The Role of Proteolytic Enzymes in Metastatic Bone Disease

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Abstract

The interaction of cancer cells and the bone microenvironment leads to an alteration of the delicate physiologic balance between bone formation and resorption. Among the factors distorted due to the dialogue between tumor cells and bone stroma are proteases, which are key regulators of bone remodeling and invasion of cancer cells. In addition to their classical degradative function on extracellular matrix (ECM), many proteases have been shown to have new substrates and roles that are, one way or another, involved in intraosseous growth of cancer cells and associated bone remodeling. Despite mounting evidence demonstrating the contribution of proteases to bone metastasis and promising preclinical data with different protease inhibitors, disappointing results in clinical trials performed in cancer patients, generally with no skeletal metastatic disease, have discouraged further investigations. In this Perspective, we describe recent advances in understanding of the role of proteases in bone metastasis and discuss strategies that need to be considered in the development of new protease inhibitors and in the implementation of clinical trials.

Introduction

Metastases to bone are particularly common in patients dying from breast, prostate, thyroid, kidney, and lung carcinomas. Autopsy studies have revealed that skeletal metastases for these cancer types combined are present in one of every two patients (1), with breast and prostate cancers accounting for metastatic incidence in bones in around 70% of cases or higher (2-4). Irrespective of the tissue of origin of the cancer, most metastases occur in the axial skeleton, where a highly vascularized red bone marrow is present in the adult (5) and blood from the breast, lungs, kidney, prostate and thyroid may drain directly through the valveless Batson vertebral-venous plexus (6;7). Complications of bone metastasis include intractable pain, pathological fractures, nerve compression syndromes, hypercalcemia, and bone marrow suppression, with median survival measured in months for lung cancer and 2-4 years for breast and prostate cancers (7). Metastatic tumor tissue in bone is detected indirectly radiographically as osteolytic and/or osteosclerotic reactions. In prostate cancer, those lesions are usually described as osteosclerotic (8), while in lung, thyroid, or renal cancer the bone metastatic lesions are osteolytic (9-11). Breast cancer metastases are often of a mixed osteosclerotic/osteolytic variety with a predominance of osteolysis in advanced disease (12). In truth, however, histological and biochemical studies have demonstrated that both osteoblast and osteoclast activity are present in most lesions (13-16). In order to metastasize to bone, tumor cells must go through a series of sequential events including local invasion, intravasation, hematogenous dissemination (or lympho-hematogenous dissemination through potential interconnections between lymphatic and hematic systems), arrest in the microvasculature of the bone and binding to capillary or sinusoidal endothelial cells, extravasation, and growth and neovascularization within the bone marrow (17-19). During this so-called metastatic cascade, various basement membranes or interstitial extracellular matrices must be traversed by cancer cells, for which different proteases work alone or in concert to
Proteases and Bone Remodeling

During the normal turnover of the adult skeleton, bone formation and bone resorption are tightly coupled to maintain an appropriate balance. Osteoclast activity starts with attachment to the bone matrix mainly through integrin \( \alpha v \beta 3 \), followed by the formation of the “actin ring” that creates a tight sealing zone with the underlying mineralized bone matrix. A highly acidified Howship’s lacuna due to HCl secretion by osteoclasts occurs as a result of the concerted action of a vacuolar proton pump (H\(^+-\)ATPase) confined to the “ruffled border”, and a chloride channel (23). The creation of this acidic milieu mobilizes the bone mineral phase and exposes the non-mineralized ECM lying underneath, which consists of about 90% type I collagen, to proteolytic degradation. Cathepsin K, a cysteine lysosomal enzyme, is considered of utmost importance in the degradation of fibrillar type I collagen in this acidic environment with a pH of about 4 (23). The inability to degrade demineralized collagen fibers and the osteopetrotic phenotype found in cathepsin K knockout mice (24) and in pycnodysostosis (25), a rare human autosomal disease due to a cathepsin K mutation characterized by short stature and abnormally dense brittle bones, confirms the importance of cathepsin K in bone resorption. MMPs have also been implicated in the degradation of type I collagen in the subosteoclastic resorption lacuna, as supported by studies in which MMP inhibitors suppressed bone ECM degradation by osteoclasts in a significant manner (26;27), and MMPs compensate for the lack of cathepsin K in osteoclasts deficient in the cysteine enzyme (28). In line with this view is the finding of MT1-MMP (also known as MMP-14) at the sealing zone of osteoclasts (29), implying a role for this membrane-bound MMP in proteolysis of bone matrix at a later stage, once the pH of the resorption lacuna has increased due to higher levels of phosphate or Ca\(^{2+}\) (30). MT1-MMP has a crucial role in skeletal development, as demonstrated by MT1-MMP knockout mice that exhibit craniofacial dysmorphism, osteopenia, and dwarfism due to inadequate type I collagen turnover (31). Although MMP-9 has also been found to be expressed by osteoclasts, its role in osteoclast activity is mostly related to its ability to facilitate the recruitment of osteoclasts towards the bone surface rather than to a degradative function (32). In addition, osteoclast-derived MMP-9 is involved in the release of vascular endothelial growth factor (VEGF) from ECM stores (33), with mice containing a null mutation in the \( MMP-9 \) gene displaying impaired vascularization and endochondral bone formation (34). In situations where cathepsin K is absent, several observations also suggest that MMP-13 synthesized by peri-osteoclastic cells participates in bone matrix collagenolysis (27).

Osteoblasts are classically associated with the synthesis and deposition of bone matrix, and stimulation of the differentiation of pre-osteoclasts into mature osteoclasts. However, osteoblasts are also able to secrete several MMPs necessary to remodel their own type I collagen-rich non-
mineralized osteoid, generating collagen fragments that recruit osteoclasts to the bone surface (35;36). In addition, since osteoclasts are not capable of attaching to non-mineralized bone ECM, it has been proposed that the degradation of the osteoid by interstitial collagenases produced by stromal cells and osteoblasts adjacent to osteoclasts is essential for osteoclastic bone resorption to take place (35;36). For instance, MMP-13 knockout mice exhibit osteopetrotic changes in trabecular bone, due in part to inefficient collagen degradation in those animals (37). However, MMP-13 is not expressed by osteoclasts but by late differentiated osteoblasts and osteocytes (38), suggesting that bone ECM degradation by MMP-13 is carried out by peri-osteoclastic osteoblasts (27,39). Osteoblasts also express MMP-2, which has been reported to have a crucial role in osteocytic canalicular formation with a contribution to bone metabolism that leads to decreased mineralization (40).

Matrix Metalloproteinases and Bone Metastases

In humans, the MMP family is currently comprised by at least 25 enzymes that include secreted and plasma membrane-tethered members. They are active at neutral pH, and require Ca\(^{2+}\) for activity. MMPs share a general structure consisting of an N-terminal signal sequence, a propeptide domain, a catalytic domain and, with the exception of only two MMPs, a hemopexin-like C-terminal domain linked to the previous domain through a flexible hinge region. Most MMPs are synthesized as inactive enzyme precursors (zymogens), in which an unpaired cysteine sulfhydryl group within the propeptide domain coordinates with a Zn\(^{2+}\) ion present in the catalytic domain, preventing binding and cleavage of the substrate. MMPs acquire proteolytic activity only after dissociation of the cysteine from the Zn\(^{2+}\) atom (cysteine switch), which occurs by chemical modification, conformational changes, or proteolytic displacement of the propeptide domain. In contrast to the majority of the MMPs, which are secreted as zymogens and are activated extracellularly, a few secreted MMPs (MMP-11, -21, and -28) and all the membrane-anchored MMPs (membrane-type MMPs (MT-MMPs) and glycosylphosphatidylinositol (GPI)-containing MMPs) are processed intracellularly by members of the proconvertases family of serine proteinases, which cleave the prodomain by catalysis of a furin-like insert located in the linker motif connecting the prodomain and catalytic domains (for review, see (41;42)). In addition to zymogen activation, MMPs are regulated by a number of cytokines and growth factors that control their gene expression and secretion (43) and by endogenous tissue inhibitors of MMPs (TIMPs), of which four forms (TIMP-1 to -4) have been described. TIMPs bind active or latent forms of MMPs, blocking their proteolytic activity. However, TIMP-2 can also form a ternary complex with MT1-MMP and pro-MMP-2 facilitating the activation of the MMP-2 zymogen. The overall balance of active MMPs and TIMPs will finally determine the net proteolytic activity (for review, see (41;42)).

Other proteases related to MMPs include ADAMs (A Disintegrin And Metalloproteinase) and ADAMTSs (ADAMs with Thrombospondin motifs type I). They differ from MMPs in that, in addition to the prodomain and catalytic domains present in MMPs, their structure involves disintegrin, cysteine-rich, EGF-like transmembrane domains, and a cytoplasmic tail, instead of a hemopexin-like C-terminal domain. ADAMTSs are similar to ADAMs, except that they contain a thrombospondin-like domain between the disintegrin and cysteine-rich domains, and that they lack a transmembrane domain and therefore are secreted. The presence of metalloproteinase and disintegrin domains endows ADAMs and ADAMTSs with features of both proteinases and adhesion molecules. Known functions include collagen processing as procollagen N-proteinase and cleavage of some matrix proteoglycans, and inhibition of angiogenesis (44).

The role of MMPs in physiological and pathological bone remodeling extends far beyond the direct degradation ability of MMP-1, -8, -13, -14, and -18 on interstitial fibrillar type I collagen (45), which constitutes the main organic component of
bone ECM. In fact, additional functions of these and other MMPs, including release of growth factors sequestered in bone ECM, shedding of membrane-bound cytokines, and angiogenesis, may contribute to bone metastases. Indirect evidence coming from preclinical studies with broad-spectrum MMP inhibitors suggests a role for MMPs in bone metastasis. For example, in experiments in immunosuppressed female nude mice injected intracardiacally with estrogen-independent human breast cancer MDA-231 cells, transfection of the cells with TIMP-2 alone led to a marked reduction of radiographically-evident osteolytic lesions and increased survival in mice compared to those injected with non-transfected MDA-231 cells. Moreover, no differences in tumor growth were observed when non-transfected and TIMP-2-transfected MDA-231 cells were injected orthotopically, suggesting a specific role for MMPs in intrasosseous tumor growth and subsequent osteolysis (46). Experiments carried out using the SCID-human experimental model for bone metastasis, in which human prostate cancer cells are injected into a subcutaneous human bone xenograft, also provided evidence that MMPs are involved in skeletal metastasis. In the SCID-human model, treatment with the broad-spectrum MMP inhibitor batimastat resulted in a significant reduction in prostate cancer cell proliferation, recruitment of osteoclasts, and marrow trabecula degradation (47). Similar results were obtained in a model of bone metastases in which nude mice were injected with MDA-MB-231 human breast cancer cells into the arterial circulation and treated with batimastat (48).

More direct evidence of the contribution of MMPs to bone metastasis comes from studies proving a functional role of specific MMPs. Although most MMPs are produced mainly by stromal cells neighboring tumor cells (21), MMPs have been found to be upregulated in some instances by cancer cells metastatic to the bone. For example, in a differential gene expression microarray analysis performed in MDA-MB-231 subpopulations with different bone metastatic ability, MMP-1 and ADAMTS-1 were found to be upregulated in highly bone-metastatic clones (49), suggesting their potential involvement in mechanisms underlying breast cancer metastasis to bone. These results are supported by recent studies showing that in a highly bone-metastatic MDA-MB-231 subline, combined knockdown of MMP-1 and ADAMTS-1 was required to reduce the incidence of bone metastasis, preserve bone integrity, and increase survival (50). Elegant studies by the authors demonstrate that overexpression of MMP-1 and ADAMTS-1 by the breast cancer cells results in proteolytic cleavage of tumor-associated epidermal growth factor (EGF)-like ligands that signal through the EGF receptor in osteoblasts, stimulating their differentiation and promoting the expression of receptor activator of nuclear factor-κB ligand (RANKL) on their surface, thereby activating osteoclasts, which express the RANK receptor that binds RANKL (50). These results are supported by clinical studies showing significantly increased serum levels of MMP-1 in breast cancer patients with bone metastases (50;51), and upregulated MMP-1 levels in bone metastatic tissue from breast cancer patients (52).

MMP-7, also known as matrilysin, has been found to be highly expressed at the tumor-bone interface in animal models and human samples of prostate and breast metastasis, mainly localized to osteoclasts in areas of osteolysis (53;54). Although MMP-7 has a broad ECM substrate specificity, it does not cleave the characteristic bone ECM constituent type I collagen. Through in vitro and animal studies using MMP-7 null mice, pre-osteoclast-derived MMP-7 was found to contribute to tumor-induced osteolysis via a proteolytic cleavage mechanism acting on the osteoclastogenic factor RANKL (53;54). The generation of soluble RANKL (sRANKL) by MMP-7 would facilitate osteoclast activation in bone metastatic sites without the necessity of direct contact between RANKL-expressing osteoblasts and RANK-expressing osteoclast precursor cells. The contribution of MMP-7 to bone metastasis is further supported by studies in breast and prostate cancer patients showing a positive association between circulating MMP-7 serum concentrations and progression towards skeletal metastasis (55;56).
Another MMP that is frequently localized to osteoclasts is MMP-9, also known as gelatinase B. It proteolytically digests many proteins in the ECM, including laminin, gelatin (denatured collagen), and types IV, V, XI and XVI collagen, but not the predominant bone type I collagen. Activation of MMP-9 has been demonstrated in the SCID-human model for bone metastasis using human PC3 prostate cancer cells and in co-cultures of these tumor cells and human bone explants (57). An increase in net MMP-9 activity in PC3 bone tumors coincided with the recruitment of osteoclasts, where most of the MMP-9 was localized. The source and function of active MMP-9 were further confirmed by ribozyme suppression of MMP-9 in PC3 cells, which did not affect the intraosseous tumor growth and osteolysis, nor the activation of MMP-9 in osteoclasts (57). A mechanism-based MMP inhibitor with high selectivity for gelatinases proved to be efficacious in inhibiting PC3 growth, osteolysis, and angiogenesis in the SCID-human model (58). However, the precise role of MMP-9 activation during bone colonization by prostate cancer cells is not well-defined. Silencing MMP-9 gene expression in osteoclast precursors demonstrated that tumor-mediated osteoclast motility is mediated by pro-MMP-9 (57). The function of active MMP-9 in intraosseous prostate growth is less clear, but its capacity to solubilize VEGF from bone ECM (59), to process and shed Kit ligand from bone marrow stromal cells stimulating mobilization and recruitment of endothelial progenitor cells to tumor deposits (60;61), and to recruit pericytes along microvascular endothelial cells (62), suggest a role in angiogenesis. This is supported by studies in which a diminished microvascularity is observed in the prostate tumor-bone microenvironment of MMP-9-null immunosuppresse mice compared with wild-type control mice (63; 64). In line with findings in prostate cancer bone metastasis, immunohistochemical studies in skeletal metastatic lesions in breast cancer patients revealed dominant MMP-9 expression in osteoclasts and macrophages (65).

It has been found that bone marrow stromal cells stimulate the invasion of prostate cancer cells through type I collagen via a process contributed mainly by increased expression of MMP-12 in cancer cells (66). The significance of this in vitro finding, in which an unbiased microarray analysis of MMP genes was used to determine which proteases were expressed and functional during the initial interaction of prostate cancer cells and bone marrow stromal cells, is further supported by immunohistochemical studies revealing increased MMP-12 expression in prostate cancer cells interacting with the bone microenvironment in vivo (66). Further studies are needed, however, to determine the precise role of cancer-associated MMP-12 in bone metastasis, whether contributing to type I collagen degradation through direct or indirect mechanisms involving activation of collagenases.

Recently, studies on gene expression profiles of bone and brain metastases from breast cancer patients were performed to identify genes that could be used as predictors of organ-specific metastasis. The authors found that MMP-13, encoding a critical protease for the degradation of type I collagen, was among the most up-regulated genes specifically in breast cancer bone metastases (67). The importance of this MMP in osteolysis associated with breast cancer cells is highlighted by another study in which MMP-13 gene and protein expressions were found to be up-regulated at the tumor-bone interface with respect to tumor alone area in mice injected with syngeneic mammary tumor cell lines (68). Although MMP-13 is expressed in this model by tumor cells, osteoblasts, and stromal cells, knocking down MMP-13 only in cancer cells was sufficient to abrogate tumor-associated osteolysis and the number of activated osteoclasts at the tumor-bone interface (68). This suggests a crucial role for tumor-derived MMP-13 in this model through a potential mechanism that, according to the authors, could regulate RANKL expression affecting osteoclast activation and tumor-induced osteolysis. Another study shows that breast cancer cells can also induce the expression of MMP-13 and RANKL in bone cells (69). Taken together, these studies suggest a role for MMP-13 at the site of tumor-associated
osteolysis in breast cancer bone metastasis, independent of the cell source. MMP-13 has also been shown to be expressed in non-small cell lung carcinoma facilitating cell shedding from the primary tumor and bone marrow microinvolvement associated with a poorer survival rate (70), and has been found to show higher expression in renal cancer bone metastases than primary tumors due to stimulation by transforming growth factor (TGF)-β1 that is abundant in the bone microenvironment and known to promote cancer-induced osteolysis (71).

MT1-MMP is a major mediator of type I collagen processing and is the only MMP whose absence produces severe skeletal abnormalities in mice. In normal bone, MT1-MMP is expressed in osteoblasts, presumably playing a role in degradation of type I-rich osteoid, and at the sealing zone of osteoclasts. In addition, MT1-MMP could also have direct or indirect systemic effects since it appears to be important in the development of many organs that could impact bone metabolism. In breast cancer patients, cancer cells are either MT1-MMP-negative or weakly positive in primary tumors, while in specimens of bone metastasis they are strongly positive for MT1-MMP (65). Similarly, MT1-MMP expression has been shown to correlate with aggressive behavior in prostate cancer, and invasion and metastasis (72-74). In primary prostate adenocarcinomas MT1-MMP-positive and -negative malignant glands are found within the same specimen (75), while a robust and uniform expression is seen in bone metastatic cells (76). The relevance of MT1-MMP to bone metastasis was demonstrated in studies in which MT1-MMP was overexpressed or silenced in human prostate cancer cell lines with absent or high baseline levels of the enzyme, respectively. Intratibial injection of those genetically modified cells in immunodepressed mice gave rise to totally opposite phenotypes, with increased intraosseous tumor growth and osteolysis in the case of prostate cancer cells overexpressing MT1-MMP, whereas diminished tumor intraosseous growth and even osteoclastic responses when MT1-MMP-silenced cancer cells were used (76). In vitro experiments demonstrated that tumor-associated MT1-MMP can directly degrade type I collagen and, importantly, can cleave RANKL expressed on the tumor cell surface mediating solubilization of RANKL and stimulating osteoclastogenesis (76). A recent study has shown that tumor-associated MT1-MMP-mediated RANKL shedding also stimulates the migration of prostate cancer cells, which express RANK, through a Src-dependent mechanism (77). These data suggest the existence of novel autocrine and paracrine pathways mediated by tumor-associated MT1-MMP that could favor bone matrix degradation and intraosseous expansion of prostate cancer, even in the absence osteoblast-osteoclast precursor interaction.

Representative MMPs and substrates in bone metastases are summarized in Fig. 1.

**Cathepsins and Bone Metastases**

The cathepsin family consists chiefly of a number of primarily lysosomal cysteine proteases, although aspartic (cathepsins D and E) and serine proteases (cathepsins A and G) are also included under the traditional term of “cathepsins.” In humans, eleven cysteine cathepsins have been described to date, including cathepsins B, C, F, H, K, L, O, S, V, X, and W, which belong to clan CA, family C1, of the papain-like cysteine proteases (78). They are synthesized as inactive glycosylatedzymogens that are converted into catalytically active cathepsins by removal of their N-terminal propeptide by other proteases or by autocatalysis usually at acidic pH (78). Cathepsin activity is regulated by endogenous inhibitors that bind tightly to enzyme, preventing substrate hydrolysis, and include cystatins, stefins, thyropins, and serine protease inhibitors (serpins) (78). In addition to their function as intracellular exopeptidases primarily involved in lysosomal protein recycling (78), cysteine cathepsins play important physiological roles, including antigen processing, prohormone and proenzyme activation, and bone remodeling (79). In certain tumors and T cells, cysteine cathepsins can be found at the cell surface or are secreted (80), as happens with cathepsin K, into the resorption pit between osteoclasts and bone (23). An association

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Fig. 1. Diagram representing the most representative roles of MMPs at bone metastatic sites. Expression of MT1-MMP by cancer cells results in proteolytic cleavage of tumor-associated membrane-bound RANKL (mRANKL), generating a soluble form of RANKL (sRANKL) that activates RANK in autocrine and paracrine manners favoring migration of tumor cells and osteoclastogenesis, respectively. Pre-osteoclasts present at the tumor/bone interface secrete MMP-7 that can also cleave mRANKL in osteoblasts or cancer cells, leading to osteoclast differentiation. Tumor cells express ADAMTS-1 and secrete MMP-1, which cleave neighboring membrane-tethered epidermal growth factor-like ligand (mEGF-like ligand). The resulting soluble form (sEGF-like ligand) signals through EGFR expressed by osteoblasts, stimulating their differentiation and expression of RANKL. In addition, MMPs assist in the degradation of organic bone matrix degradation after resorption of the mineralized components by osteoclasts. MMP-13 secreted by tumor cells, osteocytes, and osteoblasts, and MT1-MMP expressed at the sealing zone of osteoclasts, unwind and cleave type I collagen triple helices. As a consequence of this, and the action of osteoclast-derived proteases such as MMP-9, several growth factors/cytokines are released from ECM stores and, in some cases, converted into active forms.

between cathepsins and cancer has been suggested in different studies (reviewed in (79)). Here, we will focus our attention only on those cathepsins that have been associated with metastatic bone disease.

A causal role for cathepsin K in bone metastasis has been attributed in part to its exclusive ability to degrade both helical and non-helical regions of type I collagen (native collagen), and to cleave secreted protein, acidic, cysteine-rich (SPARC/osteonectin) (81), a non-collagenous bone matrix component highly expressed in bone metastatic lesions that attract and anchor tumor cells in the bone (82). In vivo studies using the SCID-human model for prostate cancer bone metastasis and in vitro coculture studies of prostate cancer and bone stromal cells have demonstrated upregulation of cathepsin K in osteoclast and stromal cells interacting with cancer cells as well as upregulation of SPARC, followed by secretion of SPARC fragments, all of which is significantly reduced by a selective proteinase K inhibitor (81).
Immunohistochemical studies in prostate cancer patient samples revealed cathepsin K immunoreactivity in both cancer cells from primary carcinomas and osseous metastases. However, in bone metastatic lesions, osteoclasts were strongly positive for cathepsin K, and sera from these patients showed higher levels of NTx (cross-linked N-telopeptides of type I collagen) than from patients with primary tumor, suggesting increased osteoclastic bone resorption (83). Similar findings showing that cathepsin K is expressed not only by osteoclasts but also by cancer cells have been reported in breast cancer skeletal metastases (84), suggesting that selective inhibitors for cathepsin K could have a two-fold effect in the treatment of bone metastasis, targeting not only osteoclasts but also intraosseous tumors. This assumption was confirmed experimentally in two different mouse models of breast cancer bone metastasis in which an intervention protocol with the cathepsin K inhibitor CKI not only reduced the progression of osteolytic lesions but also the skeletal tumor burden (85). In addition, in vitro studies demonstrated that cathepsin K inhibitors are capable of inhibiting breast cancer cell invasion, without affecting primary tumor growth in vivo (85;86).

Although the expression level of cathepsin L in osteoclasts was considerably lower than that of cathepsin K, experimental evidence suggested that various proinflammatory cytokines found in bone upregulate its expression and secretion (87). Studies using bone explants obtained from cathepsin L knockout mice clearly demonstrated a role for this cysteine protease in the activation of MMPs present in osteoclasts and involved in bone matrix degradation (28). Taken together, these findings suggest a possible contribution of cathepsin L to bone metastasis, supported by a study with a limited number of metastatic bone tumors that revealed enhanced expression of cathepsin L at the message level (88). Further studies are needed to confirm the contribution of cathepsin L to bone metastasis.

Another cysteine cathepsin that has been shown to activate MMPs either directly (89) or indirectly through degradation of TIMPs (90) is cathepsin B. Since expression of cathepsin B is enhanced in human breast stromal cells grown on collagen gels (91), it is reasonable to think that the bone microenvironment could modulate the expression of the cysteine protease synthesized by stromal and/or tumor cells in skeletal metastasis. Confocal analysis aimed at studying proteolysis by living cells showed that interaction of human prostate cancer DU45 cells with type I collagen and human bone fragments increased the level and secretion of cathepsin B by cancer cells, in agreement with high expression levels and activity of the enzyme in vivo in the SCID-human model of bone metastasis (92). Although these findings are promising, further studies are needed to determine the contribution of cathepsin B to bone metastasis, either by a direct mechanism or by the action of other proteases activated by cathepsin B.

As for the serine protease cathepsin G, it can facilitate tumor-induced osteolysis through different mechanisms. In a mouse model that mimics osteolytic changes associated with breast cancer-induced bone metastasis, it has been found that cathepsin G is upregulated at the tumor-bone interface primarily in osteoclasts and osteoclast precursor cells via their interaction with tumor cells. Cathepsin G is capable of proteolytically cleaving RANKL and shedding it in a soluble form that induces osteoclast differentiation and activation; in vivo inhibition of cathepsin G reduces tumor-induced osteolysis but does not affect tumor growth (93). Recently, cathepsin G has been found to induce the recruitment of osteoclast precursors through proteolytic activation of protease-activated receptor-1 (PAR-1) (94), and to stimulate angiogenesis through an indirect mechanism in which cathepsin G activates pro-MMP-9 and then cleaves and activates TGF-β, resulting in enhanced expression of the angiogenic factors VEGF and monocyte chemotactic protein (95). Taken together, these observations suggest important roles for cathepsin G in osteolysis and angiogenesis associated with bone metastasis.
Serine Proteases and Bone Metastases

The plasminogen activator-plasmin system is the most extensively studied system within the serine proteases. There are two plasminogen activators, namely urokinase-type plasminogen activator (uPA) and tissue plasminogen activator (tPA), which are coded by two different genes and have different structure and function. In particular, tPA has been associated mostly with intravascular fibrin degradation, whereas uPA is a fibrin-independent protease involved mainly in extracellular proteolysis related to various aspects of tumor progression such as invasion, motility, and angiogenesis. Pro-uPA is secreted as a simple chain zymogen, which classically becomes activated by plasmin cleavage. As a result of this cleavage, a two-chain active uPA held by a disulfide bond is generated (96). Pro-uPA may bind to the cell surface through a high-affinity uPA receptor (uPAR) via its N-terminal EGF-like domain, and there may be converted to uPA by plasmin or other proteolytic enzymes such as kallikrein 2 or cathepsin B. On the cell surface, uPA can activate membrane-bound plasminogen into membrane-bound plasmin, which in fact is responsible for most proteolytically-driven effects of uPA. Receptor-mediated activation of uPA has an important role in the proteolytic efficiency of the system, as uPAR-bound uPA exhibits a 40-fold lower $K_m$ (Michaelis constant) than soluble uPA, with an overall increased catalytic efficiency. There are two uPA inhibitors (PAI) of the serpin family, namely PAI-1 and PAI-2. The complex uPAR-uPA is inactivated mainly by PAI-1, which causes clearing of the trimeric complex by internalization (96). Urokinase supports the malignant phenotype through mechanisms involving direct or indirect ECM degradation, stimulation of cell motility mediated by association with uPAR and integrins, stimulation of cell proliferation, and inhibition of apoptosis (96). In prostate cancer patients, uPA expression was found to be higher in bone metastases than in primary tumors (97), and high levels of circulating uPA correlated to metastatic bone dissemination (98). Metastatic prostate cancer cells have been reported to synthesize uPA that binds to uPAR on the surface of osteoblasts, leading to uPA-mediated generation of plasmin that increases extracellular insulin growth factor-1 bioavailability, thus supporting the proliferation and survival of prostate cancer cells within the bone microenvironment (99). Moreover, uPA activates TGF-β produced by prostate cancer cells and osteoblasts that play important roles in bone metastasis (99). Heterotypic cell-cell contact studies involving prostate cancer cells and bone-derived cells have demonstrated an upregulation of uPA gene expression in the cancer cells (100; 101), and knock-down of tumor-associated uPA was sufficient to reduce intraosseous tumor growth and protect trabecular bone from destruction in an experimental model of skeletal metastasis (100). Paradoxically, tumor-bone stromal interaction led to enhanced PAI-1 expression and secretion in bone cells that stimulate prostate cancer cell migration (100). Analysis of prostate cancer cells transfected with maspin, a serpin known to interact with the cell surface uPA/uPAR system in an in vivo bone metastasis model, demonstrated a significant reduction of intraosseous growth, osteolysis, and angiogenesis (102;103). The importance of the uPA/uPAR system in bone metastasis is further supported by studies in both prostate and breast cancer patients in whom disseminated cancer cells found in the bone marrow show higher uPAR expression (104;105). A recent study using a multiplex real-time RT-PCR assay revealed a significant association between uPA gene transcripts and the presence of bone metastasis in patients with clear cell renal cell carcinoma, suggesting that this serine protease might have predictive value in guiding treatment decisions (106).

Recently, members of the type II transmembrane serine protease family, particularly matriptase, have emerged as unique enzymes that, unlike MMPs and uPA, have been found to be expressed only by epithelial and carcinoma cells (107). Matriptase is synthesized as a single chain zymogen tethered to the cell membrane via an N-terminal signal anchor, and its serine protease domain is located in the C-terminus, and is autoactivated by androgens, lysophospholipids, and suramin (108) being converted to a two-chain active
metastatic disease. However, the cells within bone and diminishing bone inhibitors in abrogating the growth of cancer demonstrate the value of various protease bone metastasis (Fig. 2). Preclinical data mechanisms by which proteases promote by proteases, suggesting indirect processed, activated, or made growth of metastatic deposits are also vital significance to bone remodeling and findings have demonstrated that factors of degradation of ECM components, new response. Moreover, in addition to direct experimental modulation ultimately affects microenvironment, and that their contribution of MMP activity at such late stages is not clear, nonexistent characterization of the protease profile (protease degradome (113)) of the tumors to be treated, which might be patient-specific, and lack of studies validating the actual inhibitory effects of MMP inhibitors during treatment.

In addition, most cancer trials did not focus on bone metastasis. To our knowledge, only two clinical trials have been conducted with MMP inhibitors in patients with hormone-refractory prostate cancer with bone metastasis. In a Phase III clinical trial, the combination of the “deep pocket” inhibitor selective for MMP-2 and -9 prinomastat (AG3340) with mitoxantrone and prednisone did not show beneficial effects compared with chemotherapy alone (114), while in a randomized phase II trial with rebimastat (BMS 275291), a broad spectrum MMP inhibitor that does not inhibit sheddases, similar conclusions were drawn, except that high doses of the drug were associated with reduced levels of N-telopeptide in serum, suggesting a slower bone turnover (115). These limited studies should not, by any means, be considered definitive to invalidate the use of MMP inhibitors in patients with bone metastasis. Their use in patient populations with unknown protease degradomes could potentially be as ineffective as using anti-HER2 monoclonal antibody for all breast cancer patients, regardless of HER2 status. Moreover, as mentioned above, the use of broad-spectrum inhibitors may hinder the inhibitory effect of the drug on specific MMPs that truly contribute to metastatic growth by blocking other MMPs with important physiological protease held by a disulfide bond (109). Its activity is regulated mainly by the Kunitz-type inhibitors hepatocyte growth factor activator inhibitor (HAI)-1 and -2 (109). Some of the substrates for matriptase include hepatocyte growth factor (HGF) and pro-uPA, which become activated upon cleavage by matriptase, as well as VEGFR-2, the G-protein-coupled protease-activated receptor 2 (PAR-2), insulin-like growth factor binding protein-related protein-1, and the GPI-anchored serine protease prostasin (109). Although a correlation between matriptase expression and increasing tumor grade has been reported in breast and prostate cancer (reviewed in (109)), recent studies indicate that loss of HAI-2 occurs during prostate cancer progression (110), suggesting that the disturbance between the protease and its inhibitor is what really influences cancer progression. A novel role has been found recently for matriptase in prostate cancer, where it mediates the proteolytic removal of the CUB domain necessary for activation of platelet-derived growth factor D, for which increasing evidence suggests a critical role in prostate cancer progression and bone metastasis (111).

Is There Still a Place for Protease Inhibitors in the Treatment of Bone Metastasis?

The data described above clearly show that proteases derived from either cancer cells or bone cells are frequently expressed and active within the tumor-bone microenvironment, and that their experimental modulation ultimately affects intraosseous tumor growth and bone response. Moreover, in addition to direct degradation of ECM components, new findings have demonstrated that factors of vital significance to bone remodeling and growth of metastatic deposits are also processed, activated, or made bioavailable by proteases, suggesting indirect mechanisms by which proteases promote bone metastasis (Fig. 2). Preclinical data demonstrate the value of various protease inhibitors in abrogating the growth of cancer cells within bone and diminishing bone responses associated with skeletal metastatic disease. However, the excitement about targeting proteases in cancer has vanished over recent years due to the failure of clinical trials, mostly involving MMP inhibitors. Several potential explanations for this failure include the use of inhibitors with low selectivity that lead to dose-limiting systemic secondary effects such as musculoskeletal pain and arthralgia, off-target inhibition of physiologically relevant MMPs that may result in neutralization of the inhibitors aimed at targeting MMPs that truly contribute to disease (112), treatments in patients with advanced metastatic disease for whom the contribution of MMP activity at such late stages is not clear, nonexistent characterization of the protease profile (protease degradome (113)) of the tumors to be treated, which might be patient-specific, and lack of studies validating the actual inhibitory effects of MMP inhibitors during treatment.

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functions, thus generating an adverse or null net effect.

Another aspect that should be considered for treatments with MMP inhibitors and could explain their failure as therapeutic drugs is the tumor-suppressing functions shown by some MMPs (116). For example, the antiangiogenic factor tumstatin is generated by MMP-9 proteolysis of collagen IV (117), and MMP-8 plays antitumor roles through its ability to regulate inflammatory responses in skin carcinogenesis (118). Therefore, a precise analysis of the expression and activity patterns of MMPs should be considered for each cancer, or even each patient, to develop a rational choice for the use of specific inhibitors that could be of therapeutic benefit. Moreover, in the design of clinical trials using MMP inhibitors, the coordinated stimulation and activation of certain MMPs occurring at specific stages of malignant progression should be taken into consideration. In the case of skeletal metastasis, the expression and activity of specific proteases occur at early stages of bone colonization by cancer cells, suggesting that MMP inhibitors could represent good preventive agents to be considered before clinical metastases are detected, rather than curative therapies at very advanced stages. This could explain the lack of beneficial effects observed with prinomastat and rebimastat in prostate cancer patients with bone metastasis, as opposed to the promising results obtained with other MMP inhibitors in preclinical models of bone metastasis.

Novel approaches to MMP inhibition involve the design of chemical inhibitors targeting extra-catalytic domains (exosites), to avoid inhibitory activities of the compounds across different MMPs due to an overall similarity of the active site cleft of MMPs, which eventually could lead to a lack of specificity and side effects (119). In that context, inhibitors targeting exosites may be useful to inhibit non-catalytic functions, such as those of MT1-MMP involved in osteoclast fusion (120), which correlates with the osteoclast functionality (121) increased in bone metastasis. For a new generation of more selective MMP inhibitors, it is of crucial
importance to define in a precise manner the MMP targets for each specific cancer, as well as the MMPs with tumor-suppressing functions, so that the right inhibitor is chosen and chances of therapeutic success are increased. For example, in prostate cancer cells metastatic to bone, it has been found that MT1-MMP is expressed and active, and facilitates intraosseous tumor growth and osteolysis (76), making it a potential target of therapeutic value not only due to its capacity to degrade the typical bone ECM component type I collagen, but to its ability to mediate ectodomain cleavage of membrane-bound RANKL (76;77) and cell adhesion molecules such as CD44 (122) and intercellular adhesion molecule ICAM-1 (123), to activate pro-MMP-2 (124) and pro-MMP-13 (125), and to promote prostate cancer cell migration (77), and angiogenesis (126). Recently, the highly selective fully human MT1-MMP inhibitory antibody DX-2400, discovered using phage display technology, has demonstrated excellent results in breast cancer models, inhibiting tumor growth, spontaneous metastasis, and angiogenesis, suggesting that this highly specific inhibitor may also prove successful in the clinic (127). Another approach to obtain MMP inhibitors with better selectivity profiles involves the use of “suicide inhibitors” in which the target molecule is covalently modified by the inhibitor binding (128). An example of these agents is SB-3CT, which has been shown to inhibit intraosseous prostate tumor growth, osteolysis, and angiogenesis in a preclinical model (58).

Several approaches have been developed to inhibit cysteine cathepsin activity, mostly with small-molecule inhibitors. Preclinical studies with cathepsin K inhibitors have succeed in reducing bone metastasis in mice, and some of them such as odanacatib (MK0822) and relacatib (GSK462795) entered clinical trials for this indication (129). A phase II trial with odanacatib in breast cancer patients with bone metastasis showed a reduction of urinary N-telopeptide marker by 77% from baseline levels over four weeks similar to zoledronate treatment (130), indicating that this investigational drug has the potential to slow the accelerated rate of bone destruction associated with bone metastasis.

A large body of proof-of-principle studies using small-molecule inhibitors of uPA, peptide inhibitors of the uPA-uPAR interaction, and antibody-like inhibitors, suggests that disrupting uPA-mediated proteolysis and/or subsequent plasmin-mediated cascades involving the activation of certain pro-MMPs and the activation of factors sequestered in the ECM could result in antitumor effects (reviewed in (131)). However, very few of these preclinical attempts have been assessed in bone metastasis models, and clinical trials are awaited for definitive confirmation in cancer patients (131;132).

The widely publicized failure of many protease inhibitors in clinical trials for cancer, mainly for MMPs, has ruthlessly hampered further studies to demonstrate their therapeutic value, unfairly leading to a general consensus that these agents are ineffective even in situations with limited or null studies, such as in patients with certain cancers metastatic to bone. This skeptical attitude could be ameliorated by a thorough understanding of the actual contribution of specific proteases and their complex interactive proteolytic pathways in bone metastasis occurring in specific cancers; discriminating between proteases that facilitate cancer progression from those with tumor-suppressing activities; identifying temporospatial and tissue-specific patterns of proteases in cancer; developing methods to assess pharmacodynamic efficacy of the inhibitors; defining the protease degradome of the cancerous lesions to be treated; and generating more selective protease inhibitors. Only then, along with better designed clinical trials, will we be able to define the real value of protease inhibitors in the treatment of patients with bone metastatic disease, for whom, still, no curative treatments exist.

Conflict of Interest: None reported.

Peer Review: This article has been peer-reviewed.
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