Tumorigenesis and Neoplastic Progression

Prostate Cancer-Associated Membrane Type 1-Matrix Metalloproteinase

A Pivotal Role in Bone Response and Intraosseous Tumor Growth

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Membrane type 1-matrix metalloproteinase (MT1-MMP) is a major mediator of collagen I degradation. In human samples, we show that prostate cancer cells in skeletal metastases consistently express abundant MT1-MMP protein. Because prostate cancer bone metastasis requires remodeling of the collagen-rich bone matrix, we investigated the role of cancer cell-derived MT1-MMP in an experimental model of tumor-bone interaction. MT1-MMP-deficient LNCaP human prostate cancer cells were stably transfected with human wild-type MT1-MMP (MT1wt). Furthermore, endogenous MT1-MMP was down-regulated by small interfering RNA in DU145 human prostate cancer cells. Intratibial tumor injection in severe combined immunodeficient mice was used to simulate intraosseous growth of metastatic tumors. LNCaP-MT1wt cells produced larger osseous tumors than Neo control cells and induced osteolysis, whereas DU145 MT1-MMP-silenced transfectants induced osteogenic changes. *In vitro* assays showed that MT1wt overexpression enhanced collagen I degradation, whereas MT1-MMP-silencing did the opposite, suggesting that tumor-derived MT1-MMP may contribute directly to bone

remodeling. LNCaP-MT1wt-derived conditioned medium stimulated *in vitro* multinucleated osteoclast formation. This effect was inhibited by osteoprotegerin, a decoy receptor for receptor activator of nuclear factor κ B ligand, and by 4-[4-(methanesulfonamido) phenoxy] phenylsulfonyl methylthiirane, an MT1-MMP inhibitor. Our findings are consistent with the hypothesis that prostate cancer-associated MT1-MMP plays a direct and/or indirect role in bone matrix degradation, thus favoring intraosseous tumor expansion. (*Am J Pathol* 2007, 170:2100–2111; DOI: 10.2353/ajpath.2007.060720)

A major clinical complication of prostate cancer patients is the development of skeletal metastasis. Intraosseous growth of cancer requires the action of proteolytic enzymes to facilitate remodeling of the bone matrix, promote angiogenesis, and increase the availability of local growth factors. Matrix metalloproteinases (MMPs) have been shown to play a pivotal role in the pathogenesis of osteolytic bone metastases.^{1,2} Previously, we and others showed that systemic inhibition of MMP activity reduces tumor cell proliferation, disrupts osteoclast recruitment, and suppresses bone degradation in animal models of bone metastasis.^{2–5} Recently, we reported up-regulation of MMP-9 activity shortly after the bone microenvironment was colonized by prostate cancer cells.⁶

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Membrane type 1-matrix metalloproteinase (MT1-MMP) is a membrane-anchored protease that has the capacity to activate pro-MMP-2 on the cell surface⁷ and to promote tumor growth and angiogenesis.^{8,9} MT1-MMP degrades several extracellular matrix components including type I collagen,¹⁰ the most abundant matrix protein in bone.¹¹ In fact, mice deficient in MT1-MMP display a prominent skeletal phenotype due to abnormalities in bone remodeling.^{12,13} Previously, in human prostate tissues, we showed that MT1-MMP is expressed in the basal cells of benign glands, in the secretory cells of prostatic intraepithelial neoplasia, and in some invasive prostate adenocarcinoma glands.¹⁴ Together, these studies suggest that tumor-associated MT1-MMP activity may promote prostate cancer progression and metastasis. In this study, we present the first description of MT1-MMP expression in human prostate cancer bone metastasis. We demonstrate that overexpression of MT1-MMP in prostate cancer cells promotes intraosseous tumor growth and an osteolytic response in an *in vivo* model, whereas down-regulation of MT1-MMP generates virtually the opposite results. Tumor-derived MT1-MMP may contribute to tumor growth and bone remodeling directly by degradation of bone matrix and indirectly by shedding of soluble receptor activator of nuclear factor κ B ligand (sRANKL), which can promote osteoclast recruitment and differentiation.

Materials and Methods

Cell Culture and Stable Transfections

The human prostate cancer cell lines LNCaP and DU145 (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium and Dulbecco's modified Eagle's medium, respectively, both supplemented with 10% fetal bovine serum, at 37°C and 5% CO₂. The human full-length wild-type MT1-MMP cDNA was constructed into the pcDNA 3.1/myc-His (–) expression vector (Invitrogen, Carlsbad, CA) using appropriate restriction sites. LNCaP cells were stably transfected with pcDNA 3.1 vector containing full-length wild-type (MT1wt) MT1-MMP using Effectene Transfection Reagent (Qiagen, Valencia, CA), based on the manufacturer's instructions. Control LNCaP cells (Neo) were transfected with the pcDNA 3.1 vector without MT1-MMP DNA insert. Stable cell lines (pooled populations) were selected and maintained in culture medium supplemented with G-418 (Invitrogen).

MT1-MMP small interfering RNA (siRNA) was designed using siRNA Target Finder (Ambion, Austin, TX; http://www.ambion.com/techlib/misc/siRNA_finder.html), and the selected target sequence was 5'-AAGTCTTCACT-TACTTCTACA-3'. The siRNA targeting the sequence was synthesized using a Silencer siRNA Construction kit (Ambion), and its silencing effectiveness was tested by treatment of the MT1-MMP-expressing DU145 human prostate cancer cells with the synthesized MT1-MMP siRNA. An MT1-MMP siRNA-expressing DNA insert was constructed into the pSilencer Hygro siRNA Expression Vec-

tor (Ambion). The vector containing the MT1-MMP siRNA (MT1si)-expressing insert was amplified, purified, and sequenced. DU145 cells were transfected with either the MT1si-expressing vector or a vector expressing a scrambled siRNA (scr-si) that did not affect MT1-MMP expression. The transfected cells were selected by hygromycin treatment, and the resistant cells were further cloned.

The murine monocytic cell line RAW 264.7¹⁵ (American Type Culture Collection) was cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium with 4 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose and supplemented with 10% fetal bovine serum.

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from LNCaP and DU145 transfectant cells using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. MT1-MMP mRNA was amplified with the primers of MT1-MMP (forward, 5'-CG-CTACGCCATCCAGGGTCTCAAA-3'; and reverse, 5'-C-GGTCATCATCGGGCAGCACAAAA-3'). RANKL primers used were 5'-TCCCATCTGGTTCCATAAA-3' (forward) and 5'-ATCCAGTAAGGAGGGGTTGG-3' (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified as an internal control to compare relative expression of the target genes among different samples. Polymerase chain reaction (PCR) products were run on a 1% agarose gel and visualized by ethidium bromide staining.

Immunohistochemistry

Bone metastasis tissue samples ($n = 20$) were obtained from rapid autopsies of prostate cancer patients.¹⁶ These patients had androgen-independent disease, as previously reported.¹⁷ Aside from two patients with no associated bone change, all samples revealed a diffuse osteoblastic reaction to prostate cancer cells.¹⁶ Five-micrometer paraffin sections were immunostained using a rabbit polyclonal antibody against the catalytic domain of MT1-MMP (Spring Bioscience, Fremont, CA) and the Vectastain Elite ABC peroxidase kit (Vector Laboratories, Burlingame, CA), following manufacturer's instructions.

Immunoblotting

LNCaP-Neo and LNCaP-MT1wt cells cultured to 80% confluence were washed twice with phosphate-buffered saline and then lysed in Nonidet P-40 lysis buffer (25 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, and 1% Nonidet P-40) in the presence of a protease inhibitor cocktail not containing ethylenediamine tetraacetic acid (Roche Applied Science, Indianapolis, IN). Protein concentrations were determined using the bicinchoninic acid method (Pierce Chemical Company, Rockford, IL). To detect MT1-MMP, equal amounts of whole-cell lysates (50 μ g) were subjected to sodium dodecyl sulfate-polyacrylam-

ide gel electrophoresis under reducing conditions followed by immunoblotting using the LEM-2/15 monoclonal antibody to the catalytic domain of human MT1-MMP (1:2000) (kindly provided by Dr. A. Arroyo, Hospital de la Princesa, Madrid, Spain). After washing, the membranes were incubated with an anti-mouse secondary antibody conjugated with horseradish peroxidase (1:3000; Cell Signaling Technology, Danvers, MA). An MT1-MMP enriched membrane preparation from nonmalignant monkey kidney epithelial BS-C-1 cells was electrophoresed as a positive control.¹⁸ The blots were stripped and re-probed with an antibody to β -actin (Sigma-Aldrich, St. Louis, MO). Membranes were subjected to enhanced chemiluminescence detection (Pierce), according to manufacturer's instructions.

Enzyme Activity Assays and Exogenous Pro-MMP-2 Activation

MT1-MMP activity in cell extracts was measured using the Biotrak MMP-14 activity assay (GE Healthcare, Little Chalfont, Buckinghamshire, UK), as described by the manufacturer. For pro-MMP-2 activation, LNCaP transfectant cells were incubated (2 hours, 37°C) with serum-free medium supplemented with 2 nmol/L recombinant pro-MMP-2, which was produced in HeLa S3 cells infected with the appropriate recombinant vaccinia viruses.¹⁹ Cell lysates were then obtained and subjected to gelatin zymography as described previously.⁶ Alternatively, recombinant pro-MMP-2 was incubated either with an equal amount of cell lysates prepared from DU145 clones or with the same number of live DU145 cells in serum-free Dulbecco's modified Eagle's medium at 37°C for 16 hours. The lysates and culture media were collected and subjected to gelatin zymography.

Three-Dimensional Collagen Growth Assay

Rat type I collagen (5 mg/ml solution; Trevigen, Gaithersburg, MD) was diluted to 2 mg/ml using a solution composed of 10 $\times$ RPMI 1640 medium culture medium, distilled water, and 1 N NaOH (4:1:4.8:0.2 ratio), at 4°C. LNCaP transfectant cells were suspended in the solution (1×10^4 cells/ml), and 1 ml of the cell suspension was dispensed per well in six-well plates. Each mixture was allowed to polymerize at 37°C for 1 hour. Growth culture medium (3 ml/well) was then added on top of the gels. After 18 days, the gels were dissolved with 2 mg/ml bacterial collagenase (Worthington Biochemical Corporation, Lakewood, NJ), and cells were counted with a hemocytometer. For DU145 clones, the assay was performed similarly, except that the collagen gels with cells were dissolved after 6 days.

Growth of Prostate Cancer Cell Transfectants in Mice

Five-week-old male C.B.-17 severe combined immunodeficient (SCID) mice (Taconic Farms, Germantown, NY)

were randomly divided into six groups of 9 to 10 animals. For LNCaP transfectants, 2×10^5 cells in 10 μ l of serum-free medium were injected into the proximal end of tibiae 4 to 5 mm down the diaphysis using calibrated Microliter syringes (Hamilton Syringe Co., Reno, NV) and 27-gauge needles under anesthesia. For DU145 transfectants (DU145-scr-si clone and DU145-MT1si clones 1, 5, and 8), 1×10^5 cells in 10 μ l of medium were injected the same way. Lukens bone wax (Surgical Specialties Co., Reading, PA) was applied over the site of injection to prevent cell leakage. Prostatic specific antigen levels were determined in plasma of mice intratibially injected with LNCaP using an enzyme-linked immunosorbent assay kit (Anogen, Mississauga, ON, Canada), according to the manufacturer's instructions. For subcutaneous growth, 5×10^6 LNCaP transfectant cells were inoculated in male SCID mice ($n = 5$). X-rays of whole mice were obtained every 2 to 3 weeks with a mammography unit. All procedures were done in compliance with the Animal Investigation Committee of Wayne State University and National Institutes of Health guidelines.

Histomorphometry

Tibiae were fixed and decalcified in formic acid/formaldehyde (Cal-Rite; Richard-Allan Scientific, Kalamazoo, MI). Five-micrometer-thick longitudinal sections were stained with hematoxylin and eosin or immunostained for cytokeratin, as described previously.⁶ Tibiae with no evident intraosseous tumor, as confirmed by immunohistochemistry for cytokeratin, were excluded from the analysis. Digital photomicrographs were captured under $\times 5$ magnification using a Zeiss Axioplan 2 microscope (Zeiss, Göttingen, Germany) equipped with a software-controlled (Axiovision; Zeiss) digital camera. All $\times 5$ -microscopic fields found in each longitudinal section cut through the middle part of the tibiae were analyzed. The jpeg images obtained were then merged to get a panoramic view of the whole sagittal section of the tibia. The percentage occupied by tumor and trabecular and cortical bone in the histological section of the entire tibia was calculated by the software based on the measurement of the corresponding areas in pixels.² In certain cases, only bone regions containing tumor cells were selected to calculate the percentage of tumor-associated trabecular bone tissues. These regions were defined by left and right edges of intraosseous tumor nests, respectively, whereas top and bottom sides were the outer edges of cortical bone included in the segment. Several tumor-associated regions can be found in a whole-bone tissue slide. In all cases, only tibiae that showed intraosseous tumor growth were selected for histomorphometrical analysis.

In Vitro Type I Collagen Degradation

The ability of LNCaP-MT1wt and LNCaP-Neo cells to degrade type I collagen was measured using their lysates (prepared as explained above) with the ENzChek Collagenase Assay kit (Molecular Probes, Eugene, OR), according to the manufacturer's instructions.

In Vitro Differentiation of Preosteoclast-Like Cells

Mouse bone marrow primary cultures or Raw 264.7 cells were used to investigate the osteoclastogenic effect of factors shed by the LNCaP transfectants. In the first case, bone marrow cells isolated from femora and tibiae from male mice were cultured in α -minimal essential medium containing 10% fetal bovine serum and macrophage colony-stimulating factor (100 ng/ml; R&D Systems, Minneapolis, MN) on LUX coverslips (Miles Scientific, Division of Miles Laboratories, Inc., Naperville, IL) in 24-well culture plates.²⁰ Three days later, nonadherent cells were removed, and the cultures were exposed to protein-normalized 48-hour conditioned medium (40%; conditioned medium volume/total culture volume) from either LNCaP-Neo or LNCaP-MT1wt cells, supplemented with fetal bovine serum to reach a final concentration of 0.5%. Seventy-two hours later, the cells were fixed and stained with Diff-Quik kit (Dade Behring, Newark, DE), according to the manufacturer's instructions. As for Raw 264.7 cells, 2×10^4 cells in complete culture medium were seeded in each well on top of LUX coverslips and incubated at 37°C for 24 hours. The coverslips were washed once and then exposed to conditioned medium from LNCaP-Neo or LNCaP-MT1wt, as described above. RANKL (50 ng/ml; PeproTech, Inc., Rocky Hill, NJ) was used as a positive control and 48-hour culture medium (40% final volume) as a negative control. Recombinant human osteoprotegerin (100 ng/ml; Leinco Technologies, St. Louis, MO) was used to block RANKL-induced differentiation of preosteoclasts into osteoclast-like cells. In a different experiment, to confirm the function of MT1-MMP on membrane-tethered RANKL shedding, conditioned media were obtained from LNCaP-Neo and LNCaP-MT1wt cells incubated for 48 hours with 10 μ mol/L 4-[4-(methanesulfonamido) phenoxy] phenylsulfonyl methylthiirane (MIK-G2), an MT1-MMP and gelatinase inhibitor,²¹ or the vehicle (culture medium supplemented with 0.3% polyethylene glycol and 0.1% dimethyl sulfoxide). LNCaP cells do not secrete gelatinases A and B (data not shown), and consequently, MIK-G2 was used in this experiment as a selective inhibitor for MT1-MMP in the LNCaP-MT1wt cells. In all of the wells in which Raw 264.7 cells were exposed to the different conditioned media, fetal bovine serum was added to reach a final concentration of 0.5%. Five days later, the cells were fixed and stained with Diff-Quik kit.

Osteoclast-like differentiation was assessed in digital photomicrographs of either bone marrow primary cultures or Raw 264.7 cells captured under $\times 40$ magnification in five fields at random using a Zeiss Axioplan 2 microscope (Zeiss). Tartrate-resistant acid phosphatase staining was performed to confirm histochemically osteoclast-like differentiation. Briefly, cells were fixed for 10 minutes with 4% paraformaldehyde in phosphate-buffered saline, washed three times with phosphate-buffered saline, and then stained for tartrate-resistant acid phos-

phatase using the Diagnostic Acid Phosphatase kit (Sigma) according to the manufacturer's instructions. All experiments were performed in triplicate.

Statistical Analysis

Data comparing differences between two groups were statistically analyzed using unpaired Student's *t*-test. Multiple comparisons were made using one-way analysis of variance with Tukey-Kramer post-testing. Differences were considered significant when $P < 0.05$.

Results

MT1-MMP Is Highly Expressed in Prostate Cancer Bone Metastasis

Paraffin sections of bone metastases obtained from 20 prostate cancer patient autopsies were immunostained for MT1-MMP. As shown in Figure 1, A and C–E, all of the specimens exhibited strong immunolocalization of MT1-MMP to prostate cancer cells. In areas of bone tissue without metastatic tumor, MT1-MMP immunoreactivity was also detected in endothelial cells, osteocytes, stromal cells, and osteoblasts (Figure 1F).^{12,22–24}

Ectopic MT1-MMP Expression in Human Prostate Cancer Cells Leads to Enhanced Intraosseous Tumor Growth and Osteolysis

LNCaP cells, which lack endogenous MT1-MMP,²⁵ were stably transfected with a vector expressing full-length wild-type human MT1-MMP. As a control, LNCaP cells were also transfected with an empty vector. Expression of MT1-MMP was confirmed by reverse transcriptase-PCR (RT-PCR) and immunoblotting (Figure 2, A and B, respectively) in the LNCaP cells transfected with MT1-MMP cDNA (LNCaP-MT1wt) but not in the control cells (LNCaP-Neo). In accordance with studies from other groups,^{25,26} MT1-MMP mRNA and protein were nondetectable in parental LNCaP cells. Whole-cell lysates of LNCaP-MT1wt cells contained 60- and 57-kD proteins representing the latent and active forms of MT1-MMP, respectively.¹⁸

An antibody-based enzymatic activity assay specific for MT1-MMP demonstrated that LNCaP-MT1wt cells exhibited significantly higher activity than LNCaP-Neo cells (Figure 2C). A series of *in vitro* assays were done to confirm the functionality of the ectopically expressed MT1-MMP. First, we tested the capacity of LNCaP transfectant cells to activate exogenous pro-MMP-2, because these cells did not express endogenous MMP-2.²⁶ As expected, only LNCaP-MT1wt cells were capable of fully activating exogenous recombinant human pro-MMP-2 (Figure 2D). This pro-MMP-2 activation can be ascribed only to the presence of catalytically active MT1-MMP, because levels of TIMP-2, necessary to form the trimeric complex for activation of pro-MMP-2, were similar in both transfectants (data not shown). Then, we studied the

Figure 1. Immunodetection of MT1-MMP in autopsy specimens of bone metastases of prostate cancer patients. **A** and **C-E**: Representative immunohistochemical staining of specimens from different patients showing strong MT1-MMP immunoreactivity in tumor cells. **B**: A serial section of **A** evaluated without the primary antibody as a negative control. **F**: Bone area devoid of tumor cells. Ob, osteoblasts; Oc, osteocytes; St, stromal cells; and En, endothelial cells. Bars = 50 μ m.

Figure 2. MT1-MMP expression and MT1-MMP-associated features in LNCaP transfectants. **A**: RT-PCR analysis of MT1-MMP. Thirty-five cycles of PCR were performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a loading control. **B**: Immunoblot analysis of MT1-MMP. LNCaP transfectant whole-cell lysates were immunoblotted using a specific antibody against MT1-MMP. An MT1-MMP-enriched membrane preparation from non-malignant monkey kidney epithelial BS-C-1 cells was electrophoresed as a positive control, and the housekeeping protein β -actin was also blotted as a loading control. **C**: MT1-MMP activity assay. LNCaP transfectant cell lysates were assessed for MT1-MMP enzymatic activity as described in Materials and Methods. Values depicted are means $\pm$ SE from triplicate determinations. * $P < 0.0003$, Student's *t*-test. **D**: Gelatin zymography of pro-MMP-2 activation process in LNCaP transfectant cell lysates.

ability of LNCaP transfectant cells to grow within a three-dimensional type I collagen lattice. The proliferation of LNCaP-MT1wt cells within the three-dimensional type I collagen matrix was fourfold higher than that of LNCaP-Neo cells ($222,800 \pm 53,620$ versus $54,000 \pm 11,890$ cells/well, respectively, $P = 0.015$). These results were in agreement with studies showing that MT1-MMP confers tumor cells with the ability to grow within a three-dimensional collagen I lattice and that such growth is dependent on the proteolysis of extracellular matrix.²⁷ Conversely, the *in vitro* growth rate of LNCaP-MT1wt and LNCaP-Neo cells cultured on plastic surfaces did not differ, because their doubling times were similar (data not shown).

To investigate the role of MT1-MMP activity in bone tumor growth, the LNCaP transfectants were injected into tibiae of male SCID mice, and intraosseous tumor growth was monitored by serial X-rays and serum prostatic-specific antigen levels. Our goal was to stop the experiment before breach of the cortex by tumor cells to avoid periosteal reactions and to ensure that we were characterizing only intraosseous tumor.

Twelve weeks after intratibial injection, obvious osteolytic lesions were observed by radiography in 89% of the mice injected with LNCaP-MT1wt cells, compared with 11% of the mice injected with LNCaP-Neo cells. The cortex appeared to be intact in all mice with no evidence of periosteal reaction. In addition, serum prostatic-spe-

Figure 3. Tumor growth and bone response in tibiae injected with LNCaP transfectants. **A:** Radiographs of tibiae from male SCID mice that received intratibial injections of LNCaP-Neo or -MT1wt transfectant cells. The mice were sacrificed 12 weeks after tumor cell injection. Note predominant osteolytic lesions in tibiae injected with LNCaP-MT1wt cells. Included are the radiographs of all of the mice involved in the study. **B and C:** Histomorphometric analysis of tibiae injected with LNCaP transfectants. **B:** Percentage of the whole-tissue cross section occupied by bone tissue in tibiae 12 weeks after tumor injection. * $P = 0.0038$, Student's t -test. **C:** Percentage of the whole-tissue cross section occupied by tumor cells in the same tibiae. ** $P = 0.0003$, Student's t -test. Only tibiae with confirmed intraosseous tumor growth (as determined by cytokeratin immunohistochemistry) were analyzed by histomorphometry. Results are presented as means $\pm$ SE. **D:** Representative histology of hematoxylin and eosin-stained longitudinal midsections of tibiae injected with LNCaP-Neo or -MT1wt cells, respectively. In the bone tissue injected with LNCaP-Neo cells, **arrows** indicate tumor cells, and **arrowheads** indicate bone trabeculae. The microscopic image in the LNCaP-MT1wt-induced bone tumor shows a predominance of tumor cells with the absence of trabeculae. Bars = 100 μ m.

sific antigen levels in the mice injected with LNCaP-MT1wt were found to be higher than that in the mice inoculated with LNCaP-Neo cells (41.7 ± 5.1 versus 11.1 ± 1.2 ng/ml, respectively). Therefore, we decided to sacrifice the mice at this time. *Ex vivo* radiological imaging of the tibiae injected with LNCaP-MT1wt cells revealed cortical expansion and intense bone degradation, whereas osteolysis was minimal in the tibiae injected with LNCaP-Neo cells (Figure 3A).

By histology, tumor was found to be contained within the marrow cavity in eight of nine and six of nine mice

intratibially injected with LNCaP-MT1wt and LNCaP-Neo cells, respectively. In mice injected with LNCaP-MT1wt, there was obvious thinning of cortical and trabecular bone. Quantitative histomorphometric analysis confirmed that bone matrix area (cortical plus trabecular) was significantly lower in tibiae injected with LNCaP-MT1wt cells (Figure 3B), demonstrating that tumor-derived MT1-MMP activity is associated with bone matrix resorption. MT1-MMP expression in LNCaP cells also significantly enhanced intraosseous tumor growth. The cross-sectional area occupied by tumor cells was higher in tibiae injected with LNCaP-MT1wt cells than in those injected with LNCaP-Neo cells (Figure 3C). Representative histological images obtained from tibiae inoculated with LNCaP-Neo and -MT1wt transfectants showed an inverse correlation between intraosseous tumor growth and bone area (Figure 3D). Despite the evidence demonstrating an association between bone degradation and MT1wt transfectants, we did not detect a difference in the number of tartrate-resistant acid phosphatase-positive cells between groups (data not shown). A likely reason for this is that osteoclasts may have come and gone by the time the mice were sacrificed. Other potential reasons include increased intraosseous tumor burden in tibiae injected with MT1wt cells and the possibility of a direct effect of cancer-derived MT1-MMP on bone degradation. Importantly, the growth advantage conferred by MT1-MMP was manifested only within the bone microenvironment because tumors generated by subcutaneous injection of the LNCaP transfectants did not differ in their growth kinetics (data not shown).

Down-Regulation of MT1-MMP Reverses Osteolysis Caused by Intraosseous Prostate Cancer Cell Growth

A specific human MT1-MMP siRNA was designed and synthesized for treatment of DU145 cells, which endogenously express substantial levels of MT1-MMP.²⁵ We found a significant reduction of MT1-MMP expression and activity in MT1-MMP siRNA-treated DU145 cells compared with scrambled siRNA-treated DU145 cells (data not shown). Based on the effective MT1-MMP silencing effect observed in the experiment, an MT1-MMP siRNA-expressing DNA insert was designed and constructed into a vector, as described in Materials and Methods. DNA sequencing analysis revealed a 100% base match between the purified insert and the design. DU145 cells were stably transfected with vectors expressing either MT1-MMP siRNA or scrambled siRNA. Then, hygromycin-resistant clones established from pooled populations were selected based on their reduced MT1-MMP expression at both the gene and protein levels. A reduction in MT1-MMP expression was confirmed by RT-PCR (data not shown) and immunoblotting in the selected MT1-MMP siRNA-expressing DU145 clones (DU145-MT1si 1, 5, and 8) compared with scrambled siRNA-transfected cells (Figure 4A). Furthermore, whole-cell lysates obtained from DU145-MT1si clones 1, 5, and 8 revealed a 71.4, 42.6, and 67.3% decrease in

Figure 4. MT1-MMP expression and MT1-MMP-associated features in siRNA-transfected DU145 clones. **A:** Immunoblot analysis of DU145 transfectant clone whole-cell lysates using a specific antibody against MT1-MMP. The housekeeping protein β -actin was used as a loading control. **B:** MT1-MMP activity in cell lysates obtained from DU145-MTsi and DU145-scr-si transfectants was measured as described in Materials and Methods. Values depicted are means $\pm$ SE ($n = 3$). ** $P < 0.001$, analysis of variance, followed by Tukey-Kramer multiple comparisons test. **C:** Enzymatic cleavage of DQ collagen I by cell lysates obtained from DU145-scr-si and DU145-MTsi1 transfectants. Values are means $\pm$ SE ($n = 3$). * $P = 0.01$, Student's t -test.

MT1-MMP activity, respectively, when compared with the control clone (DU145-scr-si) (Figure 4B). *In vitro* assays were performed to confirm the functional significance of MT1-MMP down-regulation. DU145-MT1si1, the clone that showed the greatest degree of MT1-MMP inhibition, had decreased proliferation within three-dimensional type I collagen lattices when compared with DU145-scr-si clone ($33,833 \pm 2315$ cells/well versus $52,333 \pm 882$ cells/well, respectively, $P = 0.0017$). On the other hand, when cultured on plastic surfaces, there were no significant differences in cell proliferation rates between MT1si-transfected DU145 clones and the control DU145-scr-si clones (data not shown). In addition, the degradation of DQ-collagen I achieved by DU145-MT1si1 cell lysates was lower than that by DU145-scr-si lysates (Figure 4C). Although DU145 cells themselves do not express MMP-2,²⁶ both live cells and cell lysates of DU145-MT1si1 demonstrated diminished activation of exogenous pro-MMP-2 compared with scr-si transfectant (data not shown).

The different DU145 clones were injected intratibially in SCID mice, and tumor growth was monitored radiographically. The mice were sacrificed 8 weeks later at a point at which there was no evidence of cortical breach or periosteal reaction. By radiographical analysis, DU145-scr-si cells generated varying degrees of osteolysis and osteosclerotic changes compared with control tibiae not injected with tumor cells (Figure 5A). Tibiae injected with DU145-MT1si transfectants also showed a mixed osteoblastic/osteolytic bone response, but in most cases, the osteosclerotic reaction was more intense than in the DU145-scr-si-injected tibiae. Figure 5A shows *ex vivo*

radiographs of DU145-MT1si1-injected tibiae as a representative group of all of the tibiae injected with the different DU145-MT1si clones.

Histological analysis of tibiae injected with the different DU145 clones showed tumor incidences of 8 of 10 for DU145-scr-si, 10 of 10 for DU145-MT1si1, 8 of 10 for DU145-MT1si5, and 8 of 10 for DU145-MT1si8. Tibiae injected with DU145-scr-si had marked remodeling of trabecular bone in areas adjacent to tumor cells, and a discrete marrow cavity was no longer present. DU145-MT1si-injected tibiae revealed a similar pattern of intraosseous tumor growth, but manifested thickening of the trabecular bone associated with adjacent tumor compared with DU145-scr-si-injected tibiae (Figure 5B). Consistent with this observation, the trabecular bone in both DU145-scr-si- and DU145-MT1si1-injected tibiae was increased with respect to bones not injected with DU145 cells (control) (Figure 5B), suggesting a stimulation of bone formation.

By histomorphometry, intraosseous tumor growth (as measured by tumor cell area/tissue area) tended to be diminished in DU145-MT1si-injected tibiae compared with DU145-scr-si-injected tibiae (Figure 5C). To quantify bone changes associated with MT1-MMP silencing in tumor cells, we calculated trabecular bone area percentages in regions of bone containing tumor cells. We found an increase in the percentage of tumor-associated trabecular bone area in those tibiae injected with DU145-MT1si cells, confirming the radiological and histological findings (Figure 5D). We wished to determine whether MT1-MMP silencing was associated with prevention of bone degradation or with true bone formation. For this purpose, we compared bone mass between tumor-injected bones and contralateral control bones not injected with tumor cells. We found a trend toward increased total bone mass in DU145-scr-si-injected tibiae with respect to control bones (data not shown) and a statistically higher total bone mass in DU145-MT1si-injected tibiae than in control tibiae (9.0 ± 1.0 versus 5.5 ± 0.7 mm², respectively, $P < 0.05$, Tukey-Kramer multiple comparison test). These findings strongly suggest that MT1-MMP silencing in prostate cancer cells is associated with new bone formation.

Prostate Cancer-Associated MT1-MMP Induces Type I Collagenolysis and Osteoclast Formation *In Vitro*

To investigate the mechanism(s) by which tumor-derived MT1-MMP might induce osteolysis, we focused on two known activities of MT1-MMP: its ability to degrade collagen I¹⁰ and the possibility that MT1-MMP cleaves membrane-anchored RANKL, a potent osteoclastogenesis factor.²⁸ As shown in Figure 6A, LNCaP cells expressing wild-type MT1-MMP accomplished the degradation of DQ collagen I more efficiently than LNCaP-Neo cells as a function of time. As mentioned earlier, MT1-MMP-silenced DU145 cells degrade type I

Figure 5. Tumor growth and bone response in tibiae injected with DU145 transfectants. **A:** Radiographs of tibiae from mice sacrificed 8 weeks after DU145 cell injection. Osteosclerotic changes can be observed in the majority of the tibiae injected with DU145-MT1si1 cells. Control bones are tibiae that were not injected with DU145 cells. **B:** Representative H&E-stained sections of tibiae injected with DU145-scr-si or DU145-MT1si1 cells and control tibiae with no DU145 cell injection at the end of week 8. Note the marked remodeling of trabecular bones in areas adjacent to DU145-scr-si tumor cells. New bone formation is evident in DU145-MT1si1-injected tibiae compared with the other two groups. Bars = 100 μ m. **C** and **D:** Histomorphometric analysis of tibiae injected with DU145 clones. Only tibiae with confirmed intraosseous tumor growth (as determined by cytokeratin immunohistochemistry) were analyzed by histomorphometry. Results are presented as means $\pm$ SE. **C:** Percentage of the whole-tissue cross section occupied by tumor cells in tibiae 8 weeks after DU145 injection. **D:** Percentage of trabecular bone in areas where tumor was present. T.A., tumor-associated. * $P < 0.05$, Tukey-Kramer post test ($P = 0.025$, analysis of variance).

collagen less efficiently than controls (Figure 4C). These studies suggest that tumor-derived MT1-MMP may contribute directly to bone matrix remodeling.

We next evaluated the hypothesis that tumor-derived MT1-MMP releases sRANKL, leading to preosteoclast differentiation. In the normal bone microenvironment, RANKL is produced by stromal cells and osteoblasts. However, LNCaP cells^{29,30} and primary human prostate cancer³¹ are known to express RANKL. RT-PCR analyses confirmed similar expression levels of RANKL mRNA in both LNCaP-MT1wt and LNCaP-Neo cells (Figure 6B). Immunoblot analyses of whole-cell lysates demonstrated that LNCaP-MT1wt cells exhibited reduced levels of full-length membrane bound RANKL (~40 kD) when compared with LNCaP-Neo cells (Figure 6C). An additional lower band molecular weight protein of ~37 kD was also detected. However, detec-

tion of this smaller protein was inconsistent, and its nature remains unclear.

The reduced levels of membrane-tethered RANKL protein in the LNCaP-MT1wt whole-cell lysates suggested the possibility of shedding of RANKL. Although we used several techniques [multiple specific antibodies, concentration of conditioned medium (CM), and enrichment of RANKL in CM with osteoprotegerin (OPG)-Fc chimeric protein/precipitation with protein G agarose], we were unable to detect sRANKL. The reasons for this are unknown, but we suspect that sRANKL may have biological function at concentrations too low to be detected by our techniques. Instead, RANKL shedding by MT1-MMP-expressing cells was detected indirectly by measuring the osteoclastogenic effect of conditioned medium. We first examined the ability of CM from the LNCaP transfectants to induce *in vitro* osteoclast differentiation using bone marrow primary cultures. As can be seen

Figure 6. Prostate cancer-associated MT1-MMP degrades type I collagen and sheds RANKL. **A:** Enzymatic cleavage of quenched-fluorescent derivative of type I collagen (DQ collagen I) by protein-normalized cell lysates from LNCaP-Neo (■) or -MT1wt (▲) transfectants. Values depicted are means $\pm$ SE ($n = 3$). *** $P < 0.001$, Student's *t*-test. **B:** Detection of RANKL mRNA by RT-PCR analysis. After 35 cycles of PCR, similar levels of RANKL (377-bp product) were observed in LNCaP transfectants. GAPDH loading controls are shown in the bottom panel. **C:** Immunoblot of membrane-bound RANKL (~40 kD) in whole-cell lysates, showing lower expression in LNCaP cells transfected with MT1-MMP. β -Actin was used as an internal loading control (bottom). **D:** Conditioned medium (CM) from LNCaP cells transfected with wild-type MT1-MMP enhances significantly the formation of osteoclasts (tartrate-resistant acid phosphatase-positive multinucleated cells) in mouse bone marrow cells cultured with macrophage colony-stimulating factor compared with control LNCaP-Neo CM. **E:** Tumor-associated MT1-MMP promotes osteoclastogenesis *in vitro* in cultures of the macrophage/monocyte precursor mouse cell line Raw 264.7, and this effect is inhibited by OPG. **F:** The enhanced *in vitro* osteoclast formation by soluble factors released from LNCaP cells transfected with MT1-MMP is inhibited by the MT1-MMP inhibitor MIK-G2. Values depicted are means $\pm$ SE ($n \geq 6$). For comparison between more than two groups, * $P < 0.05$; ** $P < 0.01$, and *** $P < 0.001$ Tukey-Kramer post hoc applied to significant effect of group analysis of variance.

in Figure 6D, CM from LNCaP cells transfected with wild-type MT1-MMP induced a significant increase in osteoclastogenesis compared with CM from LNCaP-Neo control cells (Figure 6D). These data were confirmed with the macrophage/monocyte precursor mouse Raw 264.7 cells,²⁹ a cell line commonly used to model osteoclast differentiation. Furthermore, as can be seen in Figure 6E, osteoclastogenesis induced by CM from LNCaP-MT1wt cells was abrogated (>50%) by the addition of OPG, a specific soluble decoy receptor for RANKL.³² The data demonstrate, albeit indi-

rectly, that the CM from LNCaP-MT1wt cells contained higher levels of biologically active sRANKL than the CM from LNCaP-Neo cells. To examine further the role of MT1-MMP activity in the release of sRANKL, we used a synthetic MMP inhibitor (MIK-G2), a derivative of SB-3CT, that was shown to inhibit both gelatinases and MT1-MMP with high affinity.²¹ As shown in Figure 6E, the CM from LNCaP-MT1wt cells collected in the presence of MIK-G2 (10 μ mol/L) significantly reduced the number of osteoclasts-like cells in the RAW 264.7 cul-

tures. This inhibition was not due to an effect of the inhibitor on the RAW 264.7 cells because MIK-G2 had no effect on preosteoclast-like differentiation in the presence of recombinant sRANKL. Furthermore, the LNCaP cells used in these assays do not express gelatinases (data not shown), indicating that the observed MIK-G2 effect was mediated by MT1-MMP. Taken together, these *in vitro* studies suggest that the osteoclastogenic effect of the LNCaP-MT1wt CM is consistent with a release of sRANKL by MT1-MMP. Although these *in vitro* observations cannot be extrapolated directly to the *in vivo* situation, they provide support to the hypothesis that MT1-MMP is a physiological mediator of osteoclastogenesis.

Discussion

It is well established that various proteases play important roles in invasion, angiogenesis, and metastasis in cancer^{33,34} and that many of these enzymes assist in osteolysis associated with bone metastases derived from different neoplastic tissues.^{35,36} In the case of prostate cancer, although most osseous metastases present an overall osteoblastic radiographical appearance, histological, radiographical, and biochemical analyses reveal that osteoblastic and osteolytic responses coexist.³⁷ Previously, we reported that broad-spectrum, pharmaceutical MMP inhibition led to diminished tumor cell proliferation and bone degradation in a model of prostate cancer-bone interaction.³ We then demonstrated that net MMP-9 tissue activity was up-regulated during early stages of bone colonization by prostate cancer cells⁶ and that its inhibition leads to significant inhibition of intraosseous prostate tumor angiogenesis and growth, as well as reduced osteolysis.³⁸ There are, however, no reports on the role of MT1-MMP in prostate cancer bone metastasis, despite the contribution of this unique membrane-bound MMP in processes that are essential in cancer progression.^{8,39} We previously reported a heterogeneous expression of MT1-MMP in primary prostate cancer tissue.¹⁴ Interestingly, some malignant glands expressed high levels of MT1-MMP protein, whereas others did not. In contrast, the results from the current study demonstrated an abundant and uniform expression of MT1-MMP in prostate cancer cells metastatic to bone. Together, these data suggest a correlation between MT1-MMP expression in prostate cancer cells and cancer progression and bone metastasis.

To evaluate further the biological consequences of prostate cancer-derived MT1-MMP in the bone microenvironment, we introduced the MT1-MMP gene into a human prostate cancer cell line with undetectable MT1-MMP mRNA and protein. We also down-regulated MT1-MMP expression by siRNA silencing in another prostate cancer cell line with high intrinsic MT1-MMP expression. The over- and underexpression of MT1-MMP in the prostate cancer cell transfectants efficiently enhanced and reduced, respectively, cell functions that usually are associated with MT1-MMP. We then inoculated these cells into the tibiae of immunodeficient mice as a means of

modeling growth and expansion of the metastatic deposit. Through these analyses, we found that prostate cancer cell-derived MT1-MMP enhanced intraosseous tumor growth and bone degradation. The observation that modulation of MT1-MMP expression did not affect subcutaneous growth of tumor or the proliferation of the cells *in vitro* suggests that tumor-derived MT1-MMP contributes a unique stimulatory effect with regard to tumor growth in the bone microenvironment. Interestingly, silencing of MT1-MMP not only inhibited the ability of DU145 prostate cancer cells to grow within bone but also promoted osteogenesis. The mechanism underlying bone formation associated with MT1-MMP inhibition remains undefined. However, because turnover of bone matrix is an ongoing process involving both bone formation and degradation, we suggest that MT1-MMP inhibition may have shifted the balance toward bone formation simply by inhibition of osteolysis/osteoclastogenesis. A caveat of the *in vivo* studies is that the animal model did not replicate primary tumor growth in the prostate and the process of hematogenous dissemination of tumor cells. In this regard, Cao et al⁴⁰ showed enhanced local tumor invasion, lymph node metastasis, and lung metastasis on orthotopic injection of MT1-MMP-expressing prostate cancer cells into the prostate.

The *in vitro* studies revealed that the level of MT1-MMP expression and activity in prostate cancer cells correlated with the ability to degrade type I collagen. These data suggest a direct effect of prostate cancer-associated MT1-MMP on bone degradation and may explain, in part, the proclivity of prostate cancer to thrive in the bone microenvironment.

Bone degradation occurs as a result of removal of both the mineral and nonmineral components of bone matrix. Although the MT1-MMP activity of cancer cells may contribute directly to the degradation of the surface osteoid layer, mainly composed of type I collagen, it is likely that osteoclasts are required for dissolution of mineralized bone matrix. We hypothesized that the enhanced osteolytic response induced by LNCaP cells overexpressing MT1-MMP could also be due to the shedding of RANKL, an essential mediator of osteoclastogenesis. Because LNCaP cells express RANKL on their membranes, nearby MT1-MMP could be responsible for sheddase activity. In fact, MT1-MMP has been reported to be implicated in RANKL ectodomain shedding in *in vitro* systems.²⁸ We found that our various LNCaP transfectants had similar expression of RANKL at the gene level, but the membrane form of RANKL was reduced in the MT1-MMP-expressing LNCaP cells at the protein level, supporting the notion that RANKL is shed from the cancer cell surface because of MT1-MMP activity. *In vitro* osteoclastogenesis assays further confirmed this hypothesis, because LNCaP-MT1wt-derived conditioned medium significantly induced differentiation of preosteoclasts. Moreover, OPG, a soluble decoy receptor for RANKL,³² reverted the osteoclastogenic effect revealed by LNCaP-MT1wt-derived conditioned medium to baseline levels. Interestingly, OPG did not completely abolish osteoclastogenesis; thus other cancer-derived factors may contribute to osteoclastogenesis, bypassing the RANKL path-

way.⁴¹ The use of an MT1-MMP inhibitor was also capable of inhibiting the osteoclastogenic effect of LN-CaP-MT1wt-derived conditioned medium, indicating that MT1-MMP activity is essential to release RANKL from the cancer cell surface. A recent report showed that MMP-7, produced mainly by osteoclasts at the prostate tumor-bone interface, can also act as a RANKL sheddase and promote osteolysis.⁴² The data in our study suggest an additional mechanism wherein both an MT1-MMP-associated sheddase activity and RANKL may be brought into the bone microenvironment by the tumor cells. Although this hypothesis needs to be corroborated *in vivo*, our studies revealing MT1-MMP expression by prostate cancer cells metastatic to bone in clinical samples together with others who have reported RANKL expression in up to 100% of prostate cancer cells in skeletal metastases from patients⁴³ strongly suggest that possibility. This implies that cell-cell contact between osteoclast precursors and osteoblast/stromal cells may not be necessary because cancer cells rather than bone cells may serve as a source of sRANKL that may reach and activate osteoclast precursors. Nonetheless, this mechanism is not inconsistent with the possibility that bone-derived MT1-MMP is involved in RANKL release from either stromal cells or tumor cells.

In summary, we showed that prostate cancer cell-associated MT1-MMP may contribute to enhanced intraosseous tumor proliferation and osteolysis. Furthermore, MT1-MMP inhibition in prostate cancer cells may result not only in protection from bone degradation but also in an osteogenic effect. Our observation that bone metastases from prostate cancer patients have high and uniform MT1-MMP expression, in concert with our previous studies revealing heterogeneous expression of MT1-MMP in primary prostate cancer tissue, strongly supports the hypothesis that MT1-MMP contributes to human clinical prostate cancer progression and metastasis. Based on our data, MT1-MMP activity may be a promising target for therapeutic intervention in prostate cancer patients.

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