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Matrix metalloproteinase-1 promotes breast cancer angiogenesis and osteolysis in a novel in vivo model

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Abstract

Matrix metalloproteinase-1 (MMP-1) is critical for mediating breast cancer metastasis to bone. We investigated the role of MMP-1 in breast cancer invasion of soft tissues and bone using human MDA MB-231 breast cancer cells stably transfected with shRNAs against MMP-1 and a novel murine model of bone invasion. MMP-1 produced by breast cancer cells with control shRNA facilitated invasion of tumors into soft tissue in vivo, which correlated with enhanced blood vessel formation at the invasive edge, compared to tumors with silenced MMP-1 expression. Tumors expressing MMP-1 were also associated with osteolysis in vivo, whereas tumors with inhibited MMP-1 levels were not. Additionally, tumor-secreted MMP-1 activated bone-resorbing osteoclasts in vitro. Together, these data suggest a mechanism for MMP-1 in the activation of osteoclasts in vivo. We conclude that breast cancer-derived MMP-1 mediates invasion through soft tissues and bone via mechanisms involving matrix degradation, angiogenesis, and osteoclast activation.

Keywords

Breast cancer; Bone; Invasion; Metastasis; MMP; Murine model

Introduction

Breast cancer cells have a high propensity to metastasize to bone [1–3]; and 65–75% of breast cancer patients with advanced disease develop bone metastases [4]. Once in the bone,

breast cancer cells stimulate bone matrix turnover, causing the release of growth factors embedded within the mineralized matrix, thereby promoting tumor growth [5]. High matrix turnover also results in osteolysis, and as the bone is destroyed, breast cancer patients suffer from painful fractures, hypercalcemia, and nerve compression [6]. Although many studies have focused on breast cancer metastasis to bone, breast cancer also directly invades the soft tissues, ribs and sternum bones of the chest. Following mastectomy, 10–20% of patients develop local/regional recurrence (LRR), a recurrent tumor in the skin, subcutaneous tissue, muscle, or bone within the chest wall [7]. The molecular mechanisms by which breast cancer cells invade interstitial tissues in LRR and induce osteolysis in patients with either metastatic disease or LRR remain unclear.

The matrix metalloproteinase (MMP) family consists of over 20 enzymes that degrade the extracellular matrix. Physiologic levels of MMPs are low, but expression is elevated in most cancers [8, 9]. Matrix metalloproteinase-1 (MMP-1) is an interstitial collagenase that is often upregulated in breast cancer, and in vitro studies demonstrate that this enzyme facilitates the invasive properties of breast cancer cells [10, 11].

Bone is predominantly comprised of mineralized fibrillar type-I collagen [12, 13], which in its soluble form, is degraded by the interstitial collagenases, namely, MMP-1 [14, 15]. Recently, Massague's group identified an osteotropism gene set in which MMP-1 is one of four genes consistently overexpressed in breast cancer cells [16]. Their data demonstrate that MMP-1 is essential for metastasis to bone and raise the question of whether breast cancer-derived MMP-1 also contributes to local invasion and to tumor-induced osteolysis once cancer cells colonize the bone.

Resorption of bone requires the dissolution of mineralized and soluble matrix components, a process that is accomplished primarily by multinucleated, bone-residing osteoclast cells, which require activation and differentiation. It is well established that osteoclasts are activated by the binding of receptor activator of nuclear factor kappa B ligand (RANKL) to its receptor, RANK, on osteoclasts [17]. In vitro studies using general MMP inhibitors and synthetic collagen suggest that interstitial collagenases also aid in this process by cleaving collagen, thereby generating collagen fragments, which then activate osteoclasts [18]. Following activation, an osteoclast attaches to the bone surface and seals a compartment, known as a lacuna, into which it secretes proteolytic enzymes [19–21], including tartrate-resistant acid phosphatase (TRAP), which degrades mineralized bone, and leaves soluble type-I collagen [17]. The lysosomal protease, cathepsin K, functions in this acidic pH to partially degrade remaining collagen products [22, 23] and MMPs, which are active at neutral pH, degrade leftover collagen after the osteoclast has retreated in in vitro experiments [24, 25]. These studies provide evidence that MMPs may aid in bone resorption, but the role(s) of tumor-derived MMP-1 in the process of bone resorption has not been investigated. Interestingly, MMP-1 has been localized to osteoclasts and their lacunae [26], and bone-resorbing agents stimulate collagenase production in the bone [27]. These findings imply that collagenases play a role in breast cancer-induced bone resorption in vivo by cleaving collagen, thereby producing collagen fragments that activate osteoclasts. To determine the function(s) of MMP-1 in the invasion and destruction of bone, we created a novel murine breast cancer xenograft model.

Several approaches have been used to study breast cancer/bone interactions and/or bone metastasis in vivo. Intratibial injections allow researchers to study interactions between tumors and cells in the bone microenvironment; this procedure, however, stimulates a bone remodeling response that can complicate interpretation of results [13]. Arguello and colleagues described a model in which tumor cells are injected into the left cardiac ventricle [28]. This technique requires cancer cells to extravasate and settle in a secondary location,

such as bone; however, these injections are technically challenging [13] and yield a low tumor incidence (~30%) over 10–12 weeks [16]. Pollard and Luckert described a rat model to investigate tumor induced osteolysis, in which prostate tumor cells that arose spontaneously in the dorsolateral lobes were transplanted to an area adjacent to bone, but the periosteum was scratched during inoculation to enhance tumor cell attachment to bone [29].

In our model, breast cancer cells are injected into the hind limb musculature, adjacent to, but not contacting, the femur. This model is technically simple, reproducible, and avoids a needle-induced bone remodeling response. Using this model and RNAi technology we investigated the role of breast tumor-secreted MMP-1 in the invasion of soft tissues and the bone. We demonstrate that MMP-1 mediates invasion of tumor cells through peritumoral soft tissues and enhances osteolysis *in vivo*. We propose that these events are facilitated by vasculature recruitment and osteoclast activation.

Materials and methods

Culturing of cells

MDA MB-231 breast cancer cells (American Type Culture Collection, ATCC, Rockville, MD) and human microvessel endothelial cells (HMVECs, Cascade Biologics, Portland, OR) were cultured as described [30]. RAW264.7 cells (ATCC) were cultured in MEM, supplemented with 10% FBS, penicillin (100 units/ml)/streptomycin (100 lg/ml), and 2 mmol/l glutamine. For serum-free conditions, cells were rinsed with HBSS and cultured in their respective media, supplemented with 0.2% lactalbumin hydrolysate (LH).

Short hairpin RNA plasmids

Construction of short hairpin RNA (shRNA) plasmids were described [31]. In this study, three different MMP-1 shRNA oligonucleotides (shRNA 5', 153, or 305) were ligated into the pSUPER.retro.puro expression vector (OligoEngine, Seattle, WA). Each shRNA targeted a specific region of the MMP-1 mRNA sequence: starting at base 26 in the 5' untranslated region of the MMP-1 mRNA, or at base 153 or 305 of the MMP-1 mRNA coding region. shRNA sequences used were: MMP-1 (5'): 5'-GGAAGC CATCACTTACCTTGC-3'; MMP-1 (153): 5'-GAGCAA GATGTGGACTTAG-3'; and MMP-1 (305): 5'-ACCA GATGCTGAAACCCTG-3'. MMP-1 shRNA (153) and shRNA (305) have been described [31, 32] and the 5' MMP-1 shRNA was designed using Block-iT (Invitrogen, Carlsbad, CA). A non-specific mammalian sequence (MAMM-X, OligoEngine) was inserted into the pSuper-retro-puro vector as an shRNA control.

Stable transfections

Each shRNA plasmid was transfected into MDA MB-231 breast cancer cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Cells were selected with puromycin-supplemented (1.5 µ/ml) DMEM 50/50-F12; surviving colonies were expanded [31] and analyzed for MMP-1 mRNA expression using quantitative real-time RT-PCR (see below). The three control shRNA clones that best mirrored parent MDA MB-231 MMP-1 mRNA levels and the three MMP-1 shRNA clones with >90% inhibition of MMP-1 (compared to the control group) were chosen for experiments. We measured MMP-2, -3, -9, -13 and -14 and 2', 5' oligoadenylate synthetase 1 (OAS1) mRNA levels in the parental cells and stably transfected derivatives as described [31]. Clones were cultured individually, but were pooled immediately prior to experiments.

Quantitative real-time RT-PCR

Real-time PCR reactions were performed using 250 ng of input RNA per reaction as described previously [30, 31, 33, 34]. mRNA was quantified using standard curves generated using serial log dilutions of a cDNA plasmid containing the gene of interest and ranged from 1 to 1,000 pg. Data were averaged and normalized to the housekeeping gene, hypoxanthine-phosphoribosyl transferase (HPRT), and are presented as a percent of the MDA MB-231 cells or fold inhibition. Primer sequences were: MMP-1: 5'-AGCTA GCTCAGGATGACATTGATG-3' (forward) and 5'-GCC GATGGGCTGGACAG-3' (reverse) and HPRT: 5'-AGCT TGCTGGTGAAAAGGAC-3' (forward) and 5'-CCAGAT GTTTCCAAACTCAACTTGA-3' (reverse). Primer sequences for other MMPs were provided previously [31].

Immunoblotting

MDA MB-231 cells were cultured in serum-free media for 24 h, media were precipitated, and MMP-1 protein was analyzed by SDS-PAGE [31, 34]. Blots were probed with an MMP-1 antibody (Chemicon, Billerica, MA) diluted at 1:5,000.

Proliferation of clones

Proliferation rates of transfected cells were compared to parental cells by pooling an equal number of cells from each clone and plating 5×10 cells per well of a 12-well culture plate. At 24, 36, 48, 72, or 96 h, cells were detached with trypsin and viable cells were counted using trypan blue exclusion.

Collagen degradation assay

Control or MMP-1 shRNA transfected cells were serumstarved (24 h), harvested, and pooled. Cells (5×10 cells/ml) were suspended in neutralized type-I collagen (1 mg/ml) (Organogenesis, Canton, MA) [35], and 1 ml was added per well of a 6-well plate. After the collagen polymerized at 37°C, 1 ml of serum-free DMEM containing ~0.0003% trypsin to activate latent MMPs was added to each well. After 24 h at 37°C, the overlying media were removed and weighed. The medium released from a collagen gel was calculated by weighing the total media collected minus the original medium added (1 g). Assays stained for MMP-1 using immunohistochemistry (IHC) were terminated at 6 h, so that gels embedded with control shRNA cells were not entirely degraded. Collagen gels were removed from wells with a rubber spatula, fixed in 10% neutral buffered formaldehyde and processed for histological staining in the Pathology Department at Dartmouth Hitchcock Medical Center (DHMC). Goat MMP-1 antibody (R&D Systems, Minneapolis, MN) was diluted to 1:100.

In vivo tumorigenesis

In groups of eight, female nude mice (*nu/nu* Charles River, Wilmington, MA) were injected intramuscularly in the left hind limb with 1×10^6 cells containing pools of control shRNA or MMP-1 shRNA clones. The femurs in *nu/nu* mice are visible through the skin; therefore it was possible to deposit cells near to, but without contacting, this bone. Cells were suspended in 100 μ l Growth Factor Reduced Matrigel (BD Biosciences, San Jose, CA). Mice were housed in the Animal Resource Center at Dartmouth Medical School until sacrificed on day 7, 14, 21, or 28 postinoculation. Upon sacrifice, the tumor-bearing and contralateral limbs were harvested for histological analysis. A portion of each tumor (~0.25 g) obtained on day 28 was placed in RNeasy Lysis buffer (Qiagen), flash-frozen in liquid nitrogen, and ground into powder using a mortar and pestle. RNeasy RLT lysis buffer (Qiagen) was added immediately, total RNA was harvested, DNase-treated and analyzed as described under

Quantitative real-time RT-PCR. The Dartmouth College Institutional Animal Care and Use Committee approved animal studies.

Histological analysis

Following sacrifice, femurs were decalcified in Nitric (Decal Chemical Corp., Tallman, NY), cut in a cross-section or longitudinal plane, and fixed in formaldehyde. Samples were processed and stained with hematoxylin and eosin (H&E). The number of invasive tumor protrusions, defined as a substantial portion of tumor (>200 μm in length) that jutted away from the round-shaped portion of the tumor, was counted per tumor. A pathologist quantified tumor invasion of the bone. A tumor was considered to have invaded the bone if tumor cells breached the outermost, or periosteal, layer of the bone. Invasion of bone was defined as a positive (1), or a null (0) event. Tumor vasculature was quantified by staining endothelial cells with a rabbit polyclonal CD31 antibody (AbCam, Cambridge, MA) diluted 1:100. Vessels were counted in three fields (20 $\times$) [36] per tumor.

Tube formation assay

To condition media, clones expressing control or MMP-1 shRNAs were pooled and plated (1×10^6 cells/100 mm dish). After 24 h, 5 ml basal medium, consisting of Medium 132 (Cascade Biologics) supplemented with 2% FBS, was added to each plate and conditioned for an additional 24 h. For the tube formation assay, 50 μl Growth Factor Reduced Matrigel[®] (BD Biosciences) was added per well of a 96-well plate and allowed to polymerize. Then, 7.5×10^4 HMVECs in 100 μl of (1) control shRNA cell conditioned media (CM), (2) control shRNA CM with an MMP-1 neutralizing antibody (1 $\mu\text{g}/\text{ml}$, Chemicon, MAB13402) or (3) MMP-1 shRNA CM were added per well. Tube formation was quantified by averaging the number of branch points in 3 views per well, with 9 wells per condition at 20 $\times$.

Osteoclastogenesis assay

To condition media, control or MMP-1 shRNA clones were pooled and plated at 1×10^4 cells/100 mm dish. After 24 h, 5 ml MEM media, supplemented with 0.2% LH, was added to each plate for 24 h. To make non-soluble, cross-linked collagen, collagen-coated plates (BD Biosciences) were subjected to ultra-violet irradiation overnight. For the osteoclastogenesis assay, murine monocytic RAW264.7 cells were resuspended (5×10^4 cells/ml), and 1 ml was plated per 24 mm-well of collagen-coated plates. After 24 h, RAW264.7 cells were left untreated in MEM with LH, or treated with (1) RANKL (50 ng/ml, R&D Systems) and M-CSF (20 ng/ml, R&D Systems), (2) control shRNA CM on soluble collagen or fixed collagen, (3) control shRNA CM with an MMP-1 neutralizing antibody (1 $\mu\text{g}/\text{ml}$, Chemicon), or a control, anti-FLAG antibody (1 $\mu\text{g}/\text{ml}$, Sigma), (4) MMP-1 shRNA CM, or (5) activated or latent recombinant MMP-1 (rMMP-1, R&D Systems). Following 3 days at 37°C, cells were stained for TRAP, using a kit as per the manufacturer's instructions (Sigma). To examine TRAP staining, counterstaining with hematoxylin was unnecessary. To identify multinucleated cells, the hematoxylin counterstain was used, as the manufacturer directs. Mature osteoclasts (TRAP⁺ and multinucleated, >2 nuclei) were counted and averaged in 9 views per triplicate group using the 40 $\times$ objective.

Statistical analysis

Student's *t* test was used to determine statistical significance. All samples were prepared in triplicate and experiments were conducted at least three times. Statistical power at $P < 0.05$ was considered significant.

Results

shRNA stable inhibition of MMP-1

We created four groups of MDA MB-231 breast cancer cells, stably transfected with one of three shRNAs targeting MMP-1, or a control sequence. A pool of three MDA MB-231 clones expressing the control shRNA maintained mRNA levels similar to parental MDA MB-231 cells (Fig. 1a). MMP-1 mRNA levels of the MMP-1 shRNA 153 group were significantly decreased to ~ 10% of control levels, while MMP-1 shRNAs 305 and 5' decreased MMP-1 mRNA levels to ~ 50% of the control, (* $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0001$) (Fig. 1a). Secreted MMP-1 protein from clones mirrored mRNA levels at 24 h (Fig. 1a). MMP-1 was secreted in its latent form, but can be autoactivated by these cells [37] or other proteinases present in vivo [38]. Since the MMP-1 shRNA 153 pool of clones was the most effective at silencing MMP-1 mRNA levels, it was used in remaining experiments (MMP-1 shRNA).

To verify shRNA specificity, we measured mRNA in our clones for MMP-2, -3, -9, -13 and -14, which are associated with tumor progression [9]. mRNA levels of these MMPs were not influenced by the MMP-1 shRNA, compared to the parental (data not shown and [31]). Further, MMP-1 was more highly expressed (copies per copy housekeeping gene) than other MMPs (data not shown and [31]). To ensure that shRNAs did not induce an interferon response [39], OAS1 mRNA was quantified [40] and was unchanged in cells stably expressing shRNAs compared to parental cells (data not shown).

MMP-1 shRNA prevented degradation of type-I collagen in vitro

Since MMP-1 contributes to breast tumor invasion by degrading type-I collagen in vivo [41], we recapitulated this in vitro using a functional assay [30, 31, 33]. Gels embedded with control shRNA cells were completely destroyed (Fig. 1b). Approximately 800 μ l of media was liberated from these gels; however, collagen gels remained intact when containing MMP-1 shRNA cells, $P < 0.0001$ (Fig. 1b). Negative values on the y-axis result from evaporation, and for this reason the original 1,000 μ l is not fully recovered when the entire gel has dissolved. To monitor secreted MMP-1 protein within the collagen matrix, the assay was stopped at 6 h and stained for MMP-1 using IHC. Control shRNA cells stained positively for MMP-1, while only a few MMP-1 shRNA cells stained partially, despite the equal number of cells in each gel (Fig. 1c). Importantly, collagen surrounding control shRNA cells displayed punctate MMP-1 staining throughout (arrowhead), indicating that these cells secrete MMP-1; in contrast, MMP-1 was not present in the matrix surrounding MMP-1 shRNA cells (arrowhead) (Fig. 1c). These results demonstrate that MDA MB-231 cells with silenced MMP-1 expression have significantly reduced MMP-1 mRNA levels, protein levels, and enzymatic activity, compared to cells expressing MMP-1. These data are consistent with the traditional role of MMP-1 in matrix degradation.

Tumor invasion through soft tissue was attenuated in tumors with silenced MMP-1

To test whether silencing tumor-derived MMP-1 decreases tumor invasion in vivo, control or MMP-1 shRNA cells were injected intramuscularly into the hind limb of nude mice, (see Materials and methods). Prior to injection, proliferation rates of clones were compared and found to be similar (Fig. 2a).

Eight mice from each group (control or MMP-1 shRNA) were sacrificed 7, 14, 21, or 28 days post-injection, and the hind limbs were removed for histological examination. H&E staining of the tumor/femur region revealed that by day 7, mice injected with control shRNA cells formed tumors with invasive fronds (Fig. 2b, upper left panel) and longer protrusions (>200 μ m) (Fig. 2b, lower left panel). These invasive outcroppings appeared to be growing

between muscle fascicles and may be positioned in parallel with the bone; however, we observed that they had a propensity to invade toward bone, rather than in any other direction (data not shown). In contrast, most MMP-1 shRNA tumors displayed a well-defined capsular space (Fig. 2b, right panels). Seven days post-injection, the number protrusive outcroppings ($>200\ \mu\text{m}$) from control shRNA tumors was significantly greater than that observed in MMP-1 shRNA tumors, $P = 0.014$ (Fig. 2c). Although control tumors maintained a more invasive phenotype on days 14 and 21 (data not shown), the overall number of protrusions was not significantly different from MMP-1 shRNA tumors. These data allow us to conclude that silencing MMP-1 expression in breast cancer cells makes them less invasive through soft tissues.

MMP-1 shRNA tumors displayed decreased blood vessel density early in tumorigenesis

Upon necropsy, MMP-1 shRNA tumors were macroscopically less vascularized than control shRNA tumors (data not shown), despite that all tumors were similar in size. Because angiogenesis provides nutrients essential for tumor invasion [42], we hypothesized that MMP-1 may allow control shRNA tumors to invade surrounding tissues by enhancing vessel formation [42–44]. Using an endothelial cell specific marker, CD31, for evaluation of tumor vasculature, we found that control shRNA tumors had a high density of vessels (see arrows, Fig. 3a, left panel) at an invasive edge (Fig. 3a, left panel), but vasculature appeared reduced in MMP-1 shRNA tumors (Fig. 3a, right panel). Quantification confirmed that microvessel density in MMP-1 shRNA tumors was significantly decreased compared to control shRNA tumors, $P < 0.0001$ (Fig. 3b). In addition, it is worth noting that the lumens of blood vessels in control shRNA tumors were greater in diameter than those in MMP-1 shRNA tumors (Fig. 3a), suggesting that control tumors were exposed to increased blood flow [45]. We postulated that the increased presence of tumorigenic factors, nutrients and chemoattractants within these vessels facilitated the invasive phenotype of control shRNA tumors. In agreement with increased vessel formation, we found that the presence of chemokine stromal derived factor-1 (SDF-1), expression correlated with vessel density, as demonstrated by IHC (data not shown).

To further examine whether tumor-secreted MMP-1 increases angiogenesis, we used a standard in vitro tube formation assay [46, 47], in which activated endothelial cells plated on Matrigel differentiate to form tube-like networks, while inactive cells do not. After 2 days, we observed that the addition of control shRNA cell conditioned medium (CM) to HMVECs stimulated the formation of more tube-like structures than HMVECs treated with MMP-1 shRNA CM or with control shRNA CM containing an MMP-1 neutralizing antibody (Fig. 3c). Quantification of branch points [45] revealed that HMVECs exposed to control shRNA CM in the absence or presence of a control antibody formed complex networks. On the contrary, the number of branch points formed by HMVECs exposed to control shRNA CM with an MMP-1 neutralizing antibody or MMP-1 shRNA CM was significantly reduced, $P < 0.05$ (Fig. 3d), confirming that MDA MB-231-secreted MMP-1 induces the activation and differentiation of endothelial cells. It has been shown that MMP-1 interacts with proteinase-activated receptor 1 on endothelial cells to promote their activation [30, 48]; thus, it is feasible that breast cancer-derived MMP-1 functions likewise.

Tumors with silenced MMP-1 levels prevented osteolysis in vivo

Tumors were macroscopically visible by day 14, and both control and MMP-1 shRNA tumors were approximately equal in size through the final time point, day 28 (data not shown). By day 28, tumor incidence was comparable for each group of mice: 8/8 control mice and 7/8 MMP-1 shRNA mice exhibited tumors (Fig. 4a). However, 6/7 (87.5%) control shRNA tumors invaded the periosteum, or outermost layer of bone, and were in direct contact with the compact bone, while only 2/8 (25%) of the MMP-1 shRNA tumors

breached the periosteum at this time (Fig. 4a). Thus, invasion of the bone was significantly decreased by tumors expressing the MMP-1 shRNA, $P = 0.009$ (Fig. 4a). H&E staining of tissue sections demonstrated that compact bone adjacent to control shRNA tumors was highly osteolytic, as characterized by the presence of scalloped resorption lacunae (arrows, [49]), which are produced by osteoclasts and represent bone degradation (Fig. 4b, left panels). After an osteoclast vacates its resorption lacuna, native type-I collagen remains in the lacuna, and collagenases have been implicated in the solubilization of this matrix [25]. Control shRNA tumor cells were present in the osteoclast-formed resorption lacunae (arrows) of murine femurs, in the absence of osteoclasts (Fig. 4b, left panels). In contrast, MMP-1 shRNA tumors conformed closely to surrounding normal soft tissues and bone architecture, and were not associated with osteolytic resorption lacunae (Fig. 4b, right panels). Not only were control shRNA tumors associated with bone osteolysis, but ~30% of these tumors were also present in the bone marrow cavity as represented in Fig. 4c. To confirm that MMP-1 shRNA tumors maintained reduced MMP-1 expression compared to control shRNA tumors in vivo, we measured MMP-1 mRNA levels in tumors on day 28 and found that MMP-1 shRNA tumors had significantly decreased levels of MMP-1 mRNA compared to control shRNA tumors, $P = 0.009$ (Fig. 4d). Furthermore, the enzymatic activity of MMP-1 in media conditioned by control tumor explants was 3–4 times greater than in media conditioned by MMP-1 shRNA tumor explants (data not shown). We conclude that tumor-derived MMP-1 is associated with bone osteolysis, and with tumor invasion of bone.

MDA MB-231 cell-secreted MMP-1 induced the activation and differentiation of osteoclasts in the presence of soluble type-I collagen

To examine the mechanism(s) by which MMP-1 produced by the breast cancer cells facilitated osteolysis and bone invasion in vivo, we tested whether MMP-1 recruits, activates, and/or differentiates osteoclasts in vitro. To analyze recruitment, RAW264.7 osteoclast precursor cells were plated in transwells and allowed to migrate through membranes toward media conditioned by control or MMP-1 shRNA cells. We found that MMP-1 in the control shRNA conditioned media (CM) did not stimulate migration or recruitment of osteoclast precursor cells in vitro (data not shown), and therefore conclude that breast cancer cells likely did not facilitate in vivo osteolysis by recruiting osteoclast precursors to the tumor/bone interface.

Next, we examined whether MMP-1 in control shRNA tumors stimulated the activation and differentiation of osteoclasts, as inhibition of the collagenases decreases osteoclast activation [18] and resorption lacunae formed in vitro [50]. RAW264.7 cells were treated with control or MMP-1 shRNA CM, and stained for TRAP, a marker for activated osteoclasts [17, 26]. Neither control nor MMP-1 shRNA CM stimulated osteoclast activation (as determined by TRAP⁺ staining) on plastic culture plates (data not shown). Interestingly, however, when osteoclast precursors were plated on soluble type-I collagen, TRAP⁺ staining was increased in osteoclasts treated with control shRNA CM, compared to osteoclasts treated with MMP-1 shRNA CM (Fig. 5a), indicating that breast cancer secreted MMP-1 activated osteoclasts in the presence of type-I collagen. Moreover, the average number of activated (TRAP⁺) and differentiated (multinucleated), or mature, osteoclasts [17] (Fig. 5b) was significantly reduced when cells were exposed to MMP-1 shRNA CM, compared to those treated with control shRNA CM, $P < 0.0001$, (Fig. 5c). To confirm MMP-1 as the key mediator in this phenomenon, we treated cells with control shRNA CM supplemented with either an isotype control antibody or an MMP-1 neutralizing antibody, and found that inhibition of MMP-1 significantly suppressed osteoclast maturation by the breast tumor cells, $P < 0.0001$, (Fig. 5d). Furthermore, addition of active rMMP-1 in MEM with LH significantly stimulated osteoclast maturation compared to latent rMMP-1, $P < 0.0001$, (Fig. 5d). Importantly,

RANKL mRNA and protein levels from RAW264.7 cells treated with either media conditioned by control or MMP-1 shRNA-expressing cells were the same and barely detectable (data not shown). These data demonstrate a direct role for MMP-1 in osteoclast maturation, and that RANKL is not involved.

Previous in vitro studies suggested that collagenase-generated collagen fragments trigger osteoclast bone resorption [18, 51]. Hence, we hypothesized that tumor-secreted MMP-1 facilitates osteoclast activation by cleaving collagen, thereby producing collagen fragments, which then trigger osteoclast activation. To test this, we cross-linked the collagen in collagen-coated plates and found that control shRNA CM-induced osteoclast maturation was significantly diminished when osteoclast precursor cells were plated on insoluble collagen, $P = 0.0002$, (Fig. 5e). Together, these results indicate that osteoclast maturation induced by tumor-secreted MMP-1 requires soluble collagen.

We conclude that while breast cancer-derived MMP-1 does not recruit osteoclasts, it activates osteoclast precursor cells (as seen by the expression of TRAP), and stimulates their multinucleation in the presence of soluble type-I collagen, providing one mechanism of osteoclast resorption (as defined by the presence of lacunae) at the interface of bone and MMP-1-expressing tumor cells. While our results suggest that tumor-derived MMP-1 activates osteoclasts in vitro, we cannot exclude the possibility of the tumor cells directly degrading bone in vivo. However, it is generally accepted that breast cancer cells cannot directly resorb bone [52, 53].

Discussion

Breast cancer lesions in bone arise most frequently from metastasis, and also, but less commonly, by direct invasion of a primary breast tumor into the ribs and/or sternum, also known as local/regional reoccurrence (LRR). Defining the mechanisms by which breast cancer cells invade soft tissues and trigger destruction of bone is important to understanding breast cancer pathology of the bone. Given its well-established role in the invasion and degradation of type-I collagen, we studied breast cancer secreted matrix metalloproteinase-1 (MMP-1) in the invasion of interstitial tissues and bone. Using a novel murine model in which MDA MB-231 breast cancer cells harboring either control or MMP-1 shRNAs were injected near to the femur, we demonstrate the importance of tumor-derived MMP-1 in the directional invasion of interstitial tissues and the stimulation of osteolysis in vivo.

Our data show that inhibiting expression of MMP-1 suppresses tumor invasion of soft tissues (Fig. 2). Interestingly, tumors expressing MMP-1 had enhanced angiogenesis on the invasive outcroppings, compared to tumors lacking MMP-1 (Fig. 3). Thus, we propose that this invasion is associated with the ability of MMP-1-secreting tumors to recruit vasculature and consequently, chemoattractive agents, such as SDF-1. Our findings support two studies showing that (1) MMP-1 expression in invasive melanoma increases angiogenesis and (2) breast cancer-derived MMP-1 contributes to the assembly of new tumor blood vessels [30, 43].

Once in bone, factors secreted by tumors initiate pathological bone resorption by activating bone-residing osteoclasts and stimulating their differentiation [13, 24, 54–56]. Interestingly, MMP-1, which is frequently overexpressed in breast cancer, is: (1) localized to osteoclasts and their resorption lacunae [26], (2) upregulated by agents that stimulate bone resorption [27], and (3) implicated in the process of normal bone resorption during remodeling [25]. Thus, we hypothesized that expression of MMP-1 by breast cancer cells also plays a role in tumor-induced osteolysis and we used a novel model to investigate this idea. At day 28, we observed scalloped lacunae at the interface of bone and MMP-1 expressing tumors, which

were absent in bone adjacent to MMP-1 deficient tumors. Tumors secreting MMP-1 invaded the periosteum, compact bone, and the bone marrow cavity, but this was diminished by tumors lacking MMP-1 expression, suggesting a direct role for MMP-1 in mediating these events.

To investigate the molecular mechanisms behind our observations, we examined the ability of MMP-1-secreting tumor cells to recruit and/or activate osteoclasts. Although MMP-1 secreted by the tumor cells did not recruit osteoclast precursor cells, it did stimulate the activation and differentiation of these cells when plated on soluble type-I collagen, but not on insoluble type-I collagen (Fig. 5). This finding expands upon previous *in vitro* data that suggested collagenase-generated collagen fragments triggered osteoclast activation [18]. Importantly, we show that inhibiting MMP-1, using a neutralizing antibody, blocks osteoclast differentiation. Because soluble type-I collagen was required for MDA MB-231-secreted MMP-1 to induce the activation and differentiation of osteoclasts, we propose that collagen fragments produced by MMP-1 are responsible for the maturation of osteoclasts. Our data demonstrate, for the first time, that breast cancer-derived MMP-1 is capable of inducing the maturation of osteoclasts and that this is accomplished only in the presence of soluble type-I collagen.

To our knowledge, an *in vivo* model suitable for investigating LRR, an event that affects up to 30% of breast cancer patients after mastectomy [57], has not been described. LRR involves the direct invasion of interstitial tissues and bones of the chest wall by residual primary breast cancer cells. Our murine model recapitulates LRR, and also allows observations of the tumor cell/bone interactions. Although these interactions are initiated at the cortex of the bone, rather than in the bone marrow cavity, we found that breast cancer cells invaded into the marrow cavity in ~30% of mice by day 28.

Our model of breast cancer invasion of bone may be especially applicable to LRR studies, but can also be used widely to study cancer-induced osteolysis and invasion, and to analyze the efficacy of potential therapeutic drugs targeting tumor/bone interactions. Our findings may be broadly relevant to tumor-driven invasion of bone by other cancers, including lung, prostate, and kidney, as these commonly metastasize to bone [6], and have increased MMP-1 [58–60].

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Abbreviations

ATCC	American Type Culture Collection
CM	Conditioned media
DHMC	Dartmouth-Hitchcock Medical Center
HMVECs	Human microvessel endothelial cells
HPRT	Hypoxanthine-phosphoribosyl transferase
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry
LH	Lactalbumin hydrolysate

LRR	Local/regional recurrence
M-CSF	Macrophage colony stimulating factor
MMP	Matrix metalloproteins
RANKL	Receptor activator of NF- B ligand
shRNA	Short hairpin RNA
TRAP	Tartrate-resistant acid phosphatase

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Fig. 1.

MMP-1 shRNAs significantly silenced MMP-1 expression and collagenolytic activity in MDA MB-231 breast cancer cells. (a) Real-time RT-PCR quantification and western blot analysis of MMP-1 levels in each of the MDA MB-231 subclones. Values are given as an average number of copies $\pm$ SD of MMP-1 per copy HPRT, and as a percentage of MDA MB-231 cells (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$, compared to the control shRNA group). (b) Functional, in vitro type-I collagen degradation assay. shRNA cells were embedded in a type-I collagen matrix and after 24 h, the media from each well was removed and the remaining gels were photographed. The media removed was weighed and converted

to microliters to quantify the extent of collagen degradation. Data indicate mean collagen degradation $\pm$ SD of three determinations per experiment (** $P < 0.0001$, compared to the control shRNA cell embedded gels). (c) To examine the presence of MMP-1 protein levels within the type-I collagen matrix before it was degraded by the control shRNA cells, the collagen degradation assay was stopped at 6 h and the gels were fixed and stained for MMP-1 using IHC. An equal number of cells are present per gel. Arrows: MMP-1 staining of cells, arrowheads: collagen matrix; scale bar, 25 μ m

Fig. 2.

Silencing MMP-1 expression in breast tumors decreases invasive phenotype. **(a)** Assessment of in vitro proliferation rates between MMP-1 shRNA cells and control shRNA cells demonstrates no difference between these groups. **(b)** Representative micrographs of tumor H&E sections from mice sacrificed on day 7 (8 mice per group). Control shRNA tumors displayed invasive fronds (arrows, upper left panel) and protrusions (arrows, lower left panel), while MMP-1 shRNA tumors maintained a more confined, and encapsulated phenotype (arrowheads, right panels). Scale bar, 50 μ m all panels, except bottom left, in which scale bar is 200 μ m. **(c)** The number of tumor protrusions (defined as outcropping >

200 μm) per tumor on day 7 was quantified (* $P=0.014$ compared to control shRNA tumor protrusion length). M: muscle, T: tumor

Fig. 3.

Suppression of MMP-1 expression in breast tumors results in significantly diminished vascular formation in vivo and in vitro. **(a)** Representative CD31 staining of control shRNA and MMP-1 shRNA tumors at invasive tumor edges proximal to the bone on day 7. Arrows highlight CD31⁺ blood vessels, scale bar, 50 μ m. **(b)** CD31⁺ vessels were quantified by counting the number of vessels in 3 fields of the proximal edges of five tumors from each group on day 7 to establish vessel density. Data are presented as the mean $\pm$ SD of the number of vessels per field (** $P < 0.001$, compared to control shRNA tumor edge proximal to bone). **(c)** Representative micrographs of HMVECs in an in vitro tube formation assay used to measure angiogenesis. HMVECs were plated on Matrigel and treated with serum-free media alone (negative control), control shRNA conditioned, serum-free media (CM) in the presence or absence of an MMP-1 neutralizing antibody, or MMP-1 shRNA CM. **(d)** In vitro angiogenesis, quantified and presented as the average number of branch points formed by HMVECs in 3 fields of 9 wells per condition demonstrates that HMVECs treated with either MMP-1 shRNA CM or control shRNA CM supplemented with an MMP-1 neutralizing antibody have significantly decreased angiogenesis compared those treated with control shRNA CM in the presence of control anti-FLAG antibody. Data express the mean $\pm$ SD of 21 determinations (* $P < 0.05$)

Fig. 4.

Attenuation of tumor-derived MMP-1 expression prevents bone resorption at the tumor/bone interface. **(a)** On day 28, mice injected with control or MMP-1 shRNA cells (8 mice per group) were sacrificed and the tumor-bearing limbs were stained with H&E at the tumor/bone interface. Mice injected with control or MMP-1 shRNA cells displayed a tumor incidence of 7/8 (87.5%) and 8/8 (100%), respectively. Control shRNA tumors 6/7 (85.7%) invaded the periosteum and compact bone, but this invasion was significantly decreased by MMP-1 shRNA tumors (** $P = 0.009$ compared to control). **(b)** Representative micrograph images from the tumor/bone interface of two different sections illustrate that compact bone

(CB) adjacent to control shRNA tumors (T) is highly osteolytic, as indicated by the scalloped niches (arrows), which were likely formed by osteoclasts, but are filled with tumor cells (upper and lower left panels). The white dotted line indicates tumor/bone boundary in MMP-1 shRNA tumors, in which the bone was not osteolytic (upper and lower right panels). (c) 2/7 (~30%) of control shRNA tumors invaded completely into the bone marrow cavity (BM) by day 28; however, MMP-1 shRNA tumors did not. For all images, scale bar, 200 μ m. (d) Real-time RT-PCR of MMP-1 mRNA levels isolated from xenograft tumors. Values are expressed as MMP-1 copies per copies of HPRT and is represented as fold inhibition (** $P=0.009$ compared to control shRNA tumors)

Fig. 5.

Tumor-derived MMP-1 activates osteoclasts in the presence of soluble collagen. **(a)** RAW264.7 cells were plated on collagen-coated plates and treated with serum-free media (no treatment, negative control), serum-free media with RANKL (50 ng/ml) and M-CSF (20 ng/ml) (positive control), control shRNA CM, or MMP-1 shRNA CM. After 4 days, cells were stained for TRAP (dark brown, punctuate staining). **(b)** An activated, mature osteoclast is easily identifiable because it is not only TRAP⁺, but also multinucleated. **(c)** The number of mature osteoclasts formed in the presence of serum-free media alone (negative control), or supplemented with RANKL and M-CSF (positive control), control shRNA CM, or MMP-1 shRNA CM was determined by quantifying the average number of TRAP⁺, multinucleated (>2 nuclei), cells (per 40× view) on collagen-coated plates (*** $P < 0.0001$ compared to control shRNA CM). **(d)** Average number of mature osteoclasts formed when cells were plated on soluble collagen and treated with control shRNA CM containing an anti-FLAG control antibody or an MMP-1 neutralizing antibody, or with MEM with LH containing either active or latent rMMP-1. Values are presented as the mean $\pm$ SD of 9 determinations (*** $P < 0.0001$). **(e)** Average number of mature osteoclasts formed when cells were plated on soluble collagen or fixed collagen and treated with control shRNA CM. Values are presented as the mean $\pm$ SD of 9 determinations (** $P = 0.0002$ compared to CM plated on soluble collagen)