Reversal of Chemotherapy-Induced Leukopenia Using Granulocyte Macrophage Colony-Stimulating Factor Promotes Bone Metastasis That Can Be Blocked with Osteoclast Inhibitors

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Abstract
Hematopoietic growth factors are used to reverse chemotherapy-induced leukopenia. However, some factors such as granulocyte macrophage colony-stimulating factor (GM-CSF) induce osteoclast-mediated bone resorption that can promote cancer growth in the bone. Accordingly, we evaluated the ability of GM-CSF to promote bone metastases of breast cancer or prostate cancer in a mouse model of chemotherapy-induced leukopenia. In this model, GM-CSF reversed cyclophosphamide-induced leukopenia but also promoted breast cancer and prostate cancer growth in the bone but not in soft tissue sites. Bone growth was associated with the induction of osteoclastogenesis, yet in the absence of tumor GM-CSF, it did not affect osteoclastogenesis. Two osteoclast inhibitors, the bisphosphonate zoledronic acid and the RANKL inhibitor osteoprotegerin, each blocked GM-CSF–induced tumor growth in the bone but did not reverse the ability of GM-CSF to reverse chemotherapy-induced leukopenia. Our findings indicate that it is possible to dissociate the bone-resorptive effects of GM-CSF, to reduce metastatic risk, from the benefits of this growth factor in reversing leukopenia caused by treatment with chemotherapy.

Introduction
Over 5% of cancer patients have chemotherapy-induced sepsis that is associated with 8.5% of all cancer deaths (1). Granulocyte macrophage colony-stimulating factor (GM-CSF) is commonly used to reverse leukopenia (2, 3). In addition to its prohematopoietic effects, GM-CSF has been shown to both increase (4, 5) and decrease osteoclastogenesis (6–8). The conflicting studies were resolved by the demonstration that GM-CSF has a biphasic effect on osteoclast induction (9). Specifically, it was shown that short-term exposure to GM-CSF promotes osteoclastogenesis, whereas long-term exposure inhibits osteoclastogenesis.

Bone metastasis is a frequent complication of cancers including breast cancer and prostate cancer (10). Both breast cancer and prostate cancer bone metastases have a bone-resorptive component (i.e., osteolytic metastases). Increased osteolytic activity promotes the development and progression of bone metastases (11). The increased osteolytic activity is due to the tumor-mediated production of proosteoclastogenic factors that induce receptor activator NFκB ligand (RANKL) expression (12). RANKL is a key inducer of osteoclastogenesis through the activation of its cognate receptor RANK that is present on osteoclast precursors (13).

Based on the observations that bone resorption promotes bone metastasis and GM-CSF induces osteoclastogenesis, it follows that GM-CSF administration to breast cancer or prostate cancer patients may induce bone resorption that promotes bone metastasis. However, the effect of GM-CSF on osteoclastogenesis in the presence of leukopenia, as occurs in patients receiving chemotherapy, is unknown. Accordingly, to recapitulate the clinical scenario, we tested if GM-CSF promotes cancer metastasis in the presence of chemotherapy-induced leukopenia in a murine model.

Materials and Methods
Cells
MDA-231-lux, T47D, and MCF-7 breast cancer cells were obtained from Dr. Stephen Ethier (Wayne State University, Detroit, MI). MDA-231-lux was established by stably transfecting MDA-231 breast cancer cells with a constitutively active promoter driving luciferase expression (14). PC-3-lux is a prostate cancer cell line that contains a constitutively active promoter driving luciferase expression (15). Cells were maintained at 37°C and 5% CO₂ in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Animal studies
All procedures were approved by the Animal Care Committee. Eight-week-old nude mice (female for breast
cancer and male for prostate cancer) were used. Recombinant murine GM-CSF (rmGM-CSF) dose was determined using a body surface area conversion program (http://www.accessdata.fda.gov/scripts/cder/onctools/animalquery.cfm) to determine that 1.4 µg per mouse is equivalent to the clinically used dose of 250 µg/m². Cyclophosphamide was administered at 3 mg per mouse through the i.p. route (16). Zoledronic acid (ZA; Novartis) was administered at 3 µg per mouse s.c. (17). Recombinant mouse osteoprotegerin (OPG)/Fc chimeric protein (R&D Systems) was administered at 2 mg/kg i.p. twice weekly (18). MDA-231-lux or PC-3-lux were injected into the left cardiac ventricle of mice as described (17, 19). This typically results in 100% of mice having tumors in the bone and 25% of total tumor in mice being at the soft tissue sites based on imaging and pathologic confirmation. To image tumor, luciferin (40 mg/mL) was injected i.p. and images were acquired 15 minutes postinjection using an IVIS Imaging System (Caliper). Soft versus bone tissue lesions were determined based on the location of luciferase-positive areas. For areas that were not clearly defined on the original image, a perpendicular image of the animal was taken. Total soft and skeletal tumor burdens per mouse were calculated using the summation of individual regions of luciferase-positive areas as described (17, 19).

For the measurement of osteoclast activity, serum tartrate-resistant acid phosphatase 5b (TRACP 5b) was quantified using mouse-specific TRACP 5b ELISA (IDS Ltd).

**Cell counts**

Blood was collected through retro-orbital puncture, and total blood counts were performed using hemocytometer. Differential counts were performed on whole blood smears stained in Giemsa. For tumor cells, an aqueous cell viability assay was used per the manufacturer’s directions (Cell Titer96 Aqueous Solution Assay, Promega). This assay measures the conversion of a tetrazolium salt (MTS) into a water-soluble formazan compound using a spectrophotometer.

**Measurement of bone lysis**

Tibiae were radiographed using a Faxitron X-Ray unit (Faxitron), were digitized, and the lytic area quantified as previously described (20). Briefly, the entire area of the lateral view of the bone is outlined to determine the total area, and the lytic areas are outlined to determine the percent lytic area. Bone mineral density (BMD) was quantified using dual-energy X-ray absorptiometry on an Eclipse peripheral Dxa Scanner using the pDEXA Sabre research software (Norland). Regions of interest were scanned at 2 mm/s and 0.1 mm × 0.1 mm resolution.

**Bone histomorphometry**

Bone samples were fixed in 10% neutral-buffered formalin at 4°C for 24 hours then dehydrated in ethanol. The bone samples were processed, stained with modified Goldner stain, and subjected to histomorphometry for osteoclast perimeter and the percent of trabecular bone volume based on the total tissue volume (BV/TV) of the tibia on a BIOQUANT system (R&B Biometrics, Inc.) as we have previously described (18). For BV/TV, the average of the right and left leg values for each animal was used.

**Bone marrow cultures for osteoclastogenesis evaluation**

To obtain bone marrow cells, tibiae were aseptically removed, ends were cut, and marrow cavity was flushed into a dish by injecting MEM into the proximal end using a 21-g needle. The bone marrow suspension was agitated to obtain a single-cell suspension; cells were washed twice, resuspended in MEM containing 10% FBS, and incubated for 24 hours (3 × 10⁵ cells/mL) in a 75-cm² flask. After 24 hours, nonadherent cells were harvested; resuspended (10⁶/mL) in MEM-FBS; and cultured in 100 µl of MEM containing 10% FBS, nonessential amino acids, 2 mmol/L L-glutamine, and sRANKL (125 ng/mL) in 96-well plates. In some instances, GM-CSF was added at 10 ng/mL as described. Cells were incubated for 14 days, and 50% of the media were refreshed every 3 day.

**Statistics**

Data were analyzed with the StatView Software (Abacus Concepts). A one-way ANOVA analysis was used with Bonferroni’s post hoc analysis for comparison between multiple groups. A Student’s t test was used for comparison between two groups. Significance was defined as a P value of <0.05.

**Results**

**Establishment of a murine model of GM-CSF rescue of chemotherapy-induced leukopenia**

To establish a model of leukopenia and GM-CSF rescue, we administered cyclophosphamide followed by rmGM-CSF 3 days postcyclophosphamide. Cyclophosphamide induced leukopenia with the nadir at 3 days (Fig. 1). Administration of rmGM-CSF at day 3 postcyclophosphamide resulted in normal leukocyte levels at 3 days after the nadir compared with 12 days for the mice that received vehicle. Administration of rmGM-CSF induced leukocytosis that normalized at 15 days postadministration. Cyclophosphamide reduced both neutrophils and monocytes, and this was reversed rapidly by the administration of rmGM-CSF. Taken together, these data show that the administration of cyclophosphamide serves as a functional model of chemotherapy-induced leukopenia that can be reversed by rmGM-CSF.

**GM-CSF promotes MDA-231 growth in the bone but not in soft tissue**

Murine and human GM-CSFs do not cross-react with the other species’ receptors (21). Thus, rmGM-CSF should have no direct effect on human breast cancer or prostate cancer cells. To confirm this, we incubated cancer cells with rmGM-CSF and found that it did not affect breast cancer or prostate cancer growth (Fig. 2A). To determine the effect...
of rmGM-CSF on breast cancer growth in vivo, MDA-231-lux were injected into the left cardiac ventricle and rmGM-CSF (or saline) was initiated. Administration of rmGM-CSF increased tumor growth in the bone, but not soft tissue, compared with vehicle administration (Fig. 2B and C). The rmGM-CSF–induced increase in tumor bone volume was associated with an increase in the number of bone but not soft tissue metastases (Fig. 2D), which reflected that all sites of skeletal disease were affected by rmGM-CSF. To determine the effect on the interaction between tumor and bone, we evaluated several parameters of bone remodeling. Specifically, radiographs were used to observe the lytic area and densitometry was used to evaluate the BMD, which is an index of how much mineralized matrix is present, and serum levels of TRACP5b that reflects overall osteoclast activity. In the absence of tumor, rmGM-CSF did not alter the lytic area, BMD, TRACP5b, or metaphyseal trabeculae (Fig. 3). Tumor alone induced osteolysis, reduced BMD, increased serum TRACP5b, and decreased metaphyseal trabeculae by ∼44%. Administration of rmGM-CSF, in the presence of tumor, increased the lytic area by ∼90%, reduced BMD compared with tumor alone, induced further increases of serum TRACP5b, and resulted in the greater loss of metaphyseal trabeculae (i.e., total of 56% versus 44%) compared with tumor alone. Taken together, these results indicate that GM-CSF administration promotes MDA-231 cell growth specifically in the bone, which is associated with increased bone-resorptive (i.e., osteoclastic) activity.
GM-CSF promotes MDA-231–induced osteoclastogenesis

To determine the role of GM-CSF directly on osteoclast activity versus tumor-induced osteoclast activity, we next determined the effect of rmGM-CSF administration on osteoclastogenesis in the context of tumor. In the absence of tumor, rmGM-CSF had no effect on osteoclast numbers (i.e., osteoclast perimeter; Fig. 4A). In contrast, tumor alone increased osteoclast perimeter, which was further increased by rmGM-CSF (Fig. 4A). To determine if GM-CSF directly affected osteoclastogenesis, we obtained marrow stromal cells from mice with or without intrafemoral MDA-231 tumor growth and incubated them with rmGM-CSF. Marrow stromal cells derived from mice with intraosseous tumor produced more osteoclasts than stromal cells derived from mice without tumor (Fig. 4B). However, administration of rmGM-CSF in vitro had no effect on the osteoclast numbers produced by marrow stromal cells derived from mice with or without tumor present in the bone (Fig. 4B). These results were consistent with the increased osteolysis and osteoclast perimeter induced by the presence of tumor; however, they seemed to conflict with the in vivo results that showed GM-CSF increased osteoclast perimeter in the presence of tumor (Fig. 4A). One possibility was that the increase of osteoclasts occurs in vivo only in the presence of tumors. To determine if GM-CSF induced an increase of osteoclast production in vivo in the presence of tumors, we injected mice with saline or MDA-231 cells, let tumors become established over 14 days, then administered GM-CSF or vehicle, and, after 24 hours, collected bone marrow cells to evaluate their osteoclastogenic potential. In the absence of tumor, GM-CSF had no effect on osteoclast numbers; however, in the presence of tumor, GM-CSF induced an increase in bone marrow-stromal–derived osteoclasts that were observed at 3 and 7 days post–GM-CSF administration (Fig. 4C and D). Taken together, these results indicate that GM-CSF promotes osteoclastogenesis indirectly with a requirement for tumor presence in vivo.

Inhibition of osteoclastogenesis blocks GM-CSF–induced MDA-231 cell growth in bone

That GM-CSF had no direct effect on breast cancer cell proliferation in vitro (Fig. 2A) suggested that it promotes breast cancer growth through an indirect mechanism in vivo. The induction of bone remodeling promotes the ability of cancers, including breast cancer and prostate cancer, to grow in bone (17, 22, 23). Accordingly, we assessed if blocking GM-CSF–induced osteoclastogenesis would prevent GM-CSF–induced breast cancer growth in bone. To accomplish this, the bisphosphonate ZA or vehicle was administered. After 2 weeks, MDA-231 cells were injected into the left cardiac ventricle, and GM-CSF or vehicle was administered 3 days later. Tumor burden was monitored over 6 weeks. As observed earlier, GM-CSF administration promoted tumor

Figure 2. GM-CSF promotes MDA-231-lux growth in the bone but not in soft tissue. A, the indicated cancer cells (plated at 5 × 10³ cells/100 mL/well in 96-well plates) were treated with rmGM-CSF (or saline vehicle). After 48 h, cell numbers were quantified using a cell viability assay that measures the conversion of a tetrazolium salt (MTS) into a water-soluble formazan compound. Results are from two experiments. Columns, mean; bars, SD. B to D, MDA-231-lux cells (1 × 10⁵ cells in 10 μL of PBS) were injected into the left cardiac ventricle, and either saline vehicle or GM-CSF (1.4 μg/mouse i.p.) was initiated. Tumor growth was monitored over every 2 wk using BLI (n = 5 mice per group). B, images of mice with tumors. Color indicates presence of tumor. C, BLI. Results are reported as mean relative light units (RLU) ± SD for either bone (left graph) or soft tissue (right graph). *, P < 0.05 versus vehicle at the same time point. D, 6 wk posttumor injection, the number of soft tissue or bone metastases detected using BLI were counted. Columns, mean number of metastases/mouse; bars, SD. #, P < 0.05 versus bone metastases in vehicle-treated mice.
growth in the bone (Fig. 5A). Pretreatment with ZA inhibited the GM-CSF–induced tumor growth. ZA alone also inhibited tumor growth. There was no difference in the levels of tumor growth between ZA alone compared with ZA in the presence of GM-CSF. Finally, ZA had no effect on MDA-231 growth in soft tissue in the presence or absence of GM-CSF (Fig. 5A). Taken together, these data indicate that ZA blocks the GM-CSF effect through a mechanism that is specific to the bone microenvironment and is consistent with the concept that GM-CSF mediates its protumorigenic effects in the bone through the induction of osteoclastogenesis.

**ZA can block GM-CSF–induced intraosseous MDA-231 growth without blocking the ability of GM-CSF to reverse leukopenia**

GM-CSF is administered during periods of chemotherapy-induced leukopenia that occurs secondary to the destruction of cycling progenitor marrow cells. It is unclear if this destruction also affects osteoclast production. To explore this uncertainty and recapitulate the clinical situation, we assessed if GM-CSF affects cancer growth in the bone in the presence of chemotherapy-induced leukopenia. Mice were treated with ZA (or vehicle), and 2 weeks later, MDA-231 cancer cells were injected into the left cardiac ventricle and

**Figure 3.** GM-CSF administration increases tumor-induced osteolysis. MDA-231-lux cells were injected (1 × 10⁶ cells/10 μL of PBS) into the left cardiac ventricle. Mice were treated with either saline or GM-CSF (1.4 μg/mouse i.p.). Tumor growth was monitored over 6 wk and mice were sacrificed (n = 5 mice/group). A, radiographs from different treatment groups. Note the black areas of osteolysis in the proximal tibiae. Radiographs were digitized, and the osteolytic area proportion of the tibia was calculated and is reported as mean ± SD% osteolytic area. *, P < 0.05 versus tumor alone. B, top, BMD of the proximal tibia was quantified. Columns, mean BMD; bars, SD. *, P < 0.05 versus no tumor. #, P < 0.05 versus vehicle with tumor. Bottom, serum tartrate-resistant acid phosphatase 5b (TRACP5b) was quantified using ELISA. Columns, mean enzyme activity (U/L); bars, SD. P < 0.05 versus no tumor. #, P < 0.05 versus vehicle with tumor. C, histologic sections of proximal tibiae. Arrows, metaphyseal trabeculae; T, tumor; M, normal marrow. Note the loss of trabeculae in the GM-CSF− group compared with the no tumor groups and the extensive loss of trabeculae in the tumor/GM-CSF+ group compared with the tumor/GM-CSF− group. D, histomorphometric measurement of trabecular bone. Trabecular bone volume (BV) and total tissue volume (TV) were averaged between both the tibia of each mouse and were reported as BV/TV (%) ± SD. *, P < 0.05 versus no tumor and no GM-CSF; #, P < 0.05 versus tumor and no GM-CSF.
cyclophosphamide was administered at the same time. Three days after the administration of cyclophosphamide, GM-CSF (or vehicle) was administered and animals were followed over 6 weeks using bioluminescence imaging (BLI). Even in the presence of cyclophosphamide, GM-CSF induced cancer growth in the bone and ZA inhibited this (Fig. 5B). To confirm the presence of leukopenia, we performed whole blood cell counts (WBC). As anticipated, leukopenia was present 3 days postcyclophosphamide administration. GM-CSF reversed the leukopenia within 6 days after its administration, whereas the mice that did not receive GM-CSF still had moderate leukopenia at this time point (neutropenia paralleled the total WBC; data not shown; Fig. 5C). By day 21, all groups had normal WBCs. To determine if the presence of osteolytic activity reflected the presence of tumor and GM-CSF in this clinical model, we measured osteoclast perimeter and TRACP5b. Even in the presence of the chemotherapy-induced leukopenia that occurred at day 3, at which time GM-CSF was administered, which resolved the leukopenia by day 9 and was associated with increased osteoclast perimeter at week 6. Furthermore, treatment with ZA before the administration of GM-CSF did not inhibit GM-CSF’s restoration of the WBC but did inhibit the GM-CSF–induced tumor growth and increase of osteoclast perimeter.

**ZA or OPG can block GM-CSF–induced intraosseous PC-3-lux growth without blocking the ability of GM-CSF to reverse leukopenia**

To determine if these results were specific to breast cancer or relevant to other cancers, we used PC-3 prostate cancer cells. Furthermore, to evaluate if the inhibitory effects on tumor growth were specific to ZA, we used an additional method to inhibit osteoclast activity. Specifically, we treated mice bearing MDA-231 with either vehicle or GM-CSF treatment. Arrows, osteoclast-like cells.

![Figure 4. GM-CSF requires the presence of tumor to promote osteolysis in vivo. A, MDA-231-lux cells were injected (1 × 10^6 cells/10 μL of PBS) into the left cardiac ventricle of mice, and either saline vehicle or GM-CSF (1.4 μg/mouse I.P.) was initiated. Tumor growth was monitored over a period of 6 wk. Mice were sacrificed, and tibiae were collected and subjected to histomorphometry for osteoclast perimeter. (n = 5/group). Columns, mean osteoclast perimeter; bars, SD. *, P < 0.05 versus no tumor. #, P < 0.05 versus vehicle with tumor. B, MDA-231-lux cells (or saline vehicle) were injected (1 × 10^5 cells/10 μL of PBS) into the proximal tibia. After 2 wk, mice were sacrificed and tibiae were flushed to obtain marrow cells. Marrow cells were plated, and either vehicle or rmGM-CSF (10 ng/mL) was added to the media. After 14 d, cells were stained with TRAP and multinucleated TRAP+ cells were quantified. Columns, mean osteoclasts per well; bars, SD. *, P < 0.05 versus no tumor. C and D, MDA-231-lux cells (or saline vehicle) were injected (1 × 10^5 cells in 10 μL of PBS) into the proximal tibia. After 2 wk, mice were treated with either saline vehicle or GM-CSF (1.4 μg/mouse i.p.) and marrow was collected by flushing the tibiae at the indicated time points. Marrows were subjected to culturing and osteoclasts were identified as in B. Points, mean osteoclasts per well; bars, SD. *, P < 0.05 versus no tumor; #, P < 0.05 versus MDA-231 and vehicle. D, demonstration of osteoclasts in cultures from mice bearing MDA-231 with either vehicle or GM-CSF treatment. Arrows, osteoclast-like cells.
an additional group with an inhibitor of RANKL, OPG (24). We and others have shown that OPG blocks tumor-induced osteoclastogenesis mediated by RANKL (18, 25). Mice were treated with ZA (1×), OPG (twice weekly for a total of 4 wk), or vehicle. Two weeks after the initiation of drugs, PC-3-lux prostate cancer cells were injected into the left cardiac ventricle and cyclophosphamide was administered. Three days postadministration of cyclophosphamide, GM-CSF (or vehicle) was administered and tumor burden was monitored over 9 weeks. Similar to the results for the MDA-231 breast

Figure 5. ZA can block GM-CSF–induced intraosseous MDA-231 growth without blocking the ability of GM-CSF to reverse leukopenia. A, ZA (3 μg/mouse s.c.) or saline vehicle was administered. Two weeks after the initiation of ZA, MDA-231-lux cells were injected (1 × 10⁶ cells in 10 μL of PBS) into the left cardiac ventricle. Mice were treated with either saline vehicle or GM-CSF (1.4 μg/mouse i.p.). Tumor growth was monitored over a period of 6 wk using in vivo bioluminescence every 2 wk. (n = 5/group). Points, mean RLUs for either bone or soft tissue; bars, SD. *, P < 0.05 versus GM-CSF– and ZA–; #, P < 0.05 versus GM-CSF+ and ZA–. B to D, ZA (3 μg/mouse s.c. once) or saline vehicle were administered to mice. Two weeks after the initiation of ZA, cyclophosphamide (or saline vehicle) was administered (6 mg/mouse i.p.) and MDA-231-lux cells were injected (1 × 10⁶ cells in 10 μL of PBS) into the left cardiac ventricle. Three days after the injection of tumors into mice, they were treated with either saline vehicle or GM-CSF (1.4 μg/mouse i.p.) and tumor growth was monitored using bioluminescence every 2 wk. There were five mice per treatment group. Points, mean RLUs for tumor growth in the bone; bars, SD. *, P < 0.05 versus GM-CSF– and ZA–; #, P < 0.05 versus GM-CSF+ and ZA–. C, whole blood was obtained at 0, 3, 9, 21, and 42 d postadministration of cyclophosphamide using retro-orbital puncture. Total WBC counts were performed. Points, mean; bars, SD. *, P < 0.05 versus GM-CSF– for each respective drug. D, at the end of the study, tibiae were harvested and subjected to histomorphometry for osteoclast perimeter (columns, mean osteoclast perimeter; bars, SD) and serum tartrate-resistant acid phosphatase 5b [TRACP5b; quantified using ELISA; columns, mean enzyme activity (U/L); bars, SD]. *, P < 0.05 versus ZA–; #, P < 0.05 versus GM-CSF– and ZA–.
cancer cell line, in the presence of cyclophosphamide, GM-CSF induced cancer growth in the bone (Fig. 6A, see GM-CSF−ZA−/OPG− versus GM-CSF+ ZA−). As previously observed for MDA-231, ZA inhibited GM-CSF−induced cancer growth (Fig. 6A, see GM-CSF+ ZA−/OPG− versus GM-CSF+ ZA+). Similarly, OPG had a potent inhibitory effect on GM-CSF−induced cancer growth as did ZA (Fig. 6A, see GM-CSF+ ZA−/OPG− versus GM-CSF− OPG+). GM-CSF reversed the cyclophosphamide-induced leukopenia in the presence of PC-3-lux, and ZA or OPG with kinetics similar to that observed for MDA-231 (Fig. 6B). These results indicate that both ZA and OPG can inhibit GM-CSF−mediated tumor growth in bone whereas having no effect on the ability of GM-CSF to reverse leukopenia. To determine if the presence of osteolytic activity reflected the presence of tumor and GM-CSF in this PC-3 prostate cancer model of the clinical scenario, we measured osteoclast perimeter and TRACP5b. Even in the presence of the chemotherapy-induced leukopenia that occurs at day 3, at the end of the study at 9 weeks, administration of GM-CSF at day 3 induced an increase in osteoclast perimeter that was observed as late as 9 weeks. Administration of ZA or OPG blocked the GM-CSF induction of osteoclast perimeter (Fig. 6C). The induction of osteoclast activity by GM-CSF and its inhibition by ZA or OPG was reflected by serum TRACP5b levels (Fig. 6D).

**Discussion**

In the current study, we uncovered a previously unidentified risk associated with the administration of GM-CSF for the treatment of chemotherapy-induced leukopenia. Specifically, our work shows that the administration of GM-CSF to reverse chemotherapy-induced leukopenia promotes the growth of breast cancer and prostate cancer in the bone through the induction of osteoclast activity. Furthermore, we identified that the administration of an osteoclast inhibitor in conjunction

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**Figure 6.** ZA or OPG can block GM-CSF−induced intraosseous PC-3-lux growth. ZA (3 μg/mouse s.c. once), OPG (2 mg/kg i.p. twice weekly), or saline vehicle were administered to mice. Two weeks after the initiation of ZA or OPG, cyclophosphamide (6 mg/mouse) or an equivalent volume of saline vehicle was administered i.p., immediately followed by injection of PC-3-lux cells (1 × 10^6 cells in 10 μL of PBS) into the left cardiac ventricle. Three days after injection of PC-3-lux cells into mice, they were treated with either GM-CSF (1.4 μg/mouse) or an equivalent volume of saline vehicle i.p., and tumor growth was monitored over a period of 9 wk using bioluminescence every three weeks (n = 5/group), A, results are reported as mean RLU ± SD for tumor growth in bone. *, P < 0.05 versus GM-CSF− and ZA− /OPG−; #, P < 0.05 versus GM-CSF+ and ZA− /OPG−. B, whole blood was obtained at 0, 3, and 21 d postadministration of cyclophosphamide. WBCs were performed. Points, mean; bars, SD. *, P < 0.05 versus GM-CSF− for each respective drug. C, at the end of the study, tibiae were harvested and subjected to histomorphometry for osteoclast perimeter. Columns, mean osteoclast perimeter; bars, SD. *, P < 0.05 versus no drug; #, P < 0.05 versus GM-CSF− and no drug. D, serum tartrate–resistant acid phosphatase 5b (TRACP5b) was quantified using ELISA. Columns, mean enzyme activity (U/L); bars, SD. *, P < 0.05 versus no drug; #, P < 0.05 versus GM-CSF− and no drug.
with GM-CSF diminishes the induction of metastasis. The ability to dissociate the prometastatic osteoclastogenic activity from the ability to reverse leukopenia provides a potential therapeutic approach to diminish the morbidity associated with chemotherapy-induced leukopenia without adding additional therapy-induced risk for tumor progression.

Granulocyte colony-stimulating factor (G-CSF) and GM-CSF are frequently administered to reverse chemotherapy-induced neutropenia (26, 27). G-CSF has osteoclastic effects and prometastatic effects in the bone (28); however, the effects of GM-CSF on osteoclastogenesis in the context of tumor were less known. Several studies have shown that GM-CSF inhibits osteoclastogenesis in vitro, whereas other reports have shown that GM-CSF stimulates osteoclastogenesis (5, 9, 29). Of particular note is that short-term administration of GM-CSF, similar to how it would be used in the clinical scenario, was shown to induce osteoclasts in a murine model (29). One potential reason for the conflicting results was that the latter studies were performed in the face of an inflammatory environment in which a variety of cytokines are present, which could confer a proosteoclastogenic effect on GM-CSF. We have previously reported that some breast cancer cells produce GM-CSF that promotes bone metastasis through the induction of osteoclastogenesis (30), which led to the supposition that the administration of GM-CSF may promote the same prometastatic osteoclastogenic activity (31). In the current study, GM-CSF was administered in the context of cancer. This suggests that the cancer cells themselves provided factors that conferred proosteoclastogenic activity on GM-CSF. Another possibility is that the tumor microenvironment itself may be proinflammatory through the stromal production of cytokines and migration of leukocytes into the tumor (32). Thus, the proinflammatory tumor microenvironment may have conferred proosteoclastogenic activity on GM-CSF.

In light of the observation that increased bone resorption promotes bone metastasis (33) and the possibility that GM-CSF induces bone resorption through osteoclastogenesis, we hypothesized that administration of GM-CSF during chemotherapy-induced leukopenia would promote bone metastasis. We showed that although murine GM-CSF had no direct effect on human cancer cells, administration of mGM-CSF promoted growth of cancer in the bone but not in soft tissue. These results are consistent with the observations that (a) ZA had no effect on prostate cancer growth in soft tissues whereas it inhibited growth in bone in vivo (34), and (b) ZA inhibited tumor-induced osteolysis and is directly proportional to tumor burden (17). This indicates that GM-CSF promotes receptivity of the bone but not soft tissue microenvironments for cancer establishment. This occurred in a background of chemotherapy-induced leukopenia, which makes it clinically relevant. This suggests that clinical administration of GM-CSF to reverse chemotherapy-induced leukopenia may ultimately promote bone metastasis in patients. Our results are consistent with the observation that G-CSF, a known inducer of osteoclast activity, was shown to induce tumor growth in the bone in a murine model through a dependency on osteoclasts (28).

Several lines of evidence indicated that GM-CSF promoted osteoclast activity in our model system including increased serum TRACP, radiographic osteolysis, osteoclast perimeter, and decreased BMD. The induction of osteoclast activity seemed critical for the development of tumor growth in the bone. That ZA inhibited both bone resorption and tumor growth in the bone, but not in soft tissue, indicated that ZA indirectly inhibited tumor growth through the bone microenvironment. This seems to be in conflict with the previous findings that ZA directly induced apoptosis in breast cancer cells (35, 36) and prostate cancer cells (37, 38) in vitro. However, the direct cytotoxic effects of bisphosphonates is controversial as several studies indicate that bisphosphonates have no proapoptotic effect (39) or were inconclusive (40) and the lack of antitumor effects upon bisphosphonates administration clinically (33). That OPG inhibited GM-CSF–induced tumor growth similarly to ZA provides further support that the anti-tumor effect is mediated through the bone microenvironment as opposed to directly by ZA. In support of this possibility is the previous report that OPG has no direct effect on prostate cancer cell growth in vitro or at soft tissue sites in vivo (18).

In summary, we have shown that the administration of GM-CSF in a model of chemotherapy-induced leukopenia promotes the establishment of cancer growth in the bone. Furthermore, we determined that the use of osteoclast inhibitors were able to dissociate the osteoclastic activity from the proleukocytic activity, allowing for the rapid restoration of leukocyte numbers while blocking the GM-CSF–induced growth of tumor in the bone. These findings suggest that careful consideration should be given for the potential effect on tumor progression that clinical use of GM-CSF (and presumably G-CSF) for chemotherapy-induced leukopenia could have. Furthermore, this study indicates that methods to reverse leukopenia without promoting osteoclastogenesis should be developed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Reversal of Chemotherapy-Induced Leukopenia Using Granulocyte Macrophage Colony-Stimulating Factor Promotes Bone Metastasis That Can Be Blocked with Osteoclast Inhibitors

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