

# Osteoprotegerin Inhibits Osteolysis and Decreases Skeletal Tumor Burden in Syngeneic and Nude Mouse Models of Experimental Bone Metastasis

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## ABSTRACT

Certain malignancies, including breast cancer, frequently metastasize to bone, where the tumor cells induce osteoclasts to locally destroy bone. Osteoprotegerin (OPG), a member of the tumor necrosis factor receptor family, is a negative regulator of osteoclast differentiation, activation, and survival. We tested the ability of recombinant OPG to inhibit tumor-induced osteoclastogenesis, osteolysis, and skeletal tumor burden in two animal models. In a syngeneic model, mouse colon adenocarcinoma (Colon-26) cells were injected into the left ventricle of mice. Treatment with OPG dose-dependently decreased the number and area of radiographically evident lytic bone lesions, which, at the highest dose, were undetectable. Histologically, OPG also decreased skeletal tumor burden and tumor-associated osteoclasts. In a nude mouse model, OPG treatment completely prevented radiographic osteolytic lesions caused by human MDA-MB-231 breast cancer cells. Histologically, OPG decreased skeletal tumor burden by 75% and completely eradicated MDA tumor-associated osteoclasts. In both models, OPG had no effect on metastatic tumor burden in a panel of soft tissue organs. These data indicate that OPG may be an effective therapy for preventing osteolysis and decreasing skeletal tumor burden in patients with bone metastasis.

## INTRODUCTION

Bone metastasis is a frequent complication of several common human malignancies, including carcinomas of the breast, prostate, and lung (1). Bone resorption inhibitors such as bisphosphonates have been shown in several animal models to inhibit the osteolysis that frequently accompanies bone metastases (2–5). The pathophysiology of bone metastasis is poorly understood, but accumulating evidence suggests that the osteotropism of certain tumors may be related to their ability to activate osteoclasts and stimulate bone resorption (6, 7). The inhibition of tumor-associated osteolysis with bisphosphonates has been associated with increased (2), decreased (3–5), or unchanged (8, 9) skeletal tumor burden. Thus, the etiological relationship between bone resorption and bone metastasis remains hypothetical. The recent discovery of a potent new bone resorption inhibitor, OPG<sup>2</sup> (10), with its distinct mechanism of action, allowed us to revisit this hypothesis.

OPG is a member of the tumor necrosis factor receptor family that antagonizes the ability of OPG ligand (OPGL; Ref. 11), also known as ODF (12), RANK ligand (RANKL; Ref. 13), or TRANCE (14), to bind to its receptor RANK (15). RANK is a receptor on osteoclasts and preosteoclasts that is essential for their differentiation, activation, and survival (16, 17). RANK knockout mice lack osteoclasts and have severe osteopetrosis due to a lack of bone resorption (17). Mice lacking OPG develop severe early onset osteoporosis (18), whereas mice overexpressing rOPG have osteopetrosis (10). In animal models of humoral hypercalcemia of malignancy, rOPG prevents and rapidly

reverses hypercalcemia (19, 20). The parathyroid hormone-related protein-producing Colon-26 tumor, grown s.c., induces a robust systemic osteoclast response that is completely reversed by OPG treatment (20). In this study, we tested the ability of OPG to inhibit the localized osteolysis in two murine models of metastatic bone disease using murine adenocarcinoma and human breast cancer-derived cell lines.

## MATERIALS AND METHODS

**Cancer Cell Lines.** MDA-MB-231 human breast cancer cells were generously provided by Dr. Toshiyuki Yoneda (University of Texas, San Antonio, TX) and maintained in culture in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (CanSera, Rexdale, Canada) and 1% penicillin-streptomycin-glutamine (Life Technologies, Inc.). Colon-26 murine colon adenocarcinoma cells (21) were obtained from the Tumor Repository of the National Cancer Institute (Bethesda, MD) and maintained in culture in DMEM (Life Technologies, Inc.) containing 10% fetal bovine serum, 1% penicillin-streptomycin-glutamine, and 1% nonessential amino acids (Life Technologies, Inc.).

**Intracardiac Injections of Cancer Cell Lines.** The protocols for all animal studies were approved by Amgen's Institutional Animal Care and Use Committee. MDA-MB-231 cells ( $1 \times 10^5$ ) were injected into the left ventricle of athymic BALB/c-*nu/nu* female 7–8-week-old mice (Harlan Sprague Dawley, Houston, TX). Colon-26 cells ( $1 \times 10^5$ ) were similarly injected into the left ventricle of a syngeneic host, 7–8-week-old BALB/c  $\times$  DBA/2 (CDF1) mice (Charles River, Wilmington, MA). All intracardiac injections were performed under light isoflurane anesthetic, as described previously (22).

**Treatment with rOPG.** The rOPG used for these studies comprised the ligand-binding domain of human OPG fused to the Fc domain of human IgG, as described previously (10, 23). Treatment was initiated within hours of the intracardiac injection of tumor cells. For both studies, each group included 10 mice. For the Colon-26 study, mice were treated i.v. with either vehicle (PBS) or OPG (0.3, 1.0, or 3.0 mg/kg) on days 0 (tumor inoculation), 3, 6, and 9. For the MDA-MB-231 study, mice were treated i.v. with either vehicle or OPG (25 mg/kg) three times per week for 4 weeks. After the final treatment, mice were sacrificed and radiographed with a Faxitron X-ray system (Model 43855A; Faxitron X-ray Corp., Buffalo Grove, IL). Bones (left and right femur, tibia, and humerus) and soft tissues (heart, lung, brain, kidney, liver, spleen, pancreas, adrenal glands, and ovaries) were harvested for histological analysis.

**Image Analysis of Osteolytic Lesions.** Radiographs were scanned, magnified  $\times 500$ , and analyzed for tumor-induced osteolysis. Lesions visible in the long bones (Fig. 1) of the skeleton were counted, and the margins were traced to determine lesion number and area using a MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA).

**Histomorphometric Analysis of Metastatic Tumor Burden.** The tibia, femur, and humerus were chosen for histomorphometric measurement of skeletal tumor burden based on radiographic evidence of significant tumor involvement in untreated mice. Bones were processed as described previously (10). Briefly, midline longitudinal sections were stained for TRAP activity (leukocyte acid phosphatase kit; Sigma Chemical Co., St. Louis, MO) and counterstained with hematoxylin. In this staining procedure, osteoclasts are red, whereas the remaining tissue is stained blue. Two nonserial sections of each bone were assessed. The total tissue section area and the tissue area occupied by tumor cells were measured using an Osteomeasure bone analysis program (Osteometrics Inc., Decatur, GA). Osteoclasts were scored based on an association with tumor cells and TRAP-positive staining within a defined area of tumor.

Soft tissue organs were collected at necropsy and fixed in 10% buffered zinc formalin. One representative midlevel section from the same area of each

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<sup>2</sup> The abbreviations used are: OPG, osteoprotegerin; rOPG, recombinant OPG; TRAP, tartrate-resistant acid phosphatase.

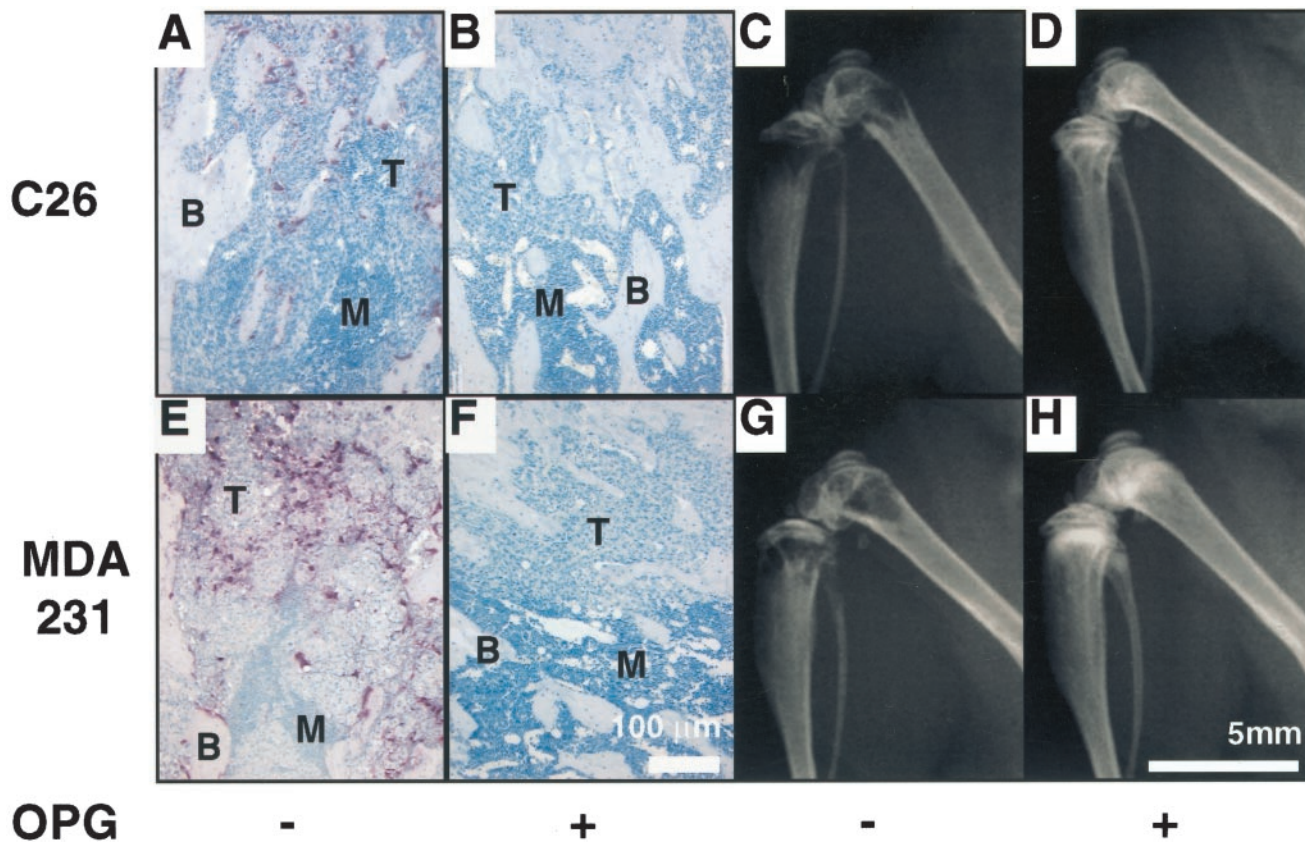


Fig. 1. Histological appearance of skeletal tumors and radiographic appearance of osteolytic lesions. Histological sections were stained for TRAP and counterstained with hematoxylin. *T*, tumor tissue; *M*, normal marrow; *B*, bone. Colon-26 tumors and MDA-MB-231 tumors in vehicle-treated mice (*A* and *E*, respectively) were associated with numerous TRAP-stained osteoclasts (purple) and loss of bone. OPG treatment at the highest doses (*B* and *F*, respectively) decreased osteoclasts to subphysiological levels while preserving the trabecular bone. Radiographically, both Colon-26 and MDA-MB-231 tumors in vehicle-treated mice were associated with numerous large osteolytic lesions (*C* and *G*, respectively). OPG treatment at the highest doses (*D* and *H*, respectively) completely prevented the radiographic appearance of osteolytic lesions. In *H*, note the increased radio-opacity of the tibial and femoral metaphyseal growth plate region associated with OPG treatment. This increase is a normal pharmacological response to OPG treatment by growing mice, whereby resorption of the secondary spongiosa is inhibited, and trabecular bone accumulates.

organ was stained with H&E and evaluated using light microscopy by a pathologist blinded to the treatment conditions. The extent of the tumor infiltration was assessed for each entire section using the following scoring system: (a) 0, no tumor; (b) 1, one or more small foci; (c) 2, up to one-third of the tissue infiltrated; (d) 3, between one-third and two-thirds of the tissue infiltrated; (e) 4, more than two-thirds of the tissue infiltrated; and (f) 5, all of the original tissue replaced by tumor. The spleen was sampled by the same method, and no tumor foci were found with either cell line, with or without OPG treatment.

**Statistical Analysis.** Statistical analysis was performed using JMP Statistical Software (SAS Institute, Inc., Cary, NC). Radiographic and histomorphometric data were analyzed by Dunnett's test for multiple treatment groups. For tumor burden data, comparisons between OPG treatment and vehicle treatment were analyzed by one-way ANOVA. Differences with a  $P < 0.05$  were considered statistically significant.

## RESULTS

Within 12 days of intracardiac tumor inoculation, Colon-26 cells had colonized the skeleton of vehicle-treated mice and caused significant localized bone destruction (Figs. 1 and 2). Treatment of mice with OPG caused a dose-dependent decrease in both the size and the number of radiographically evident osteolytic lesions (Fig. 2). These effects translated to a significant decrease in total lesion area/mouse at doses as low as 0.3 mg/kg, and at the highest administered dose (3 mg/kg), tumor-injected mice had no radiographic evidence of lytic lesions in the skeleton ( $P < 0.005$ ; Fig. 2).

We used histomorphometry to examine the effects of OPG treat-

ment on skeletal tumor burden and on the osteoclast density of tumors. OPG treatment significantly decreased the number of osteoclasts within the tumor, with a maximal reduction of 90% at 3 mg/kg ( $P < 0.01$ ; Fig. 3A). OPG treatment caused a modest decrease in the number of skeletal tumor nests/mouse, but this difference was only significant at a dose of 1 mg/kg ( $P < 0.05$ ; Fig. 3B). OPG treatment at 1 and 3 mg/kg reduced the average area of tumor nests within the skeleton, with a maximum decrease of 60% at 3 mg/kg (Fig. 3C;  $P < 0.01$ ). The percentage of bone tissue occupied by tumor cells was also significantly reduced after treatment with OPG at 1 and 3 mg/kg ( $P < 0.05$ ; Fig. 3D).

An examination of soft tissue organs was conducted to determine whether the highest dose of OPG (3 mg/kg) had any influence on the pattern or extent of metastatic tumor burden. A pathologist blinded to the treatment conditions analyzed histological sections of liver, lung, heart, brain, adrenal glands, ovaries, kidneys, pancreas, and spleen. The spleen and pancreas were tumor free in all mice. The remaining organs had variable levels of tumor involvement, and there was no effect of OPG on the distribution or extent of metastases in any of the organs analyzed (Table 1).

Intracardiac injection of MDA-MB-231 human breast cancer cells into nude mice is a well-established model of experimental bone metastasis (3–5). Skeletal lesions develop more gradually in this model compared to the syngeneic Colon-26 model, which allows for a more prolonged treatment period. In a 4-week MDA-MB-231 tumor study, we treated mice with a high dose of OPG (25 mg/kg) to

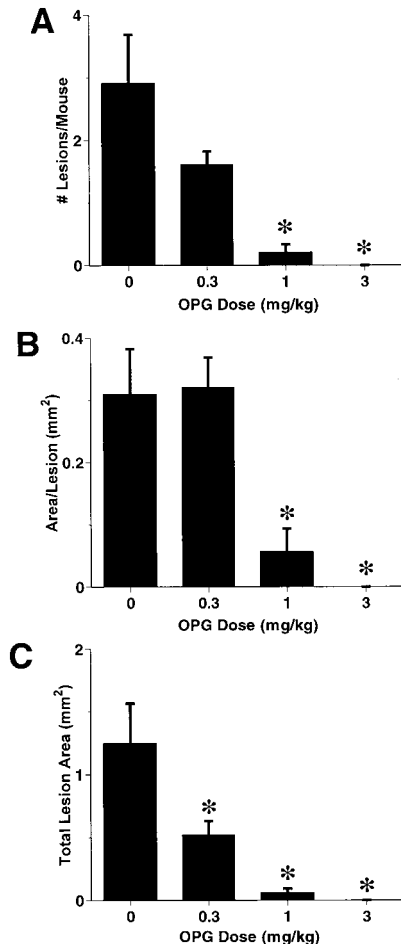


Fig. 2. Effect of OPG treatment on osteolytic lesions resulting from intracardiac injection of Colon-26 tumor cells. Mice were treated on days 0, 3, 6, and 9 with OPG at the indicated doses ( $n = 10$  mice/group). Mice were sacrificed on day 12, and X-rays were analyzed morphometrically to determine (A) the number and (B) the area of osteolytic lesions. C, total lesion area/mouse. OPG at doses of 0.3–3.0 mg/kg significantly reduced the total area of osteolysis ( $P < 0.01$ ).\*, significantly different than 0 mg/kg OPG. Data represent the means  $\pm$  SE.

overcome the potential immune response to the human OPG protein that may occur with prolonged dosing. In vehicle-treated mice, multiple osteolytic lesions were radiographically evident 4 weeks after MDA tumor inoculation. Treatment with OPG completely prevented the appearance of osteolytic lesions (Fig. 4A;  $P < 0.001$ ). Histologically, OPG treatment caused significant decreases in the average number of skeletal tumor nests/mouse, and OPG treatment also reduced the average size of these tumor nests (Fig. 4, B and C). These treatment effects translated into a significant 80% decrease in total skeletal tumor burden, as determined by the percentage of bone tissue area occupied by tumor (Fig. 4D;  $P < 0.05$ ). In vehicle-treated mice, MDA tumor nests in bone were associated with a robust osteoclast response. In contrast, tumor nests in the skeleton of OPG-treated mice were virtually devoid of osteoclasts (Fig. 4E;  $P < 0.001$ ).

DISCUSSION

In this study, we demonstrate that OPG prevents the osteoclastogenesis and bone destruction associated with metastasis of tumor-derived cell lines of human and of murine origin. In addition, treatment of both syngeneic and nude mice with OPG significantly decreased total skeletal tumor burden. In both models, OPG treatment had no significant effect on tumor burden in other organs. These results are consistent with the hypothesis that bone resorption contributes to the growth of tumor cells within bone and that the inhibition of bone resorption might selectively limit metastatic tumor growth in bone.

The Colon-26 mouse colon adenocarcinoma is an established tumor model that causes hypercalcemia when injected s.c. (20, 21). We recently demonstrated that this parathyroid hormone-related protein-secreting tumor, when injected s.c., causes systemic osteoclast activation and bone resorption that can be both prevented and rapidly reversed by OPG treatment (20). We now demonstrate that injection of this tumor cell line into the systemic circulation of mice causes aggressive metastases to bone and to other organs within 10–14 days. OPG treatment significantly decreased skeletal tumor burden in this model. This effect appeared to be related more to a reduction in the

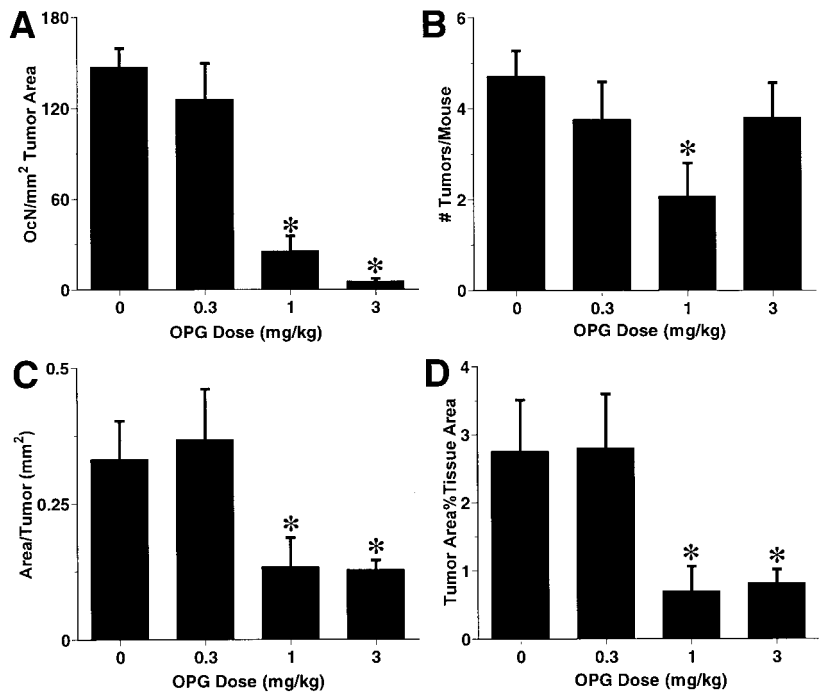


Fig. 3. Effect of OPG treatment on histomorphometric indices of skeletal Colon-26 tumor burden. Two nonserial sections of each tibia and femur were analyzed for (A) number of osteoclasts/mm<sup>2</sup> tumor area ( $OcN/mm^2$  Tumor Area), (B) number of tumors/mouse, (C) average area of each tumor, and (D) skeletal tumor burden (tumor area percentage of tissue area). OPG treatment significantly reduced the area/tumor, the number of tumors/mouse, and the tumor area percentage of tissue area ( $P < 0.05$ ). OPG treatment also dose-dependently decreased the number of osteoclasts/mm<sup>2</sup> tumor area ( $P < 0.01$ ).\*, Significantly different from 0 mg/kg OPG. Data represent the means  $\pm$  SE ( $n = 10$  mice/group).



Table 1 Metastatic tumor burden in soft tissue organs, as assessed by semiquantitative histomorphometry

No significant differences exist between OPG-treated and untreated groups (one-way ANOVA).

Cell line	OPG	Organ <sup>a</sup>							
		Liver	Lung	Heart	Brain	Adrenal	Ovary	Kidney	Pancreas
C26	-	0.6 ± 0.2	0.8 ± 0.2	3.2 ± 0.4	0.8 ± 0.4	1.8 ± 0.2	1.2 ± 0.5	1.4 ± 0.5	0
C26	+	1.0 ± 0.5	1.8 ± 0.5	2.4 ± 0.2	0.2 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	1.2 ± 0.2	0
MB-MDA-231	-	0	0.2 ± 0.2	2.2 ± 0.5	2.2 ± 1.4	1.6 ± 0.4	0.2 ± 0.2	0.2 ± 0.2	0.4 ± 0.2
MB-MDA-231	+	0	0.6 ± 0.4	1.4 ± 0.5	1.4 ± 0.5	1.0 ± 0.5	0	0	0

<sup>a</sup> One section of each organ from each mouse ( $n = 8-10$  mice/group) was analyzed by standard light microscopy using a scoring system described in "Materials and Methods." Data represent means  $\pm$  SD,  $n = 10$  mice/group. No metastases were observed in the spleen of any animals (data not shown).

average size of tumor nests rather than a reduction in the number of tumor nests. These data suggest that inhibiting bone resorption at the time of tumor cell inoculation does not alter the ability of these cells to hone to the skeleton. The significant decrease in the average size of skeletal tumor nests, coupled with the profound reduction in osteoclast number in OPG-treated mice, suggests that the inhibition of bone resorption has a negative influence on the growth of Colon-26 tumor cells after they localize to bone.

In the MDA-MB-231 study, we observed that OPG treatment was associated with significant reductions in both the frequency and the size of skeletal tumor nests. These changes translated into a significant reduction in total skeletal tumor burden. Examination of soft tissues demonstrated that the decreased skeletal tumor burden associated with OPG treatment did not result in a redistribution of metastatic cells to other sites for either of these cell lines. These results both compare

and contrast with those obtained in metastasis models using bisphosphonates. OPG and bisphosphonates have different mechanisms of action, but in the MDA-MB-231 tumor model, both OPG (present study) and bisphosphonates (3, 5) effectively inhibited osteolysis and also decreased skeletal tumor burden. These data support the notion that tumor-induced bone resorption may promote bone metastasis through the release of bone matrix- or bone cell-derived growth factors and cytokines (6, 24). It is also apparent from other studies that bone resorption is not the only regulator of bone metastasis. Effective inhibition of bone resorption in various studies has been associated with increased (2), decreased (3-5), or unchanged (8, 9) skeletal tumor burden. Important differences between these models, which include the class of therapeutic, the tumor and host species, as well as spontaneous *versus* experimental modes of bone metastasis, could be invoked to explain the disparate effects of inhibited bone resorption

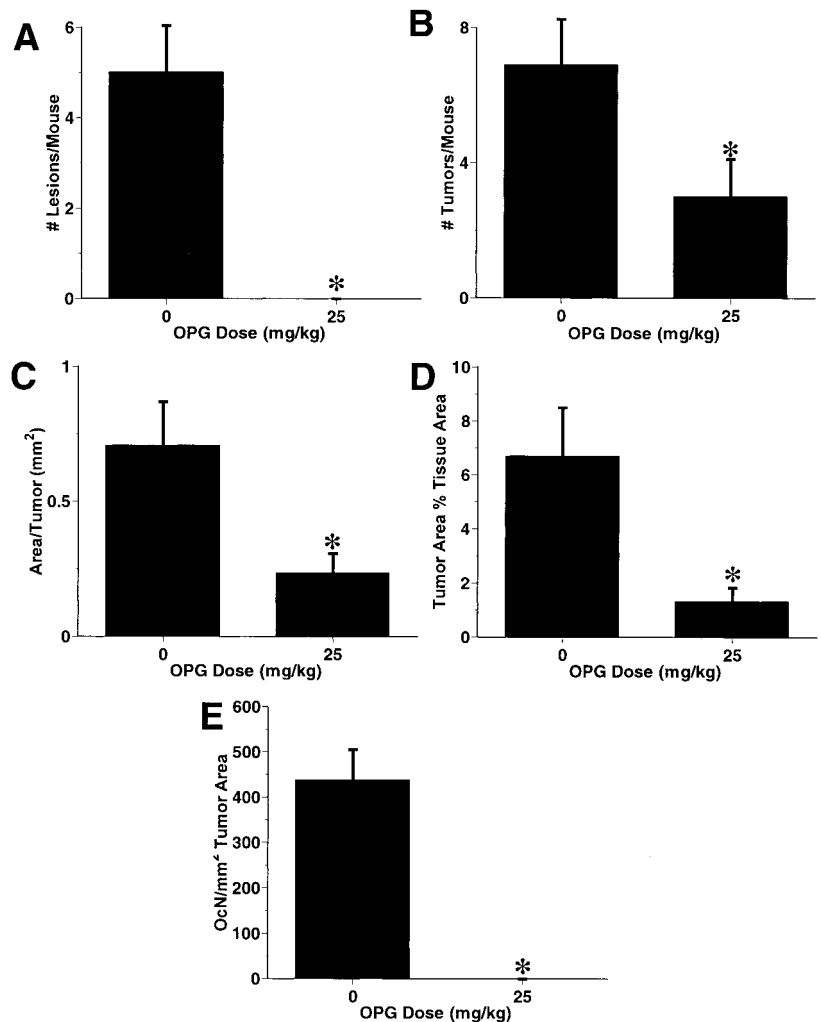


Fig. 4. Effect of OPG on osteolytic lesions and histomorphometric indices of skeletal MDA-MB-231 tumor burden. X-rays of mice were analyzed morphometrically to determine the number of osteolytic lesions in tumor-bearing mice (A). Vehicle-treated mice had an average of five lesions, whereas no lesions were detected in OPG-treated mice ( $P < 0.01$ ). The lack of osteolytic lesions precluded the analysis of the average area of the lesions. Two nonserial sections of each bone were analyzed for parameters of skeletal tumor burden. Histologically, OPG caused significant reductions in the number of skeletal tumors/mouse (B) and in the average area of bone tumors (C), which translated into a significant decrease in skeletal tumor burden (tumor area percentage of tissue area; D,  $P < 0.05$ ). In vehicle-treated mice, MDA-MB-231 tumors in bone were associated with a robust osteoclast response that was completely prevented by OPG treatment (E,  $P < 0.01$ ). \*, Significantly different than 0 mg/kg OPG. Data represent the mean  $\pm$  SE ( $n = 10$  mice/group).

on tumor burden. Despite the lack of experimental consensus, the present study adds further support to the notion that the inhibition of tumor-associated osteolysis may also decrease skeletal tumor burden.

In conclusion, OPG is an effective treatment for the prevention of tumor-associated osteolysis in models of experimental bone metastasis, using both human and murine tumor-derived cell lines. This effect was associated with and may be caused by the virtual eradication of tumor-associated osteoclasts in OPG-treated mice. The antiresorptive effect of OPG is associated with significant decreases in skeletal tumor burden with human tumor cells in immunocompromised mice and with murine tumor cells in immunocompetent mice. These beneficial effects were not associated with any changes in metastatic tumor burden in a large panel of soft tissue organs. The data collectively suggest that OPG may have clinical utility in the treatment of patients with bone metastasis.

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