Properties of Bisphosphonates in the 13762 Rat Mammary Carcinoma Model of Tumor-Induced Bone Resorption

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ABSTRACT

Bone metastasis from primary tumors is a clinically important complication of neoplastic progression. The role of parathyroid hormone-related protein (PTHrP) and transforming growth factor (TGF)-β1 in this process has been clearly established. The current study describes an in vivo model of 13762 rat mammary carcinoma tumor cell-induced osteolysis in which PTHrP and TGF-β1 expression is observed. Exposure of in vitro-cultured 13762 cells to doxorubicin, cis-platinum, carboplatin, methotrexate, 5-fluorouracil, paclitaxel, alendronate, risedronate, or pamidronate for 72 h resulted in varying effects on cell proliferation (IC₅₀ values of 0.005, 0.4, 1.9, >40, 17.9, 0.003, >40, >40, and 33.6 μM, respectively). Tumor cells were implanted into the intramedullary space of the proximal tibia of rats, and the time course of tumor progression was evaluated using radiographic and microcomputed tomography scanning techniques. Trabecular bone mineral density, cortical bone mineral density, and whole bone mineral density were measured (in mg/cm³). In untreated animals, radiographic evidence of osteolysis was evident 7 days after implantation. Trabecular bone mineral density and whole bone mineral density were significantly decreased by 21 days after implantation (48% and 26%, respectively). Bisphosphonates showed broad protective activity against tumor-driven osteolysis, Immunohis- tochemical evaluation of s.c. and intratibially implanted cells demonstrated the expression of PTHrP and TGF-β1. The results of this study demonstrate the ability of 13762 rat mammary carcinoma cells to elicit a measurable osteolysis and that bisphosphonates inhibit the tumor-induced bone resorption in this model.

INTRODUCTION

Tumor-induced osteolysis is an important contributor to morbidity and mortality among cancer patients. Tumor metastasis to bone results in the establishment of an additional tumor burden distant from the originating lesion. This additional tumor burden leads to pain and associated bone pathology. Metastatic breast cancer, multiple myeloma, and renal cell carcinoma are commonly identified tumors that promote bone resorption (1–4). Therapeutic approaches have directly targeted tumor growth (by bisphosphonates or other agents) or osteoclastic activity by the use of bisphosphonates (5–9). The interaction between neoplastic tissue and bone is complex, involving numerous mediators (10–17). Currently accepted paradigms of tumor-induced osteolysis have identified neoplastic cell interaction with the osteoclast bone cells as a critical component of this process. These models point to the pivotal role of TGF-β1 and PTHrP (18–20). The role of PTHrP in osteolytic bone resorption has been studied extensively (12, 21–24). Initial effects attributed to PTHrP included humoral hypercalcemia of malignancy, which was observed in a number of cancer patients (25). PTHrP, which is produced by the tumor cells, stimulates osteoblasts to produce factors (e.g., TGF-β1) that further stimulate osteoclast differentiation and bone resorption. In addition to the direct effects of PTHrP on bone resorption, the effects of factors released from bone as a result of osteoclastic bone resorption. One factor is TGF-β1, a multifunctional cytokine that is stored in bone, which is released and activated by osteoclastic bone resorption (26–29). TGF-β1 has been shown to influence many cellular processes including tissue development, cell proliferation, and matrix deposition (30, 31). In the metastatic bone environment, the released TGF-β1 can serve as a positive feedback on the tumor cells to induce further production of PTHrP (32–34) and further increase osteoclast differentiation mediated through osteoblasts.

In our study, we describe the use of an intraosseous model involving the 13762 syngeneic rat mammary carcinoma tumor line to induce osteolytic bone resorption. This model is consistent with our current understanding of tumor-bone interactions at a metastatic site, where tumor cells interact with osteoclasts. Tumor cells were locally implanted into the intramedullary space in the proximal tibiae of female Fisher 344 rats. The tumors and resulting bone destruction were evaluated using © 2003 American Association for Cancer Research.

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The abbreviations used are: TGF, transforming growth factor; TBMD, trabecular bone mineral density; CBMD, cortical bone mineral density; WBMD, whole bone mineral density. PTHrP, parathyroid hormone-related protein; MicroCT, microcomputed tomography.
radiographic and computed tomography scanning modalities. Imaging was able to visually demonstrate and quantify the local destruction of trabecular, cortical, and whole bone by the implanted tumor. Tumor-implanted animals were evaluated for their response to bisphosphonate therapy. In addition to the local osteolytic response at the site of tumor implantation, the animals also demonstrated significant tumor seeding of the lungs, leading to pulmonary metastases. To compare the expression of PTHrP and TGF-β1 when the tumor was implanted in different sites, immunohistochemical evaluation was performed. This evaluation confirmed a similar expression pattern regardless of tumor implant site. The tumor cells were also evaluated for their in vitro sensitivity to standard chemotherapeutic agents and bisphosphonates.

MATERIALS AND METHODS

Cell Line

The 13762 rat mammary carcinoma line was initially developed by Segaloff (35). The line was established in Fisher 344 female rats by administration of 7,12-dimethylbenz(a)anthracene. The 13762 line is a spontaneously metastatic syngeneic rat tumor, which has been extensively characterized for both its in vitro and in vivo growth (36–42). The 13762 rat mammary carcinoma cells were grown as a monolayer culture in 10% fetal bovine serum supplemented DMEM (Life Technologies, Inc., Grand Island, NY). The medium was also supplemented with 1% penicillin-streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, and 1% sodium bicarbonate. Cells were routinely maintained under a humidified, 5% CO2 atmosphere.

Cell Proliferation Assay

13762 rat mammary carcinoma cells were plated at a concentration of 1.0 × 10⁴ cells/well in 96-well plates. The cells were exposed to the following agents in complete media for 72 h: risedronate; pamidronate; alendronate; doxorubicin; cisplatinum; carboplatin; methotrexate; 5-fluorouracil; gemcitabine; vincristine; and paclitaxel (0.01 μM). The 13762/OVA line was established in Fisher 344 rats (Taconic Farms, Germantown, PA) weighing between 140 and 160 g were used. Animals and Lilly Research Laboratories Institutional Animal Care and Use Committee guidelines. Animals were maintained throughout the study. All animals were housed and handled in accordance with The Guide for the Care and Use of Laboratory Animals and Lilly Research Laboratories Institutional Animal Care and Use Committee guidelines. Animals were maintained in microisolator cages in temperature- and light-controlled rooms (12-h cycle). Animals had access to food and water ad libitum.

Tumor Implantation. 13762 cells were grown to 80% confluence, harvested using 0.25% Trypsin-EDTA (Life Technologies, Inc.), counted for cell viability using a trypan blue exclusion assay, and then resuspended in serum-free media for intratibial or s.c. implantation. For intratibial tumor cell implantation, the rats were sedated using acepromazine maleate (Boehringer Ingelheim, St. Joseph, MO) at a dose of 2.5 mg/kg i.p. Anesthesia was induced and maintained with 2–3% isoflurane/100% oxygen. The stifle and proximal tibial region were clipped and surgically prepared. Using a 25-gauge needle, the proximal tibia was slowly penetrated via a medial approach to the bone.
The approach required a distal-proximal trajectory for the needle to deliver the cells into the intramedullary space. A total of 2.0 x 10^7 cells suspended in plain DMEM (final volume, 50 μl) were implanted into the tibial intramedullary space. Animals were monitored through the recovery period and returned to their cages.

Radiology. On a weekly basis, over a period of 4 weeks, animals were anesthetized using 2.5% isoflurane/100% oxygen and radiographed using a Faxitron unit (Faxitron X-Ray, Inc., Wheeling, IL). Animals were placed in ventral recumbancy, and radiographs were captured using Green light-sensitive 3M SE+ Veterinary X-ray film in 3M Asymetrix Fast Detail Green Light Emitting Screens. (3M, St. Paul, MN).

Tissue and Blood Collection. At the end of each weekly radiography session, five tumor-bearing and five non-tumor-bearing animals were euthanized via CO2 asphyxiation. Tibiae, lungs, blood serum, and plasma were harvested from each animal. Serum was separated from blood collected in additive-free tubes. Tibiae were fixed in 10% buffered formalin for 24 h and then stored in 70% ethanol until scanned. For immunohistochemical examination, tibiae were decalcified over a 24–48 h period (Decalcifier I; Surgipath, Richmond, IL) before processing. At euthanasia, the lungs were perfused with 10% buffered formalin, kept in 10% formalin for 24 h, and then stored in 70% ethanol.

Drug Treatment. A similarly tumor-implanted cohort of rats underwent a random assignment (5 days after implantation) into the following groups: untreated tumor-bearing controls; pamidronate (80 μg/kg/day); risedronate (80 μg/kg/day); and alendronate (10, 1.0, 0.1, and 0.01 μg/kg/day). The drugs were dissolved in 0.9% sterile saline and injected s.c. (daily on days 1–48) period. Age- and sex-matched normal non-tumor-bearing controls were similarly housed. These animals were radiographically evaluated on a weekly basis after tumor implantation for 4 weeks. Controls were similarly housed. These animals were radiographed using a Faxitron unit in 3M Asymetrix Fast Detail Green Light Emitting Screens. (3M, St. Paul, MN).

MicroCT Analysis of Rat Tibiae. Right proximal tibiae from rats were scanned with an MS-8 Small Specimen MicroCT Scanner (Enhanced Vision Systems Corporation, Ontario, Canada). This scanner uses cone-beam geometry to acquire the projection images for the entire volume by rotating through 180° plus the cone angle. The use of an area detector with 1:1 pitch allows the reconstruction of isotropic voxels. In this study, the voxel dimension was 22.6 μm. The scanner settings used were as follows: tube voltage = 55 kV; tube current = 141 μA; exposure time/projection = 1 s; gain on Hamamatsu detector = 63; number of consecutive frames integrated by the Hamamatsu detector array = 2; angle increment between views = 0.749°; and number of views = 262. The bones were scanned in a 50% ethanol/saline solution, and an SB3 fanthom block was included in each scan. SB3 is a bone standard that mimics the X-ray attenuation properties of cortical bone. Each reconstructed volume was calibrated using the gray level values of ethanol/saline and SB3. The slice 3 mm from the growth plate was located by slicing through the volume to find the edge of the growth plate and then moving 133 slices from there. All analyses were derived from this slice. A semiautomated deformable boundary technique was used to detect the inner and outer cortical wall, allowing analysis of the trabecular region alone, the cortical region alone, and the whole bone slice. For these three regions, the bone mineral density (mg/cm^3) was calculated. Statistical differences between groups were compared using one-way ANOVA with Tukey’s multiple comparison test.
RESULTS

Cell Proliferation Assay. 13762 rat mammary carcinoma cell proliferation was not affected by exposure to alendronate, risedronate, or methotrexate. Pamidronate inhibited 13762 proliferation (IC$_{50}=33.62$). When exposed to doxorubicin, cis-platinum, carboplatin, 5-fluorouracil, gemcitabine, or paclitaxel, the tumor cells were growth inhibited. These agents achieved IC$_{50}$ values of 0.005, 0.38, 1.92, 17.91, 0.007, and 0.003, respectively.

Growth Factor Production. Evaluation of cytokine release into cell culture medium from 13762 cells maintained for 24 h in serum-free medium demonstrated that tumor necrosis factor $\alpha$, vascular endothelial growth factor, and basic fibroblast growth factor were undetectable in the media. The TGF-$\beta_1$ concentration in the medium was 1.35 ng/10$^6$ cells. Plasma TGF-$\beta_1$ levels from non-tumor-bearing and tumor-bearing rats harvested on day 28 after implantation ($n=5$) were an average concentration of 15.98 and 24.98 ng/ml, respectively. The difference between both groups was not statistically significant.

Imaging Studies. Radiographic evidence of changes consistent with tumor-induced osteolysis was evident 7 days after tumor implantation. On radiographs, the proximal tibiae show radiolucent lesions at the site of tumor implantation. The lesions increase in size in subsequent weeks throughout the experimental period (Fig. 1). MicroCT scanning of the tibia detected TBMD loss at day 21 after implantation (Fig. 2). For tumor-implanted bones, the mean TBMD was 380.5, 382.6, and 207.8 mg/cm$^3$ on days 7, 14, and 21, respectively. TBMD at day 21 represents a 48% reduction over non-tumor-bearing controls. CBMD was 1204.5, 1232.5, and 1227.4 mg/cm$^3$ on days 7, 14, and 21, respectively. WBMD was 703.0, 701.7, and 548.3 mg/cm$^3$ on days 7, 14, and 21, respectively. This represents a 26% reduction in total WBMD over non-tumor-bearing control animals (Fig. 3). Tumor-bearing animals at day 28 after implantation did not offer enough mineral content in any of the three parameters tested to adequately quantify the available bone density.

Alendronate (10 $\mu$g/kg, days 5–21) preserved TBMD to a level of 60% ($P<0.05$) of the untreated non-tumor-bearing animals (Fig. 4). At 1, 0.1, or 0.01 $\mu$g/kg (daily on days 5–21), there was no statistically significant difference in TBMD between treated and tumor-implanted animals. Pamidronate (80 $\mu$g/kg, days 5–21) showed a 52% protection of TBMD when compared with the untreated non-tumor-bearing control. Risedronate (80 $\mu$g/kg, days 5–21) was able to maintain TBMD at a level of 93% when compared with non-tumor-bearing animals. This effect was significant ($P<0.001$) when compared with tumor-implanted untreated rats. When WBMD was evaluated, alendronate-treated animals (10 $\mu$g/kg, days 5–21) showed a 52% protection of TBMD when compared with the untreated non-tumor-bearing control. Risedronate (80 $\mu$g/kg, days 5–21) was able to maintain TBMD at a level of 93% when compared with non-tumor-bearing animals. This effect was significant ($P<0.001$) when compared with tumor-implanted untreated rats. This level of mineral density protection was statistically significant ($P<0.001$) when compared with tumor-implanted untreated rats. The use of bisphosphonate therapy in this model was able to demonstrate preservation of both TBMD and WBMD. CBMD remained unchanged for all groups.

Clinical Pathology. Representative animals from the studies ($n=3$) were evaluated for total protein, alkaline phosphatase, alanine aminotransferase, blood urea nitrogen, creatinine, phosphorus, glucose, and Ca$^{2+}$. After evaluation of these
parameters, no clinically relevant changes were noted across all groups.

**Histopathology.** Tumors growing in several anatomical locations were evaluated for the expression of TGF-β1 and PTHrP. Tumors grown s.c., intratibially or as a result of either hematogenous or lymphatic spread, consistently demonstrated the expression of both TGF-β1 and PTHrP (Fig. 5). When grown in vivo, the tumor demonstrates an aggressive growth pattern, and the tumor tissue, regardless of anatomical site, shows broad areas of nondifferentiation as well as localized zones of glandular differentiation consistent with an epithelial tumor of mammary origin. Gross metastatic dissemination to the lungs was evident as early as day 14 and progressed until day 28 (Fig. 6).

**DISCUSSION**

The tumor-bone microenvironment is a complex system influenced by many factors and cell types. In this study, we used several techniques to characterize the behavior of the 13762 rat mammary carcinoma cells as a model of tumor-induced osteolysis, which is a complex pathology. A number of important mediators of the breast cancer-induced osteolytic process have been described (21, 44) and include PTHrP and TGF-β1, which play major roles in the tumor-bone relationship. Several in vivo models have been described that attempt to recapitulate the effects of tumors on bone (45–52), and all of them add to our understanding of complex tumor-bone interactions. Using immunohistochemical techniques, we demonstrate that the 13762 mammary carcinoma cells express both PTHrP and TGF-β1 when grown as s.c., intratibial or pulmonary nodules. Establishment of the presence of these mediators in the tumor-bone microenvironment allows for the characterization of the mechanistic pathways in this model. Although it is generally accepted that the model of tumor-induced bone lysis is mechanistically active due to the secretion of PTHrP by the tumor, which in turn activates osteoclastic resorption resulting in the release of TGF-β1, our model also demonstrates an active production of TGF-β1 by the tumor tissue itself. At this point, the possibility cannot be dismissed that TGF-β1 is being produced locally by the tumor and serves in an autocrine fashion, thus promoting the production of PTHrP.

When grown in cell culture and exposed to several routinely used chemotherapeutic agents, 13762 cells demonstrate sensitivity to many standard chemotherapeutic agents. In contrast, the cell line did not show biologically significant in vitro sensitivity toward alendronate, risedronate, pamidronate, or methotrexate when exposed for up to 72 h at concentrations of up to 40 μM. The relatively low activity of these agents against a mammary carcinoma is consistent with the relatively low cytotoxic profile of bisphosphonates when compared with more traditional cytotoxic agents. Generally, methotrexate is not considered an effective agent against breast tumors. The lack of cell-based cytotoxic activity of the bisphosphonates against 13762 cells in culture was not predictive of the in vivo results obtained. When the tumor-implanted rats were treated with bisphosphonates, significant bone preservation was maintained. In principle, this effect can be attributed to the direct modification of osteoclastic activity by the bisphosphonates. Although we have not excluded the possibility of a direct effect on the implanted tumor cells by bisphosphonates, the antiresorptive activity observed in this study is consistent with the more multifaceted effects of bisphosphonates on bone turnover and directly on osteoclast activity (53–56). Previous work has demonstrated elevated concentrations of bisphosphonates associated with osteoclastic surfaces in vivo (57). This localization is limited to the active resorption space. In the 13762 model of
tumor-driven osteoclastic resorption, it is likely that a similar localization occurs. This localization, by nature of the histological setting, does not appear to be directly adjacent to the tumor mass, thus exposure to significantly increased concentrations is unlikely to directly affect the tumor cells. The tumor itself is exposed to the lower circulating plasma concentrations of the agents and not the high local concentrations associated with the osteoclastic space.

When implanted into the tibiae of rats, 13762 tumor cells induced radiographic evidence of resorption, as noted by the presence of lytic lesions on radiographs taken at 7 days after implantation. These lesions, although apparent in radiographic film, did not become quantifiable until day 21 using MicroCT scanning. This discrepancy in detection can be attributed to the intrinsic differences between the imaging methods used. Radiography allows for visualization of the osteolytic process by evaluating the entire affected bone. The use of MicroCT technology permitted the quantification of bone parameters in the selected slice of bone being evaluated. The MicroCT slice selection established in this model identified a bone slice 3 mm distal to the tibial growth plate to determine BMD. This distance corresponds to 133 slices from the growth plate, a standard used throughout the study. Whereas the early stages of tumor implantation remain focally localized, as the invasive and osteolytic process progresses, more of the total bone volume is affected. It is at this point that the imaging techniques can clearly discriminate between the progress of the lytic lesion and the distally induced lesion induced at implantation. As seen in both the

Fig. 3 Tibial TBMD (A), CBMD (B), and WBMD (C) from control and tumor-bearing rats in which tumors had been implanted for 7, 14, or 21 days. MicroCT analysis was used to evaluate the bone mineral density, and units are expressed as mg/cm³. Each tumor-bearing group is matched by a non-tumor-bearing group (n = 5). Group means are shown (± SE).

Fig. 4 Determination of TBMD (A), CBMD (B), and WBMD (C) using MicroCT imaging in treated and untreated tibially implanted animals (n = 10). Alendronate, 10, 1.0, 0.1, and 0.01 μg/kg/day, s.c., days 5–20; pamidronate and risedronate, 80 μg/kg/day, s.c., days 5–20. Groups were harvested on day 21. Group means are shown (± SE).
radiographs and the whole bone MicroCT images, the bulk of the proximal third of the tibia becomes lysed by the latter time points. It is at the later stages of progression when accurate and reproducible quantification of osteolysis can be achieved using MicroCT. This scanning method was able to accurately and reproducibly detect the progressive loss of both TBMD and WBMD. Due to its higher relative density, CBMD remained relatively unaffected during most of the study period. CBMD was only lost in animals after 28 days of tumor growth. On treatment with bisphosphonates, tumor-bearing animals show clear protection against resorption of TBMD and WBMD. The use of risedronate in this model was able to preserve the tibiae from tumor-induced osteolysis. Until the time of euthanasia, animals did not demonstrate external signs of tumor growth, lameness, or distress throughout the experimental period (up to day 21). Also important to this model is the relatively normal clinical profile of the animals throughout the experimental period. The lack of significant alterations to the measured clinical parameters allows an additional way to discriminate potential adverse effects of test compounds in this model. Although tumor-bearing animals demonstrated expression of PTHrP in the tumor tissue, no significant elevation of serum Ca\(^{2+}\) was observed. We hypothesize that this result could be attributed to the relatively low tumor burden carried by the animals, which could potentially produce insufficient quantities of PTHrP to cause systemic alterations. Another possible reason for the lack of systemic effects is the possibility that PTHrP is being actively degraded or sequestered in bone. PTHrP levels in circulation were measured but found to be negligible (data not shown). Also noted was the lack of an increase in circulating levels of TGF-\(\beta\)1 even with a strong immunohistochemical signal from the tumors at the different implant sites. Again, we hypothesize that the effects and localization of the secreted TGF-\(\beta\)1 remain compartmentalized to the affected site. Animals allowed to

Fig. 5 Photomicrographs showing staining for PTHrP (R87) and TGF-\(\beta\)1 of 13762 rat mammary carcinoma tumor grown in various anatomical sites (A, s.c. implant; B, intratibial implant; C, pulmonary metastasis from intratibial implant).
progress to day 28 did show an increase of anesthetic-induced complications due to the overwhelming pulmonary tumor burden. This burden produces a total decrease in respiratory capacity. Pulmonary nodules arising from this model can be severe. It remains unclear how these lesions originated. In principle, they could have arisen from one of two routes. First, lesion can arise by direct injection to the bone marrow, allowing for hematogenous/lymphatic spread. Second, the lesion can arise by direct metastatic spread from the tumor formed at the injection site. The 13762 tumor has been previously demonstrated to be spontaneously metastatic in rats, and this would suggest that either of the above-mentioned routes would be a likely method for its spread.

Breast cancer induced bone lysis remains a challenging unmet medical need for which the promise of mechanistic-based therapies remains high. This intraosseous syngeneic model represents a novel system to evaluate the activity of agents with activity against tumor-mediated osteolysis. This model offers an advantage over xenograft systems because it uses a syngeneic tumor model in conventional animals, allowing for ease of husbandry and manipulation. We have demonstrated that the model is consistent with our understanding of PTHrP-TGF-β1-dependent tumor-induced osteoclastic bone resorption. This rat model offers the advantage that numerous relevant parameters can be evaluated simultaneously, in a pathophysiologically relevant manner. These parameters include total bone resorption, cytokine characterization, efficacy of antiresorptive therapies, and toxicity assessment. Development of targeted therapies for tumor-driven bone resorption will be enhanced by this novel multifunctional model.

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Fig. 6  Number of pulmonary nodules in animals implanted with 13762 rat mammary carcinoma cells intratibially. Pulmonary lesions were individually identified on organ surface and counted. Group means are shown (±SE).


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