Matrix Metalloproteinase Activity, Bone Matrix Turnover, and Tumor Cell Proliferation in Prostate Cancer Bone Metastasis

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Background: The metastasis of prostate cancer to bone is associated with a substantial increase in bone matrix turnover. Matrix metalloproteinases (MMPs) play roles in both normal bone remodeling and invasion and metastasis of prostate cancer. This study was designed to determine the role of MMP activity in prostate cancer that has metastasized to bone. Methods: Single human fetal bone fragments were implanted subcutaneously in immunodeficient mice. Four weeks later, PC3 human prostate cancer cells were injected directly into some of the implants, and daily treatment was begun with batimastat (a broad-spectrum MMP inhibitor). There were six mice (i.e., six implants) in each of four experimental arms: bone alone with and without batimastat and bone injected with PC3 cells with and without batimastat. Bone implants were harvested after 14 days of treatment and analyzed for MMP expression, bone histomorphometry, osteoclast counts, blood vessel density, and tumor cell proliferation and apoptosis. Complementary data were obtained from bone biopsy samples from patients and a bone organ coculture system. All statistical tests were two-sided. Results: MMPs were detected in tumor and stromal cells of clinical specimens and experimental bone implants. In vivo, MMP inhibition reduced the number of osteoclasts per millimeter in PC3-injected implants—from 8.2 (95% confidence interval [CI] = 7.9 to 8.5) to 3.0 (95% CI = 2.3 to 3.7) (P = .006). In addition, it prevented degradation of marrow trabeculae within the bone implants (cross-sectional area of implant occupied by mineralized trabeculae: untreated implant = 29.1% [95% CI = 27.1% to 31.1%], PC3-injected implant = 14.0% [95% CI = 10.9% to 17.1%] [P = .005 versus untreated], and batimastat-treated PC3-injected implant = 27.2% [95% CI = 22.4% to 32.0%] [P = .03 versus PC3 injected alone]). MMP inhibition reduced proliferating tumor cells from 20.8% (95% CI = 19.9% to 21.7%) to 7.4% (95% CI = 5.2% to 9.6%) (P = .006), without affecting angiogenesis or apoptosis. In vitro, MMP inhibition had no toxic effect on PC3 cells but prevented calcium release from bone fragments cocultured with PC3 cells. Conclusions: MMP activity appears to play an important role in bone matrix turnover when prostate cancer cells are present in bone. Bone matrix turnover and metastatic tumor growth appear to be involved in a mutually supportive cycle that is disrupted by MMP inhibition. [J Natl Cancer Inst 2002;94:17–25]

Prostate cancer metastasis has two defining clinical characteristics: a tendency to involve bone and a marked response of the bone to the presence of metastatic cells. Prostate cancer bone metastasis causes tremendous morbidity, including pain, impaired mobility, pathologic fractures, spinal cord compression, and other problems. Gross, clinically significant metastatic deposits develop in bone long before metastases to soft viscera become apparent (1–3). The marked increase in the turnover of bone matrix associated with the presence of prostate cancer cells in bone is often recognized as an osteosclerotic response on radiographic imaging studies. It is well accepted, however, that both bone formation and bone breakdown are present within metastatic deposits (4–6). Little is known about the biologic mechanisms underlying the stimulation of bone turnover by metastatic cells. Improved understanding of the interactions between prostate cancer cells and bone is a necessary prerequisite for the development of treatment strategies that specifically target prostate cancer bone metastasis.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that play a major role in proteolytic degradation of structural components of extracellular matrix (7). These enzymes have thus been the focus of intense investigation with regard to tumor invasion and metastasis (8). MMP-2 and MMP-9, in particular, have been found to be specifically associated with prostate cancer metastasis. In prostate cancer patients, high levels of MMP-2 and MMP-9 in the plasma and urine have been correlated with metastasis (9,10). Synthetic MMP inhibitors reduced local invasion of human prostate cancer cells implanted into immunodeficient mice (11) and metastasis of rat prostate cancer cells implanted into immunodeficient mice (12). Inhibition of MMP-9 expression by ribozyme technology reduced the metastatic potential of prostate cancer cells in mice (13). Other MMPs are less well studied but are also likely to be involved in prostate cancer metastasis. For example, we (14) showed that the location of membrane type 1 (MT1)-MMP in prostatic epithelium is altered during the progression from be-
nign epithelium to prostatic intraepithelial neoplasia to cancer. This membrane-bound enzyme is involved in the activation of pro-MMP-2 and is a potent collagen I-degrading protease (15). Thus, these data support the hypothesis that MMPs are likely involved in multiple pathways in the overall multistep process of prostate cancer metastasis.

MMPs also play a role in normal bone remodeling. For example, MMPs are involved in osteoclast recruitment to sites of bone remodeling (16,17). Mineralized bone matrix itself is degraded by a variety of osteoclast-associated enzymes, including both cysteine proteases and MMPs (18–21). A number of synthetic MMP inhibitors have been shown to inhibit bone resorption in a variety of different bone culture systems, and bone resorption can be specifically reduced by the chemical inhibition of MMP-2 and MMP-9 (22,23). Furthermore, the MMP-9 knockout mouse has abnormal skeletal development, and the MT1-MMP knockout mouse has severe skeletal defects (24,25).

Although MMPs are clearly involved in the overall process of prostate cancer metastasis, little is known about their role in the establishment and growth of metastatic prostate cancer colonies in bone. We (26) described the SCID (severe combined immunodeficient)–human model of prostate cancer metastasis, in which a variety of human and mouse organ environments were implanted into SCID mice to serve as a target for human prostate cancer cells. We found that a variety of human prostate cancer cell types preferred to form tumors in the human bone environment than in other human or mouse organ environments. We also demonstrated a bone response ranging from mostly osteolytic to mostly osteoblastic, depending on the type of prostate cancer cells introduced into the bone. Bone tumors formed by the PC3 prostate cancer cell line were consistently osteolytic in nature, suggesting that this model could be used to study the degradative portion of the bone turnover cycle.

Herein, we investigate the effect of MMP inhibition on bone matrix turnover and the growth of PC3 cells in the SCID–human model of prostate cancer bone metastasis.

MATERIALS AND METHODS

Cell Lines, Cell Culture, and Reagents

The PC3 human prostate cancer cell line was purchased from the American Type Culture Collection (Manassas, VA) and maintained in a mixture of RPMI-1640 medium and 5% fetal bovine serum. All of the culture reagents were purchased from Life Technologies, Inc. (GIBCO BRL), Rockville, MD. Cells with a low passage number were used in all experiments. For in vivo experiments, batimastat (BB-94; British Biotech Pharmaceuticals Ltd., Oxford, U.K.) was prepared in phosphate-buffered saline (PBS) (pH 7.4) containing 0.01% Tween 20. An identical solution without batimastat was prepared for control (vehicle) injections into mice. For in vitro experiments, batimastat was dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in culture media in all experiments was 1% or less.

In Vitro Toxicity of Batimastat

To test for a direct effect of batimastat on tumor cell growth, we plated PC3 cells in 96-well plates (2000 cells per well) in complete growth medium. Twenty-four hours after seeding, the medium was replaced with medium containing 20, 10, 5, 1.0, 0.1, or 0 mM batimastat. After a 4-day incubation, MTT (i.e., 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) dissolved in medium was added to each well to achieve a final concentration of 0.5 mg/mL. After a 4-hour incubation, 25% sodium dodecyl sulfate (pH 2.0) was added to dissolve the blue precipitate, and the absorbance of the reaction product was read at 595 nm.

Animal Care and Human Tissue Implantation

Male homozygous C.B-17 scid/scid mice, aged 5 weeks, were purchased from Taconic Farms, Germantown, NY. The mice were maintained according to the National Institutes of Health standards established in the Guidelines for the Care and Use of Experimental Animals (http://grants.nih.gov/grants/olaw/olaw.htm), and all experimental protocols were approved by the Animal Investigation Committee of Wayne State University, Detroit, MI. Human male fetal tissue was obtained by a third-party, nonprofit organization (Advanced Bioscience Resources, Alameda, CA) and written informed consent was obtained from the donor, consistent with regulations issued by each state involved and the federal government. Methoxyflurane anesthesia was used during all surgical procedures. The mice were implanted with human fetal bone fragments as described previously (26). Briefly, femurs and humeri of 16–22 weeks’ gestation were divided in half longitudinally and then again in half transversely into four fragments approximately 1 cm long and 3–4 mm in diameter. Thus, these fragments had both cortical bone and cancellous, marrow-containing bone. Each mouse was implanted subcutaneously with a single fragment through a small skin incision in the flank, with the opened marrow surface facing the mouse muscle and the cortical surface against the under surface of the mouse skin.

Production of Prostate Cancer Bone Tumors and Batimastat Treatment

Tumor cells were injected into the bone implants 4 weeks after implantation. Suspensions of PC3 cells (1 × 10^4 cells in a volume of 20 μL of RPMI-1640 medium) were injected by insertion of a 27-gauge needle through the mouse skin directly into the marrow surface of the previously implanted bone in two of the four experimental arms as described below. Beginning on the day of tumor cell injection, the mice were treated daily for 14 days with an intraperitoneal dose of batimastat at 30 mg/kg per day or vehicle control. This dose of batimastat was chosen because it has been shown to yield blood levels 10-fold higher than the reported concentrations of batimastat giving 50% inhibition of activity for MMP-1, MMP-2, and MMP-9 (27).

There were six bone implants in each of the following four experimental arms: 1) bone alone with vehicle treatment, 2) bone plus PC3 cells with vehicle treatment, 3) bone plus PC3 cells with batimastat treatment, and 4) bone alone with batimastat treatment. At the end of the treatment period, all of the mice were killed by cervical dislocation under CO2 anesthesia, and the human bone implants were removed for analysis.

Immunohistochemical Localization of MMPs

Freshly harvested bone implants were fixed overnight in 10% buffered formalin. The tissues were then decalcified in 10% EDTA (pH 6.5) with gentle shaking. These tissues were changed to fresh EDTA daily for 7–9 days until they became soft. The tissues were then rinsed in PBS, embedded, and sectioned.
MT1-MMP immunostaining was done with polyclonal anti-serum (RFNEEELRVDSEYPNIK) derived from the amino acid sequence of human MT1-MMP. The anti-human MMP-2 (clone CA-4001, Ab-2, product IM53) and MMP-9 (clone 56–2A4, Ab-3, product IM37L) antibodies were purchased from Calbiochem (La Jolla, CA). Sections of human bone containing human prostate cancer metastases were cut from archival paraffin blocks containing bone core biopsy specimens. These specimens were obtained from patients with known bone metastases, who gave written, informed consent according to the guidelines of a protocol approved by the Wayne State University Human Investigations Committee.

Paraffin sections of human bone implants harvested from the SCID mice or archival human tissue sections were deparaffinized and rehydrated through a graded alcohol series. For MMP-9 and MT1-MMP staining, antigen was retrieved by placing slides in 10 mM sodium citrate (pH 6.0) and boiling by microwave heating for 3 minutes. Nonspecific sites were blocked by incubation with Superbloc (ScyTek, Logan, UT). Sections were incubated with anti-MMP-2 (5 µg/mL in a mixture of PBS and 2% bovine serum albumin) or anti-MMP-2 (1:1000 dilution) monoclonal antibodies for 45 minutes at room temperature. Control slides received no primary antibody. Sections were then incubated with rabbit anti-mouse immunoglobulin G (1:40 dilution), followed by alkaline phosphatase/anti-alkaline phosphatase monoclonal antibody (APAAP) (1:40 dilution). For MT1-MMP staining, nonspecific sites were blocked with Superbloc, and sections were incubated with anti-MT1-MMP antisera at a 1:20 dilution. Immunoreactive sites were detected with a biotin/avidin enzyme kit (Vectastain kit; Vector Laboratories, Inc., Burlingame, CA). Positive immunoreactive sites for both staining protocols were visualized with the Sigma Fast Red (Sigma Chemical Co., St. Louis, MO) substrate. Sections were briefly counterstained with hematoxylin and mounted in aqueous medium.

Oligonucleotide Probes and In Situ Hybridization

Colorimetric in situ hybridization analysis of SCID–human bone tumors for MMP-2 and MMP-9 messenger RNA (mRNA) was performed with a commercially available kit (HybriProbe kit; Biognostik, Göttingen, Germany). The antisense and control oligonucleotides were designed and manufactured by Biognostik. The antisense MMP-9 probe used in this application was a random sequence oligonucleotide (5'-TACCTATTCCGACAGGTGGTCGGAAACCTAG-3', 5'-AGGGTCAAGTACTCCTTACCCAGGAAAGTG-3', and 5'-TCGAAAGATGTCTACGGTGCAAGGCATCTGC-3'). The antisense MMP-2 probe was a single fluorescein isothiocyanate (FITC)-labeled oligonucleotide (5'-TGATCCTTCTTCGGTACTGGGAAGACGTC-3', 5'-AGGGTCAAGTACTCCTTACCCAGGAAAGTG-3', and 5'-TCGAAAGATGTCTACGGTGCAAGGCATCTGC-3'). Random sequence oligonucleotides with matching length and the same content of guanosine and cytidine were used as negative hybridization controls (MMP-9 controls = 5'-TACCTATTCCGACAGGTGGTCGGAAACCTAG-3', 5'-TCCCTACGTTCTCTGTACGGTGCAAT-3', and 5'-TCACCTATCCGGACAGGTGGGAACCTAG-3'; MMP-2 control = 5'-GCTGACTACGGAGACAAAGTACCATTG-3').

Paraffin sections of bone implants were mounted on ProbeOn slides (Fisher Scientific Co., Pittsburgh, PA), deparaffinized, and rehydrated. The sections were digested with proteinase K solution (20 µg/mL) for 15 minutes at room temperature, fixed for 5 minutes in 0.5% parafomaldehyde, washed in water, and air-dried. The sections were prehybridized with HybriBuffer (Biognostik) for 3 hours at 30 °C in a humidified chamber. The prehybridization solution was drawn off and replaced with fresh HybriBuffer containing equal amounts of the antisense or control oligonucleotides. After overnight incubation at 30 °C, the slides were rinsed at room temperature, two 30-second rinses in 1x standard saline citrate (SSC) and one 2-minute rinse in 1x SSC. Two final 15-minute rinses were done in 0.1x SSC at 40 °C.

For immunodetection of the FITC-labeled probes, the sections were blocked for 20 minutes with 2% bovine serum albumin in PBS and incubated with peroxidase-conjugated anti-FITC monoclonal antibody (MAB 045P; Chemicon, Temecula, CA) for 2 hours at room temperature. Immunoreactive sites were detected by incubation with dianinobenzidine tetrahydrochloride, and nuclei were counterstained with hematoxylin.

Histomorphometric Analysis

For the measurement of the in vivo effects of tumor cells and/or batimastat on bone turnover, paraffin sections of human bone implants were stained with hematoxylin–eosin and analyzed with histomorphometry. The water bath temperature was strictly controlled during the sectioning process to prevent distortion of the paraffin-embedded sections. Low-power, digital, color photomicrographs of stained sections were analyzed with imaging software (Photoshop, version 6.0; Adobe Systems, Mountain View, CA). Areas of each image judged to be mineralized trabecular bone were manually highlighted. Cortical bone was excluded from the analysis. For each experimental arm, a mean percentage of the tissue cross-sectional area composed of mineralized bone trabeculae was calculated from 10 sections taken from each implant spaced throughout the thickness of the specimen.

Measurement of Calcium Release In Vitro

To measure the effect of batimastat on breakdown of mineralized bone, we performed short-term bone organ coculture with PC3 cells and four experimental arms analogous to the in vivo experiment. Similar fetal bones were divided into identical-sized fragments (eight fragments per experimental arm) and distributed narrow side up into 24-well plates. The fragments were cultured overnight in serum-free BGM medium (Sigma Chemical Co.) with or without PC3 cells. The medium was removed and replaced with plain BGM medium with or without 10 mM batimastat. After culture for 4 days at 37 °C, the medium was collected and the calcium concentration was measured by use of a clinical chemistry kit (Sigma Chemical Co.). Gelatin zymography was performed on conditioned medium to test for the secretion of MMPs as described previously (28).

Measurement of Osteoclast Recruitment

For the determination of the effect of batimastat on osteoclast recruitment, sections of SCID–human bone tumors were deparaffinized, rehydrated through a graded alcohol series, and subjected to antigen retrieval. The sections were then blocked with Superbloc for 10 minutes at room temperature and incubated with anti-tartrate-resistant acid phosphatase (TRAP) monoclonal antibodies (1:50 dilution; Zymed Laboratories, South San Francisco, CA) for 45 minutes at room temperature. (TRAP is a
specific marker for osteoclasts.) Immunoreactive sites were detected with the APAAP staining method described above. The number of osteoclasts adhering to endosteal surfaces was determined by visual inspection of digital images of stained tissue sections at a magnification of x20. Only bone surfaces near tumor cell nests (within three cell widths) were scored. At least 1 mm of endosteal surface was scored for each of the six implants in each experimental arm.

**Measurement of Tumor Cell Proliferation and Apoptosis**

Tumor cell proliferation was measured with immunohistochemical staining for the Ki-67 nuclear antigen. Sections of SCID–human bone tumors were deparaffinized, subjected to antigen retrieval, and incubated with monoclonal antibody Mib-1 (directed against the Ki-67 antigen; Coulter Immunotech, Miami, FL) at a 1 : 50 dilution. Immunoreactive sites were detected with the APAAP staining method described above. Nuclei were counterstained briefly with hematoxylin. At least 1000 tumor cell nuclei were scored as Mib-1 positive or negative from each of the six implants in the batimastat- and vehicle-treated arms.

Apoptotic tumor cells were detected in tissue sections by use of a commercially available terminal deoxynucleotidyltransferase-mediated uridine 5'-triphosphate end labeling (TUNEL) assay kit (TumorTACS; R & D Systems, Minneapolis, MN). Tissue sections were deparaffinized, rehydrated, and incubated for 15 minutes with proteinase K (20 μg/mL) at room temperature. Apoptotic nuclei were detected by use of the manufacturer’s protocol and visualized colorimetrically with diaminobenzidine. Sections were counterstained with methyl green, and the proportion of positive tumor cell nuclei was determined in the batimastat- and vehicle-treated arms.

**Measurement of Vessel Density**

For immunofluorescent staining of endothelial cells, the sections were deparaffinized, rehydrated through a graded alcohol series, and subjected to antigen detection. The sections were then blocked with Superblock (as above) and incubated with rabbit anti-von Willebrand factor antisemur (factor VIII-related antigen; Sigma Chemical Co.) at a dilution of 1 : 1000. This antibody reacts with both human and mouse endothelial cells as stated in the product literature. Immunoreactive sites were detected by incubation with a biotinylated anti-rabbit immunoglobulin, followed by FITC-conjugated streptavidin (Vector Laboratories, Inc.). The sections were mounted in aqueous medium containing 4',6-diamidino-2-phenylindole (nuclear counterstain; Vector Laboratories, Inc.) and analyzed by fluorescence microscopy to visualize endothelial cells in blood vessels.

The vessel density within vehicle- or batimastat-treated PC3/bone implants was measured by counting the number of vessel profiles (identified by factor VIII staining) per square millimeter of tissue within the confines of the surrounding capsular structure (29). All positively stained structures with an identifiable lumen were counted equally.

**Statistical Analysis**

All data were analyzed by use of Microsoft Excel 98 software (Microsoft Corp., Redmond, WA). The data were expressed as mean and 95% confidence interval (CI). For the *in vivo* experiments, there were six bone implants in each experimental arm (one implant per mouse). For the *in vivo* calcium release experiments, there were eight samples in each arm. Bone turnover assays (histomorphometry, osteoclasts counts, and calcium release) were evaluated in all four experimental arms. Tumor-related assays (tumor cell proliferation, apoptosis, and vessel density) were evaluated in the two experimental arms involving PC3 cell injection into bone implants. Comparisons between experimental arms were done with two-tailed Student’s *t* tests. Statistical significance was *P* < .05. All statistical tests were two-sided.

**RESULTS**

**Expression of MMPs in Clinical Prostate Cancer Bone Metastases**

We examined the expression of MMP-2, MMP-9, and MT1-MMP in sections from 18 core bone biopsy specimens from patients with documented, bone-metastatic prostate cancer. All of the sections contained prostate cancer cells, and the bone exhibited a response ranging from mostly lytic to mostly blastic, as judged by routine staining (data not shown). Although there was some variation between specimens regarding the intensity of staining, all of the clinical specimens stained positively for MMP-2, MMP-9, and MT1-MMP (Fig. 1). The immunoreactivity was localized primarily to the tumor cells, although some staining was seen in adjacent stromal fibroblasts, osteoblasts, and osteoclasts with all three of these antibodies.

**Expression of MMPs in Experimental Bone Tumors**

In the SCID–human model, bone tumors can be created in which the bone response ranges from mostly osteolytic to mostly osteoblastic, depending on the prostate cancer cell line injected into the implanted bone. To examine the role of MMPs in bone degradation, we used the PC3 prostate cancer cell line, which produces experimental bone tumors that are primarily osteolytic in nature. Immunostaining of PC3 bone tumors demonstrated immunoreactivity for all three proteinases (Fig. 2). As in the clinical specimens, the immunoreactivity for MMP-2, MMP-9, and MT1-MMP was localized primarily to the tumor cells; again, some staining was seen in the surrounding bone stromal cells with all three antibodies.

![Fig. 1. Matrix metalloproteinase (MMP) immunostaining of a bone biopsy specimen from a representative patient with metastatic prostate cancer. A) MMP-2. B) MMP-9. C) membrane type 1 (MT1)-MMP. Red = positive immunoreactivity; T = tumor cells; B = bone marrow trabeculae. Scale bar = 50 μm.](image-url)
To determine which cells produced MMP-2 and MMP-9, we performed in situ hybridization on sections containing SCID–human bone tumors. Strong positive signals for the mRNAs of both enzymes were observed in the cytoplasm of tumor cells and neighboring bone stromal cells (Fig. 2), which suggests that MMP-2 and MMP-9 are produced by both PC3 tumor cells and bone stromal cells.

**Effect of MMP Inhibition on Bone Turnover**

We showed previously that large bone tumors develop 6 weeks after the injection of PC3 cells into the marrow cavity of the implanted bone. At this relatively late time, the mineralized trabeculae are almost completely replaced by a desmoplastic tumor that consists of PC3 cells admixed with bone stroma and nonmineralized collagen fibers (26). In the present study, we harvested the bone implants only 2 weeks after injection of PC3 cells. At this earlier time, tumor cells are found easily within the marrow, but the size of the bone implants has not changed and substantial amounts of well-organized trabeculae are still present. We chose this time to study interactions between tumor cells and a relatively intact bone environment.

We used quantitative histomorphometric analysis to measure changes in mineralized trabecular bone area induced by MMP inhibition with batimastat, a broad-spectrum MMP inhibitor. In sections of plain human bone implants, 29.1% (95% CI = 27.1% to 31.1%) of the cross-sectional area was mineralized trabeculae (Fig. 3, A). As expected in the mice treated with vehicle alone, 2 weeks after the injection of the osteolytic PC3 cells into the bone implants, the proportion of mineralized bone was statistically significantly reduced to 14.0% (95% CI = 10.9% to 17.1%) ($P < .005$ versus plain implant). Treatment with batimastat for 2 weeks after the PC3 cell injection prevented the tumor-associated osteolysis (27.2% [95% CI = 22.4% to 32.0%]; $P = .03$ versus untreated PC3 bone tumors), and the percentage of mineralized bone was similar to that of control bone implants ($P = .64$ versus plain bone). Batimastat treatment of mice bearing bone implants without tumor cells resulted in no statistically significant change in the percentage of mineralized bone.

As a second measure of bone degradation, we measured the release of calcium into the medium of a bone organ coculture system using identical human fetal bone fragments and PC3 cells. The addition of PC3 cells to the organ culture system increased the amount of calcium released into the medium by approximately fivefold compared with control levels ($P < .001$). Addition of batimastat prevented this release ($P < .001$) (Fig. 3, B). In the absence of PC3 cells, addition of batimastat to the bone organ culture system had no statistically significant effect on the amount of calcium released. Gelatin zymography of the coculture medium indicated that latent and active MMP-2 and MMP-9 were present whether or not batimastat was added (data not shown). Thus, inhibition of MMP activity prevented mineralized bone breakdown induced by the addition of PC3 prostate cancer cells to this in vitro coculture system.

One of the steps involved in the cycle of bone turnover is the migration of osteoclasts to bone surfaces; MMP activity has been shown to be required for osteoclast migration in bone (17). To examine the effect of MMP inhibition on osteoclast recruitment, we immunostained sections of SCID–human bone tumors for TRAP, a specific osteoclast marker, and counted the numbers of osteoclasts per linear millimeter of trabecular surface (Fig. 4). Treatment of mice with batimastat statistically significantly reduced the number of osteoclasts per millimeter on trabecular surfaces near nests of tumor cells from 8.2 (95% CI =
7.9 to 8.5) to 3.0 (95% CI = 2.3 to 3.7) \( (P = .006) \), suggesting that MMP inhibition prevents osteoclast recruitment within bone metastases. Reduced numbers of osteoclasts may explain, in part, the reduction of osteolysis observed with MMP inhibition.

**Effect of MMP Inhibition on Tumor Growth**

Next we examined the effects of batimastat on bone tumor growth. When mice bearing PC3-injected bone implants were treated with batimastat for 2 weeks, a statistically significant reduction in the proportion of proliferating tumor cells from 20.8% (95% CI = 19.9% to 21.7%) to 7.4% (95% CI = 5.2% to 9.6%) \( (P = .006) \) was detected (Fig. 5). *In vitro* proliferation studies demonstrated that proliferation rates were the same for PC3 cells cultured alone or in 0.1–20 \( \mu \)M batimastat, concentrations that were two to 400 times the level reached in serum *in vivo* after a dose of 30 mg/kg (27) (data not shown). To investigate other mechanisms by which batimastat could inhibit the growth of tumor cells in the SCID–human model, we asked whether batimastat was affecting tumor angiogenesis and/or apoptosis. We stained sections of the SCID–human bone implants with factor VIII antibody to assess vessel density and used the TUNEL assay to determine the number of apoptotic tumor cells. When we compared the PC3/bone implants in batimastat- and vehicle-treated mice, the vessel density (approximately 43 ves-
sels per square millimeter) and the percentage of apoptotic cells (approximately 1.1%) were nearly identical in each arm. Thus, these results suggest that the batimastat-induced suppression of tumor cell proliferation in bone is not due to a direct toxic effect on tumor cells, an inadequate blood supply, or increased apoptosis.

**DISCUSSION**

Prostate cancer metastasis has distinctive characteristics. Bone is the most frequent target organ, and the presence of metastatic cells leads to a tremendous increase in bone matrix turnover. It has been proposed that stimulation of bone matrix turnover by metastatic cells may be responsible for the tendency of prostate cancer cells to thrive within the bone environment. The hypothesis is that a vicious cycle exists whereby tumor cells stimulate matrix turnover and matrix turnover stimulates tumor growth. More specifically, communication between tumor cells and bone cells leads to increased bone metabolism, and the release of stimulatory substances during bone matrix turnover enhances growth of cancer cells that have colonized bone. Because MMPs appear to play critical roles in both prostate cancer metastasis and bone matrix turnover, we hypothesized that inhibition of MMP activity may disrupt the cycle of bone matrix turnover and tumor cell growth.

Herein, we demonstrated that prostate cancer cells produce MMPs when in bone. Consistent results were found in both clinical samples and SCID–human bone tumors. Not surprisingly, MMPs were also found in neighboring bone stromal cells. Other authors have noted expression of MMPs by prostate cancer epithelial cells. In primary prostate tumor tissue, prostate cancer cells were found to be capable of producing MMPs (14,30–32). With regard to cancer cells and bone, Sanchez-Sweatman et al. (33) showed in an *in vitro* experiment that PC3 cells produce and secrete MMPs when they are placed on bone surfaces. Although we assayed only for MMP-2, MMP-9, and MT1-MMP, it is likely that the production of other MMPs may be stimulated as well. Thus, these data suggest that there is a generalized increase in MMP levels within the bone environment when cancer cells are present due, in part, to production of MMPs by the cancer cells themselves.

Although MMP protein levels may be increased by the presence of prostate cancer cells in bone, it is conceivable that net MMP enzymatic activity may not change because of concomitant increases in natural MMP inhibitors (e.g., tissue inhibitors of metalloproteinases). Thus, we turned to pharmaceutical inhibition of MMP activity to test for any functional effects that might be due to the observed increases in MMP production. Our experimental strategy was designed to maximize the possibility of observing an effect. We chose a bone tumor model where osteolysis is the predominant phenotype (26). In addition, we chose a relatively unsensitive agent that inhibits most MMPs, and we administered it at a single-dose level predicted to be high enough to produce broad-spectrum inhibition of MMP activity. Although a dose–response type of experiment might have provided additional information, lower tissue levels might lead to selective inhibition of a smaller subset of MMPs, making the data difficult to interpret.

Baseline histomorphometric analysis of bone implants and *in vitro* calcium release experiments demonstrated that broad-spectrum MMP inhibition had little to no effect on bone in the absence of prostate cancer cells. However, in the presence of PC3 cells, we found that MMP inhibition prevented both mineralized trabeculae degradation *in vivo* and calcium release *in vitro*. These observations suggest that increased MMP activity (whether from tumor cells or bone cells) plays a critical role in osteolysis within metastatic tumor deposits.

The exact mechanism underlying the prevention of matrix degradation by MMP inhibition is unclear. The prevailing hypothesis is that increased osteoclast activity is responsible for bone degradation within metastatic deposits (34). Because MMP activity is crucial for osteoclast recruitment, we counted osteoclasts within the experimental bone implants. We found that inhibition of MMPs reduced the numbers of osteoclasts along trabecular surfaces, suggesting that osteoclast recruitment within metastatic deposits is dependent on MMP activity. MMP activity has been shown to be crucial to osteoclast recruitment in non-tumor systems. Invasion of collagen by osteoclastic cell protrusions can be retarded by synthetic and endogenous MMP inhibitors (16). In addition, migration of osteoclast precursors to the bone marrow cavity is decreased by various MMP inhibitors but not by a cysteine proteinase inhibitor, an inhibitor of carbonic anhydrase, or a bisphosphonate (17). Another mechanism by which MMP inhibition can inhibit osteoclast recruitment is by reducing the amount of osteoclastic chemoattractants (35) or osteoclast-stimulating cytokines (36) released from bone during the remodeling process.

Inhibition of MMP activity may prevent osteoclastic bone degradation by mechanisms besides inhibition of recruitment. In nontumor systems, dissolution of mineralized bone matrix is thought to occur only in the osteoclast resorption lacuna. Mineralized matrix is resorbed through the action of cysteine proteinases and MMPs, both of which are produced by osteoclasts (37). Early in the dissolution process, the lacuna is acidic, and matrix dissolution is probably accomplished primarily by cysteine proteinases (most likely, cathepsin K), which are active at low pH. It appears that MMPs play a temporally delayed role in the osteoclast resorption lacuna, degrading collagen late in the process after a shift toward a more neutral pH (18,38). Electron microscope studies show that MMP inhibitors dramatically reduce degradation of the extracellular matrix in the resorption zone (38). Thus, the prevention of matrix degradation observed in the present study may be due, at least in part, to MMP inhibition of osteoclastic bone degradation within the bone tumors. MMPs are not the only molecules involved in the recruitment and/or stimulation of osteoclasts. Other candidates include transforming growth factor-β, epidermal growth factor, interleukin 1, interleukin 6, interleukin 11, tumor necrosis factor-α, prosta-glandins, and parathyroid hormone-related peptide (39,40).

As mentioned above, we found that prostate cancer cells produce MMPs when in bone, raising the possibility that these cells participate directly in the bone turnover process via these degradative enzymes. Consistent with the *in vivo* results presented in this study, Sanchez-Sweatman et al. (33) reported in an *in vitro* system that PC3 cells directly degrade mineralized bone matrix and that the degradation was reduced by generalized inhibition of MMP activity. Other studies (41,42) have suggested that other tumor cells can also degrade mineralized bone matrix directly, possibly via the secretion of MMPs.

Even if tumor-associated MMPs do not directly digest mineralized matrix, they may participate in osteolysis via a mechanism that is analogous to a known osteoblast function. It is believed that all endosteal surfaces are covered with a layer of
nonmineralized matrix. In areas of bone formation, this layer is called osteoid and it is quite thick and easily observed in stained sections (43). Evidence is accumulating that degradation of this layer must occur before the attachment of osteoclasts to the underlying mineralized matrix (Chamber’s hypothesis) (44). In normal remodeling, digestion of this layer is apparently accomplished by MMPs produced by osteoblasts (19,23,45–49). It is conceivable that prostate cancer cells in bone adopt this osteoclast function; thus, the possibility that MMPs produced by prostate cancer cells enhance osteoclastic degradation by prior degradation of nonmineralized matrix is currently under investigation in our laboratory. Furthermore, degradation of this nonmineralized layer may be partially responsible for the recruitment and activity of osteoclasts due to release of osteoclast attractants/stimulants. Bone resorption by osteoclasts is augmented experimentally by coating mineralized matrix with collagenase-cleaved collagen fragments (19). As nonmineralized matrix is degraded, osteoclasts may be exposed to extracellular proteins, such as fibronectin, vitronectin, osteopontin, bone sialoprotein, or other cryptic epitopes. Osteoclasts bind to these proteins via integrins (50). This strong adhesion may promote establishment of the resorption lacuna, an hypothesis that is supported by a study (51) in which bone sialoprotein was shown to increase both osteoclast adhesion to bone surfaces and bone resorption. Thus, we hypothesize that MMPs produced by metastatic prostate cancer cells enhance osteoclastic bone degradation by prior degradation of nonmineralized matrix in a manner similar to osteoclasts. This degradation leads, in turn, to enhanced recruitment and enhanced osteoclastic activity.

Finally, we found that MMP inhibition reduced tumor cell proliferation but had no effect on tumor cell apoptosis or angiogenesis within the experimental bone implants. In addition, the MMP inhibitor, even at high concentrations, had no direct toxic effect on PC3 cells in vitro. With regard to angiogenesis, we (29) previously demonstrated a moderate amount of new blood vessel formation in large PC3/bone tumors. In the present study, bimatostat treatment did not affect vessel density. It should be noted, however, that we harvested the implants only 2 weeks after tumor cell injection, so that large angiogenic-dependent tumors did not have time to develop (29). In any case, our results suggest that mechanisms besides angiogenesis inhibition appear to play a role in the antiproliferative effect of the MMP inhibitor bimatostat, at least during the early steps of bone colonization by prostate cancer cells.

In conclusion, we found that prostate cancer cells produce MMPs when in bone in both clinical specimens and in an experimental model of bone metastasis. In the predominantly osteolytic PC3/human bone model, inhibition of MMP activity reduced osteoclast recruitment, prevented bone matrix degradation, and reduced tumor cell proliferation. MMP inhibition had no direct toxic effect on tumor cells and did not affect tumor angiogenesis or apoptosis. To our knowledge, this is the first study to measure bone and tumor phenotypic changes induced by a clinically tested MMP inhibitor within an experimental model of prostate cancer bone metastasis. Thus, these observations suggest that MMP activity plays an important role in the increase in bone matrix turnover associated with the presence of prostate cancer cells in bone. Although the exact mechanism of diminished tumor cell proliferation is unclear, the data are consistent with the hypothesis that bone matrix turnover and tumor cell proliferation are linked to each other in a positive feedback cycle that can be disrupted by inhibition of MMP activity. A relationship between tumor growth and bone turnover may provide one explanation for the predilection for prostate cancer to grow in bone. Additional experiments are necessary to determine whether particular MMPs produced by cancer cells and/or bone cells are crucial to this process. In addition, the role of MMP activity in bone tumors that tend to be more osteoblastic in nature must be examined.

REFERENCES


