real-time PCR using a Rotor-Gene (Corbett Research, Sydney, Australia) with sense primer GCATTCTCTAGGTGTTCCTCT, antisense primer CTGGGCTTTGTGTTTCC, and sybergreen for detection. The OPG signal was normalized to levels of glyceraldehyde-3-phosphate dehydrogenase message (sense primer TGAGCACCACACTGCTTAGC and antisense primer GGCGATGGACTGTGGTCATGAG). Relative quantities of the OPG and glyceraldehyde-3-phosphate dehydrogenase messages were calculated based on a standard curve using PC-3 cDNA.

Conditioned Media. Transfected cells were grown in RPMI 1640 with 10% fetal bovine serum to ~80% confluence. Media were then replaced with serum-free RPMI 1640, and conditioned media (CM) were harvested after 48 hours.

Western Blot. Total protein was extracted from transfected cells using lysis buffer [10 mmol/L Tris, 50 mmol/mL NaCl, 0.25% Triton X-100, 0.5% CHAPS, 0.25% dithiothreitol, and Complete protease inhibitors (Amersham, Piscataway, NJ), pH 7.4] for Western blot analysis of OPG expression. Thirty micrograms of total protein were separated on 4% to 20% SDS-PAGE and transferred onto a PVDF membrane. Western blots were done as described previously (24). In addition, CM of transfected cells was concentrated 10-fold for Western blot analysis of OPG in media. OPG was detected using a mouse anti-human OPG antibody (Biocarta, Carlsbad, CA) at a concentration of 1 μg/mL. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham).

Osteoprotegerin ELISA. pcDNA-C4-2 and OPG-C4-2 cells were grown in 6-well plates under standard conditions (10% fetal bovine serum). Media were collected after 4 days and OPG levels were determined using a human OPG ELISA kit (ALPCO, Windham, NH) following the manufacturer’s recommendations.

Proliferation of Transfected Cells. Transfected cells were plated in 96-well plates at a density of 3,000 cells well per well in RPMI 1640 with 10% fetal bovine serum. The effects of transfection on the proliferation of C4-2 cells were evaluated using the Quick Cell Proliferation Assay (BioVision Laboratories, Mountain View, CA) as described previously (18).

Cell Cycle Analysis. Transfected cells were grown under standard conditions, harvested and stained with 4',6-diamidino-2-phenylindole (10 μg/mL in 0.1% NP40) for determination of cell cycle distribution by flow cytometry (Cytoflex INFLUX Cytometer, Seattle, WA).

Effects of Conditioned Media from Transfected Cells and Coculture with Transfected Cells on Osteoclast Formation. Five thousand RAW 264.7 cells per well were plated in 24-well plates in media containing human RANKL (5 ng/mL, PeproTech, Rocky Hill, NJ). Either 200 μL of 10× concentrated CM were added to the culture or 2 × 104 OPG-C4-2 or pcDNA-C4-2 cells were cocultured with RAW 264.7 cells in Trans-well plates (Costar, Corning, NY) for 6 days, with half of the media replaced on day 3. Osteoclasts were stained on day 6 using a tartrate-resistant acidic phosphatase assay (Sigma Chemical Co., St. Louis, MO) and counted. To confirm that inhibition of osteoclast generation was due to OPG expression, coculture experiments were repeated with daily addition of a neutralizing anti-human-OPG mouse monoclonal antibody (1 μg/mL, R&D Systems, Minneapolis, MN). Experiments were done three to five times in duplicate.

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Induced Apoptosis. OPG-C4-2 and pcDNA-C4-2 cells were treated with 100 or 200 ng/mL tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, R&D Systems) for 3 days. Effects of TRAIL administration on proliferation were determined by the Quick Cell Proliferation Assay (BioVision Laboratories) after 3 days of exposure, as described above. Determination of effects on apoptosis was done 3 days post-TRAIL treatment using an Annexin V-FITC Apoptosis detection kit (Oncogene, San Diego, CA) according to the manufacturer’s instructions.

Animal Studies

All procedures were done in compliance with the University of Washington Institutional Animal Care and Use Committee and NIH guidelines. Four- to 6-week-old male SCID mice (Fox Chase SCID mice, Charles River, Wilmington, MA) were used.

Subcutaneous Tumors. Animals (10 per group) were injected subcutaneously with 2 × 105 OPG-C4-2 or pcDNA-C4-2 mixed 1:1 with Matrigel (BD Biosciences, San Jose, CA). Tumor volume was measured twice weekly and calculated as L × H × W × 0.5236. Blood samples were harvested every week for determination of serum prostate-specific antigen (PSA) levels (IMX Total PSA assay, Abbott Laboratories, Abbott Park, IL). Animals were sacrificed when tumors reached ~1,000 mg or when animals were becoming compromised. Sacrifice serum was harvested for determination of serum OPG levels (Osteoprotegerin ELISA, ALPCO). Tumors were harvested for further analyses.

Intratibial Tumors. Animals were injected with approximately 105 cells in 10 μL into tibiae as described previously (25, 26). Two groups of 20 animals were used: (a) pcDNA-C4-2 cells and (b) OPG-C4-2 cells. Blood samples were drawn from animals weekly for determination of PSA serum levels (see above). Ten animals from each group were sacrificed at 3 weeks after tumor cell injection, and the remaining 10 animals in each group were sacrificed at 8 weeks after tumor cell injection or when becoming compromised. This interval was chosen based on previous experiments, which showed that at 8 weeks, C4-2 tumors were still growing within the tibia with no large tumor mass in the muscle. Bone mineral density (BMD) was measured at 3 and 8 weeks after tumor cell injections using a PIXImus Lunar densitometer (GE Healthcare, Waukesha, WI). Tibiae with tumors from five animals of each group were denaturalized and embedded in paraffin. The remaining five tumored tibiae from each group were embedded in methacrylate (27) for bone histomorphometrical (BHM) analysis.

Immunohistochemistry. Five-micrometer sections of paraffin-embedded tumors were used for determination of PSA and OPG immunoreactivity as described previously (28). A rabbit polyclonal anti-PSA antibody at 3 μg/mL (Dako, Carpentaria, CA) and a mouse monoclonal anti-human-OPG antibody (1.25 μg/mL, Biocarta) in combination with biotinylated anti-rabbit or anti-mouse polyclonal antibodies (1:150, Zymed, South San Francisco, CA) were used. Control sections were stained with a rabbit immunoglobulin G or MOPC-21, an unrelated monoclonal antibody; at the same concentrations as anti-PSA and anti-OPG antibodies, respectively.

Bone Histomorphometry. Six-micrometer sections of undecalci- fied tibiae embedded in methylacrylate and stained with Goldner stain (27) were used. BHM analysis was done on one longitudinal section in the middle of the tibia from five animals per group in the area adjacent to the growth plate (0.525-1.225 mm below the growth plate, site of injection, n = 5 per group) by Skeletech, Inc. (Bothell, WA). The percentages of bone volume and tumor volume in the tissue volume (%BV/TV and %Tvu/TV, respectively) were calculated. The trabecular thickness in μm, the trabecular volume fraction, and percent osteoid thickness, as well as the connectivity density, were determined.

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number per mm, the trabecular separation in μm, the ratio of osteoblast surface to bone surface as a percentage, and the ratio of osteoclast surface to bone surface as a percentage were also determined.

Data Analyses. Analyses of results were done using paired and unpaired Student's t tests as appropriate. The statistical significance level was \( P \leq 0.05. \)

Results

Characterization of Transfected Cells In vitro. OPG-C4-2 cells expressed large quantities of OPG mRNA, whereas control pcDNA-C4-2 cells had minimal levels of OPG mRNA (Fig. 1A). OPG protein was detected by Western blots in cell lysates and CM of OPG-C4-2 cells but not in pcDNA-C4-2 cells (Fig. 1B and C). OPG levels in the media of the transfected cells were also quantified by OPG ELISA. CM of OPG-C4-2 cells contained 8.0 ng OPG per mL per \( 10^6 \) cells, whereas no measurable levels of OPG were detected in the CM of pcDNA-C4-2 cells. OPG transfection did not affect proliferation of the cells; OPG-C4-2 and pcDNA-C4-2 cell growth rates were similar (Fig. 1D). The cell cycle distribution was also unaffected by OPG expression (data not shown).

Effects of Conditioned Media from Transfected Cells and Coculture with Transfected Cells on Osteoclast Formation. It is firmly established that OPG decreases recruitment of osteoclasts in vivo and in vitro. In studying the effects of OPG overexpression on prostate cancer cells, it is important to show that the overexpressed OPG exhibits biological activity. To determine this, we evaluated the effects of CM from OPG-C4-2 and pcDNA-C4-2 cell cultures on osteoclast formation and did cocultures of osteoclasts with the transfected cells. Addition of 200 μL 10× OPG-C4-2 CM to RAW 264.7 cells significantly decreased osteoclast formation versus addition of 200 μL 10× pcDNA-C4-2 CM (Fig. 2A). Comparable results were seen following coculture of the OPG-C4-2 and pcDNA-C4-2 cells and RAW 264.7 cells (Fig. 2B and C). Addition of a neutralizing anti-OPG antibody to the coculture experiments interfered with the inhibition observed, confirming that the inhibition observed was related to the presence of active OPG (Fig. 2D).

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Induced Apoptosis. We wished to characterize our OPG-overexpressing prostate cancer cells by determining whether the OPG had biological effects similar to those seen in earlier work (29). Treatment of pcDNA-C4-2 cells with TRAIL for 3 days decreased cell numbers from 100.0 ± 3.7% (mean ± SE) to 69.4 ± 4.7%, \( P = 0.069 \) (100 ng/mL TRAIL) or 68.4 ± 0.8%, \( P = 0.0011 \) (200 ng/mL TRAIL), whereas no significant inhibition was detected with OPG-C4-2 cells (100 ± 3.9 versus 100.6 ± 2.7%, \( P = 0.9115 \), and 86.9 ± 3.8%, \( P = 0.0728 \)). Apoptosis caused by 3 days’ treatment with 100 ng/mL TRAIL was decreased by OPG overexpression from 29.8% of cells (pcDNA-C4-2) to 16.9% (OPG-C4-2; Fig. 3B).

Subcutaneous Tumors. OPG overexpression did not alter take and growth rates of subcutaneous (SC) tumors. Take rates of OPG-C4-2 and pcDNA-C4-2 cells were 90%, and tumors grew with similar characteristics (Fig. 4A). Because serum PSA levels are used to evaluate tumor growth and response to treatments in patients, we measured serum PSA levels during growth of the OPG-C4-2 and pcDNA-C4-2 tumors. PSA levels followed tumor growth and were indistinguishable between the OPG-C4-2 and pcDNA-C4-2 tumors. There were no significant differences in PSA index (ng PSA per mL per mm\(^3\) tumor ± SE) between these two groups at days 14, 21, and 35 after tumor cell injection (14 days: 1.18 ± 0.12 and 0.87 ± 0.14, \( P = 0.1203 \); 21 days: 1.18 ± 0.20, and 0.97 ± 0.11, \( P = 0.3592 \); 35 days: 0.97 ± 0.15, and 0.84 ± 0.15, \( P = 0.5760 \)), again suggesting that OPG overexpression did not affect tumor growth (Fig. 4A and B). Immunohistochemistry and measurement of serum OPG levels by ELISA confirmed overexpression of OPG by the OPG-C4-2 tumors: OPG-C4-2 tumors exhibit intense OPG immunoreactivity, whereas pcDNA-C4-2 tumors are negative (Fig. 4C). Serum OPG levels in animals bearing OPG-C4-2 tumors were much higher than in the animals bearing pcDNA-C4-2 tumors (82.1 ± 5.7 ng/mL versus 6.1 ± 3.3 pg/mL, \( P = 0.0001 \)) (Fig. 4D).

Intratibial Tumors. In an earlier study (20), we reported that OPG mRNA was detected in normal prostate samples and primary prostate cancer, whereas the OPG protein was abundantly present in prostate cancer bone metastases, but absent in primary prostate cancer and soft-tissue metastases. C4-2 cells, a subline of the LNCaP prostate cancer cell line (which was originated from a prostate cancer lymph node metastasis), were used in the experiments described in the present article. OPG mRNA is detected at low levels...

Figure 1. Characterization of transfected C4-2 cells. A, real-time PCR analysis of OPG mRNA levels. OPG-transfected cells expressed large amounts of OPG mRNA. Levels of OPG mRNA were evaluated using real-time PCR as described in Materials and Methods. Standard curves for determination of relative quantities of OPG and GAPDH mRNA were generated using PC-3 cDNA diluted from 1- to 8-fold. The OPG signal was normalized to the GAPDH signal for analysis. B, Western blot analysis of OPG levels in cell lysates. OPG immunoreactivity was detected in cell lysates of OPG-overexpressing C4-2 cells but not the pcDNA-transfected C4-2 cells. Thirty micrograms of total protein isolated from transfected cells were separated by 4% to 20% SDS-PAGE and transferred to a PVDF membrane for Western blot analysis. C, Western blot analysis of OPG levels in conditioned media. OPG-C4-2 cells secreted OPG into the cell culture media. Twenty microliters of 10× CM from transfected cells were separated by 4% to 20% SDS-PAGE and transferred to a PVDF membrane for Western analysis. OPG immunoreactivity was detected using an anti-OPG antibody. D, proliferation assays. Overexpression of OPG did not alter the proliferation of C4-2 cells in vitro. Cell proliferation was assessed using the Quick Proliferation Assay as described in Materials and Methods. Cells were seeded at \( 3 \times 10^4 \) cells per well in 96-well plates and grown for 6 days. Experiments were done thrice in triplicate. Representative results from one experiment (mean ± SE).
in these cells, but no OPG immunoreactivity is seen in SC C4-2 tumors; this pattern parallels the expression in patients’ primary tumors and lymph node metastases. We have recently published a detailed characterization of the bone response to C4-2 cells (26).

OPG overexpression did not alter the take rates of tumor cells in the bone environment, which were 100% for both transfected C4-2 strains. Growth of prostate cancer cells in the bone was monitored by serum PSA levels. At 3 weeks, 95% of animals in both groups had measurable serum PSA levels. There were no significant differences between the BMD of tibiae with OPG-C4-2 and pcDNA-C4-2 tumors (P = 0.9) at this early time point. The BMD of both OPG-C4-2 and pcDNA-C4-2 tibiae was significantly lower versus contralateral nontumored tibiae (~4-6%, P = 0.0020 and 0.0005, respectively; Fig. 5A). At 8 weeks after injection of tumor cells, the BMD of tibiae with OPG-C4-2 tumors was higher than that of pcDNA-C4-2 tibiae (increase of 11.2%, P = 0.0074). Importantly, at 8 weeks, the BMD of tibiae with OPG-C4-2 tumors was not different from that of contralateral nontumored tibiae, and was 8.2% higher than the BMD of OPG-C4-2 at 3 weeks after tumor cell injection (P = 0.0238; Fig. 5B).

At 8 weeks after tumor cell injection, the pcDNA-C4-2 cells, like parental C4-2 cells (26), replaced and filled the bone marrow cavity and destroyed almost all bone trabeculae. In contrast, tibiae with OPG-C4-2 tumors contained numerous large bone trabeculae and

![Figure 2. Determination of effects of OPG expressed by C4-2 cells on osteoclast formation in vitro. A, conditioned media. OPG-C4-2 and control pcDNA-C4-2 CMs were generated as described in Materials and Methods. RAW 264.7 cells were cultured in the presence of 5 ng/mL RANKL and 200 μL of 10−1 CM. Osteoclasts were visualized by TRAP staining. Media were changed on day 3. Columns, mean; bars, ±SE. OPG-C4-2 CM inhibited osteoclast formation in comparison with CM from control pcDNA-C4-2 cells. B, coculture of transfected cells and RAW 264.7 cells. RAW 264.7 cells and OPG-C4-2 or control cells were cocultured in Trans-well plates in the presence of 5 ng/mL RANKL for 6 days. Osteoclasts were visualized by TRAP staining. Columns, mean; bars, ±SE. OPG-C4-2 cells inhibited osteoclast formation from RAW 264.7 cells in comparison to control pcDNA-C4-2 cells. C, representative example of osteoclasts generated from RAW 264.7 cells in the presence of OPG-C4-2 and pcDNA C4-2 cells. Top, osteoclasts generated from RAW 264.7 cells cultured in the presence of 5 ng/mL RANKL and pcDNA-C4-2 cells were stained for TRAP activity and appear as large multinucleated cells (arrow). Bottom, no osteoclasts were observed in the presence of OPG-C4-2 cells. D, effects of neutralizing anti-OPG antibody. A neutralizing anti-OPG antibody was added to the coculture experiments as described in Materials and Methods. Daily addition of 1 μg of anti-OPG antibody minimized the inhibitory effects of OPG-C4-2 CM on osteoclast formation, confirming that the inhibition of osteoclast formation observed in the presence of CM or OPG-C4-2 cells is due to OPG.

![Figure 3. OPG overexpression protected C4-2 cells from TRAIL-induced apoptosis. A, OPG-C4-2 and pcDNA-C4-2 cells were treated with 100 or 200 ng/mL TRAIL. Growth of OPG-C4-2 cells was not as strongly inhibited by TRAIL as was growth of control pcDNA-C4-2 cells. B, transfected cells were treated with 100 ng/mL TRAIL and stained for apoptosis using an Annexin V kit as described in Materials and Methods. Apoptosis was detected in OPG-C4-2 cells after treatment with TRAIL but to a lesser extent than in control pcDNA-C4-2 cells. Representative results are shown.]

small numbers of bone marrow cells together with tumor cells. Representative examples of histology of the OPG-C4-2 and pcDNA-C4-2 tibiae are shown in Fig. 6A. The presence of prostate cancer tumors was confirmed by PSA immunoreactivity in both types of tibiae (Fig. 6B), and overexpression of OPG in OPG-C4-2 cells, but not by pcDNA-C4-2 cells, in the bone microenvironment was confirmed by strong cytoplasmic immunoreactivity (Fig. 6C).

Levels of OPG in serum of mice bearing pcDNA-C4-2 tumors in bone were 19.2 ± 3.4 pg/mL, which was higher than the 6.1 ± 3.3 pg/mL detected in serum of animals bearing SC pcDNA-C4-2 tumors, although these were larger than the intratibial tumors. Moreover, our immunohistochemistry of pcDNA-C4-2 in tibiae showed low but detectable immunoreactivity of OPG, whereas SC tumors were negative. We conclude that OPG expression is increased in these cells when present in bone in comparison to SC tumors. These observations provide further indications that this model is analogous to the patient situation.

At 3 weeks after tumor cell injection, BHM analysis revealed that tumor volume and bone characteristics of the tibiae with OPG-C4-2 and pcDNA-C4-2 were not significantly different (data not shown), but significant effects of OPG overexpression on prostate cancer tumor and bone were detected at 8 weeks after tumor cell injection. Results of BHM analysis of tibiae with OPG-C4-2 and pcDNA-C4-2 tumors are presented in Table 1. BHM analysis in the area adjacent to the growth plate show that tumor volume (%TU/V) in OPG-C4-2 tibiae was significantly lower than tumor volume in pcDNA-C4-2 tibiae (48.67 ± 1.11 and 77.40 ± 5.97, respectively; P = 0.0117). To reduce the possibility that analysis of this area is not representative of the total tumor volume, analysis of tumor volume was done on the whole longitudinal section of the tibiae. The decreases in tumor volume observed in OPG-C4-2 tibiae versus pcDNA-C4-2 tibiae were similar to the results in the area adjacent to the growth plate (32.85 ± 2.50 and 54.71 ± 3.67, respectively; P = 0.004; Fig. 7A and B). Surprisingly, serum PSA levels in
animals bearing OPG-C4-2 tumors were similar to those in animals bearing pcDNA-C4-2 tumors in tibiae (Fig. 7C). OPG expression in C4-2 cells caused significant increases in %BV/TV, trabecular thickness, and trabecular number, and significant decreases in osteoblast surface/bone surface and osteoclast surface/bone surface (Table 1).

**Discussion**

Understanding the biology of prostate cancer osteoblastic metastases should lead to recognition of potential treatment targets. We have recently shown that OPG, a critical regulator of osteoclastogenesis, is expressed by prostate cancer cells (20), and it has been reported by us and others that OPG levels are increased
in patients with prostate cancer bone metastases (21–23). Treatment with recombinant Fc-OPG has decreased establishment of prostate cancer tumors and their growth in the bone environment (15–17). The studies reported herein were designed to delineate further the roles of OPG expression in prostate cancer metastases in bone.

Whereas administration of exogenous Fc-OPG inhibited tumor growth (15–17), OPG overexpression in breast cancer cells MCF-7 increased their growth. Animals inoculated with OPG-overexpressing cells developed large tibial tumors of a less-differentiated phenotype (30). Moreover, OPG expression in MDA-MB-231 cells caused a 25% survival advantage following intracardiac injection (31). These results suggest that endogenous OPG exhibits autocrine effects on breast cancer cells. In our study, overexpression of OPG in C4-2 prostate cancer cells did not directly alter growth of prostate cancer cells in vitro or growth of SC prostate cancer tumors. The differences between our results and the effects seen in breast cancer may be related to differences in the biology of breast and prostate cells. OPG has been shown to be involved in mammary development (32), whereas no similar findings have been reported in prostate cancer. Therefore, our results do not support the concept that OPG regulates prostate cancer cell proliferation through autocrine pathways, at least in the C4-2 model.

OPG has been associated with prostate cancer progression and bone metastases (21–23). Our observation that OPG expression inhibits tumor growth therefore seems to present a paradox. However, it is important to draw a distinction between correlation and causation. In the absence of relevant data, one might hypothesize that OPG expression contributes to tumor growth, but an equally valid hypothesis is that OPG expression is an adventitious effect of disruption of signaling pathways associated with tumor growth. For example, we have previously shown that OPG expression is increased in the PC-3 prostate cancer cell line by exposure to bone morphogenetic proteins (24). The bone environment is a rich source of bone morphogenetic proteins, which have fundamental roles in growth, development, and bone formation, apart from regulation of OPG. Thus, in this model, OPG expression in prostate cancer bone metastases is not a selected trait, but a characteristic due mainly to the bone environment. In support of this hypothesis, we have previously shown that expression of OPG by normal prostatic epithelium is lost in primary prostate tumors, but restored in the bone environment. Under this model, there is no a priori reason to expect OPG to stimulate growth of prostate cancer bone metastases, and the observation that OPG-expressing C4-2 cells grow more slowly than control C4-2 cells is not paradoxical.

Treatment with Fc-OPG decreased the take rate of prostate cancer tumors in some studies (15, 16) but not in others (17). In the present study, overexpression of OPG did not inhibit the establishment of the OPG-C4-2 tumors in the bone environment.

### Table 1. Effects of OPG overexpression in C4-2 cells on bone

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<th>BV/TV (%)</th>
<th>Tb.Th (μm)</th>
<th>Tb.N (mm⁻¹)</th>
<th>Tb.Sp (μm)</th>
<th>ObS/BS (%)</th>
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<td>OPG-C4-2</td>
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<td>1.14 ± 0.22</td>
<td>914.00 ± 160.71</td>
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NOTE: BHM analysis was performed in the area adjacent to the growth plate (0.525-1.225 mm below the growth plate; site of injection, n = 5 per group). Percentages of bone volume and tumor volume in the tissue volume (%BV/TV and %TuV/TV, respectively).

Results are presented as mean ± SE. Statistical significance was evaluated using Student’s t test.

Abbreviations: Tb.Th, trabecular thickness in μm; Tb.N, trabecular number in mm⁻¹; Tb.Sp, trabecular separation in μm; ObS/BS, ratio of osteoblast surface to bone surface as %; OcS/BS, ratio of osteoclast surface to bone surface as %.

![Figure 7](image-url)
The differential effects could be due to heterogeneity in the response of various prostate cancer models as well as differences in the levels of OPG. Doses of Fc-OPG used for treatment have been quite high; such levels were not reached with OPG overexpression and are probably not reached in prostate cancer patients with bone metastases. Therefore, we conclude that OPG expression in prostate cancer cells does not adversely affect the establishment of tumor cells in the bone environment.

Although the take rate of the C4-2 tumor cells seems unaffected by OPG, the results presented here suggest that overexpression of OPG by the prostate cancer cells does result in an overall reduction in tumor volume in bone. In keeping with the known biology of OPG activities in bone and the failure to observe inhibition of growth of SC prostate cancer tumors, our results indicate that inhibition of prostate cancer growth in the bone environment is due to suppression of recruitment and activity of osteoclasts by OPG. This results in decreases in both bone lysis and release of growth factors, and also in restriction of the space for tumor growth within the bone marrow cavity.

Surprisingly, serum PSA levels did not change in concert with the inhibition of tumor volume in bone exerted by OPG overexpression. Our results showed that serum PSA levels closely followed the volume of both OPG-C4-2 and pcDNA-C4-2 SC tumors, that the PSA index (ng PSA per mL per mm³ tumor) was similar for both groups, and that PSA mRNA levels in SC tumors as determined by real-time reverse transcription-PCR were also indistinguishable between OPG-C4-2 and pcDNA-C4-2 (data not shown). Therefore, we considered the possibility that there may be an artifact in measuring tumor volume by BHM adjacent to the growth plate, although this procedure has been successfully used by us in earlier studies (17, 26). To address this possibility, we measured tumor volume in the whole longitudinal section of tibiae. These results confirmed the overall decrease in OPG-C4-2 tumor volume. In none of the tumor-bearing mice was there significant extraosseal tumor growth that could confound the data. Therefore, we attribute the PSA results to another phenomenon. We have observed *in vitro* that semiconfluent proliferating prostate cancer cells secrete lower levels of PSA than nonproliferating confluent tumor cells (our unpublished data). We speculate that the OPG-C4-2 cells, confined to a limited space, cease proliferating and therefore produce higher levels of PSA than pcDNA-C4-2 cells, which can proliferate for longer periods as they fill larger marrow spaces due to degradation of trabecular bone. According to this scenario, similar PSA serum levels in the two models might be achieved despite differences in tumor volume. However, based on our results, we cannot rule out the possibility that the discordance of tumor volume and PSA levels might be specific to the bone environment and due to phenotypic changes, or the technical limitation of determination of tumor volume in one section of the tibiae.

OPG affects bone remodeling by inhibiting bone lysis. These effects were observed with OPG-C4-2 tumors, but not immediately after injection of the OPG-expressing cells. At 3 weeks after injection of tumor cells, the BMD of tumored tibia (OPG-C4-2 and pcDNA-C4-2) was actually lower than that of contralateral control tibia. This decrease could be due to tumor cells acting in combination with processes associated with the repair after injection through the growth plate. Evidently, the levels of OPG that had accumulated by that point were not sufficient to inhibit osteoclastogenesis. At 8 weeks after injection of tumor cells, however, the BMD of tibiae with OPG-C4-2 tumors was higher than the BMD of tibiae with pcDNA-C4-2 tumors, suggesting that OPG levels had become sufficient to inhibit osteolysis associated with C4-2 tumors. In fact, at this point, the BMD of the OPG-C4-2 tibiae had risen to a level similar to the BMD of contralateral nontumored tibiae. Moreover, the BMD of OPG-C4-2 tibiae at 8 weeks was significantly higher than at 3 weeks. BHM results showed that bone volume of OPG-C4-2 tibiae at 8 weeks after tumor cell injections was higher than that of pcDNA-C4-2 tibiae, and also higher than bone volume of contralateral nontumored tibiae. We hypothesize that C4-2 cells express other as-yet unidentified factors that stimulate an osteoblastic reaction, and when osteolysis is inhibited, the osteoblastic factors predominate.

The results presented herein bear on the effects of OPG on prostate cancer cells in the bone environment. Our data show that OPG overexpression in prostate cancer cells does not inhibit proliferation of these tumors directly, but affects the growth and characteristics of prostate cancer tumors in bone in an indirect manner. Because bone remodeling is a balance between bone degradation and bone formation, we hypothesize that the inhibition of tumor-associated osteolysis by OPG might be a significant event contributing to the classic osteoblastic response seen in prostate cancer bone metastases.

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**References**


Osteoprotegerin in Prostate Cancer Bone Metastasis


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