Expression of Heparanase by Primary Breast Tumors Promotes Bone Resorption in the Absence of Detectable Bone Metastases

Thomas Kelly, Larry J. Suva, Yan Huang, Veronica MacLeod, Hua-Quan Miao, Ronald C. Walker, and Ralph D. Sanderson

Abstract

Heparanase is an enzyme that cleaves heparan sulfate and through this activity promotes tumor growth, angiogenesis, invasion, and metastasis in several tumor types. In human breast cancer patients, heparanase expression is associated with sentinel lymph node metastases. However, the precise role of heparanase in the malignant progression of breast cancer is unknown. To examine this, a variant of MDA-MB-231 cells was transfected with the cDNA for human heparanase (HPSE cells) or with vector alone as a control (NEO cells). Transfection produced a 6-fold increase in heparanase activity in HPSE cells relative to NEO cells. When injected into the mammary fat pads of severe combined immunodeficient mice, the tumors formed by HPSE cells initially grow significantly faster than the tumors formed by NEO cells. The rapid growth is due in part to increased angiogenesis, as microvessel densities are substantially elevated in primary HPSE tumors compared with NEO tumors. Although metastases to bones are not detected, surprisingly vigorous bone resorption is stimulated in animals bearing tumors formed by the HPSE cells. These animals have high serum levels of the C-telopeptide derived from type I collagen as well as significant elevation of the active form of tartrate-resistant acid phosphatase (TRAP)-5b. In contrast, in animals having a high tumor burden of Neo cells, the serum levels of C-telopeptide and TRAP-5b never increase above the levels found before tumor injection. Consistent with these findings, histologic analysis for TRAP-expressing cells reveals extensive osteoclastogenesis in animals harboring HPSE tumors. In vitro osteoclastogenesis assays show that the osteoclastogenic activity of HPSE cell conditioned medium is significantly enhanced beyond that of NEO conditioned medium. This confirms that a soluble factor or factors that stimulate osteoclastogenesis are specifically produced when heparanase expression is elevated. These factors exert a distal effect resulting in resorption of bone and the accompanying enrichment of the bone microenvironment with growth-promoting factors that may nurture the growth of metastatic tumor cells. This novel role for heparanase as a promoter of osteolysis before tumor metastasis suggests that therapies designed to block heparanase function may disrupt the early progression of bone-homing tumors.

Introduction

Metastasis to bone and osteolytic lesions caused by the overactivity of tumor-stimulated osteoclasts are major complications of breast cancer. These events are regulated by several growth-regulatory factors, including heparan sulfates. Heparan sulfates are present within the tumor microenvironment as components of heparan sulfate proteoglycans or as free heparan sulfate chains. These highly anionic sugars act to fine-tune the activities of growth factors and chemokines, such as those that regulate tumor cell growth, angiogenesis, and osteoclastogenesis. Syndecan-1 is a major heparan sulfate proteoglycan of breast cancer cells and is also found in the bone marrow. Clinically, high levels of syndecan-1 expression in human breast tumors are associated with poor prognosis and an aggressive phenotype. Syndecan-1 can also be induced in reactive stroma responding to breast cancer. Moreover, when breast cancer cell lines were grown together with mouse fibroblasts, the fibroblasts were induced to express syndecan-1 to high levels. The heparan sulfate present in tumors of the breast apparently promotes growth and may be structurally and functionally different than the heparan sulfate derived from normal breast. For example, the heparan sulfate of breast cancer promotes formation of fibroblast growth factor (FGF)-2 receptor complexes to a greater degree than heparan sulfate from normal breast epithelium. This may be due in part to the action of heparan sulfate-modifying enzymes, such as heparanase.

Heparanase cleaves heparan sulfate chains with an endo-β-D-gluconuronidase activity releasing activated fragments of heparan sulfate that apparently mediate its growth and angiogenic effects by acting on tumor cells and endothelial cells. Heparanase is synthesized by cells as a Mr 65,000 protein that is processed to a fully active Mr 50,000 form. The active enzyme cleaves the glycosidic bonds of heparan sulfate at relatively few places producing fragments that are usually 10 to 20 sugar residues in length. These fragments are large enough to interact with growth factors but are not bound to the extracellular matrix or to cell surfaces. In addition, the cleavage of heparan sulfate contributes to erosion of basement membrane barriers, thereby facilitating invasion and metastasis. Indeed, heparanase has been directly implicated in promoting invasiveness, angiogenesis, and metastasis. Heparanase seems to play an important role in human breast carcinomas, where expression of heparanase correlates with large tumor size and enhanced metastatic potential. Elevated heparanase has also been reported to be associated with a poor prognosis in several other human cancers, including gastric, endometrial, pancreatic, and bladder cancers.
We have shown previously that the expression of heparanase in myeloma cells implanted s.c. in severe combined immunodeficient (SCID) mice will promote tumor metastasis to bone (23). In the present study, we extended this model to test the effects of heparanase expression on metastasis of breast cancer cells to bone. Although direct evidence of metastases to bone were not detected in this model, a marked enhancement of osteoclastogenesis and bone turnover was discovered in animals bearing tumors that expressed heparanase compared with controls. This novel finding suggests an important role for heparanase in promoting bone resorption even when tumors are not evident in the bone.

Materials and Methods

Reagents, cell lines, and culture conditions. Tissue culture plasticware was purchased from Fisher (Pittsburgh, PA) and all tissue culture reagents were analytic grade and purchased from Sigma (St. Louis, MO). The MDA-MET cell lines (NEO and HPSE) were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in sterile culture dishes as described previously (24). Both cell lines were certified to be Mycoplasma free. Cells were subcultured by trypsinization in 0.5% trypsin (Sigma) and 0.5 mmol/L EDTA in HBSS without calcium or magnesium in a laminar flow hood during their logarithmic phase of growth. Conditioned medium (incubated for 48 hours; containing serum) from cells was collected, diluted 50% in mMEM, and added to cultures of human peripheral blood mononuclear cells (PBMC; as described below).

Preparation of heparanase-expressing human breast cancer cells. MDA-MET cells were derived from MDA-MB-231 human breast adenocarcinoma cells based on their ability to hom and to grow in bone (25). Heparanase cDNA (HPSE1) was subcloned in the sense direction into pIRE2-EGFP (Clontech, Palo Alto, CA) vector, which allowed both the heparanase gene and the enhanced green fluorescent protein (EGFP) to be translated from a single bicistronic mRNA as described previously for multiple myeloma cells (23, 26). The pIRE2-EGFP/HPSE1 construct was stably transfected into MDA-MET human breast cancer cells using Lipofectin reagent (Invitrogen, Carlsbad, CA) and Opti-MEM 1 (Life Technologies, Grand Island, NY) with 10 μg DNA (pIRE2-EGFP vector only for NEO transfections or pIRE2-EGFP/HPSE1 for HPSE transfections) following the manufacturer's instructions. The cells were selected with G418 (800 μg/mL) and at least 2.0 × 10^6 cells were sorted by green fluorescence using a flow cytometer. The sorted cells were allowed to grow and again examined for green fluorescence after 1 week. A minimum of three sorting runs were done to achieve a minimum of 66% cells exhibiting green fluorescence in the transfectants.

Heparanase activity assay. The heparanase activity assay used an immobilized [3H]heparan sulfate substrate and was done as described (25, 26). Purified recombinant heparanase (46 ng) was used as the positive control and buffer was used as the negative control. Each sample was normalized to equal volume and tested in triplicate on at least two separate occasions.

Western blots. Cells were extracted in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 0.2% bovine serum albumin, 0.02% NaN₃, 0.1% phenylmethylsulfonyl fluoride, and 10 μg/mL leupeptin for 30 minutes on ice. Extracts were centrifuged at 16,000 × g at 4°C for 10 minutes and the supernatants were analyzed for protein concentration using a BCA Protein kit (Pierce, Rockford, IL). Western analysis was done using 6 μg protein per lane were added to the wells of SDS-PAGE gels and following transfer to a polyvinylidene fluoride membrane, the sections were washed with PBS and stained with biotinylated rabbit anti-mouse IgG (Vector, Burlington, CA) for 30 minutes at 22°C. Microvessel densities were determined as described previously (26, 29).

To localize heparanase in tumor tissues, the same procedure as described above was followed using an antibody to heparanase (27, 28), except that a HRP-conjugated goat anti-mouse antibody (Vector) was used to detect this mouse monoclonal antibody (26).

Osteoclasts were identified in mouse long bones that were excised, fixed in 10% neutral-buffered formalin for 2 days, and decalcified in 5% formic acid with agitation until deemed clear by the ammonium oxalate end point test (18). The decalcified specimens were then dehydrated through graded ethanol and cleared in methyl salicylate before paraffin infiltration. Subsequently, they were embedded in paraffin, sectioned (5 μm), and stained with H&E as described previously (25, 32) and tartrate-resistant acid phosphatase (TRAP) using the Acid Phosphatase Leukocyte kit (Sigma) as described (24).

Bone resorption. Serum levels of the COOH-terminal type I collagen telopeptides was determined using the RatLaps ELISA kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark) following the manufacturer's instructions. Mouse serum samples (20 μL/well) were tested in triplicate. The Mouse TRAP assay (Assmen Bioanalytiikkka Oy, Turku, Finland) was used to determine serum levels of TRAP-5b as directed by the manufacturer. Mouse serum samples were diluted 1:4 (25 μL/well) and tested in triplicate.

Isolation and culture of peripheral blood mononuclear cells and their differentiation to osteoclasts. Peripheral blood was collected from healthy donors (approved by the UAMS Institutional Review Board) using
Heparanase-transfected cells have high heparanase activity but exhibit in vitro growth kinetics similar to NEO control cells. A, density plots generated by flow cytometry of EGFP fluorescence show the percentage of cells positive for EGFP (top right quadrant of density plots). Control (nontransfected), 4%; NEO, 75%; HPSE, 66%. B, Western blot of cell extracts of NEO and HPSE cells probed with a mouse monoclonal antibody to heparanase. Two bands are evident in the HPSE cell extract, the heparanase precursor (65 kDa), and the highly active processed form (50 kDa). C, representative growth curve for NEO and HPSE cells in culture showing linear growth over 72 hours. Points, total viable cells from three different cultures using the MTT assay.

Results

To investigate the role of heparanase in an animal model of breast cancer, a subline of MDA-MB-231 human breast adenocarcinoma cells called MDA-MET were employed. MDA-MET cells were selected for their ability to colonize bone when injected into arterial blood (25). These cells were transfected with the cDNA for human heparanase (HPSE cells) or with the empty vector only (NEO cells). Transfected cells were enriched by G418 selection; because a bicistronic vector coding for EGFP was employed, further enrichment of transfectants was accomplished by fluorescence cell sorting. After multiple rounds of sorting, enriched cell populations containing at least 66% fluorescent cells were obtained for use in subsequent experiments (Fig. 1A). Western blots confirm that NEO cells constitutively express low levels of heparanase compared with HPSE cells that show high levels of the 50-kDa processed and highly active form of the enzyme and low levels of the 65-kDa unprocessed form (Fig. 1A).

Immediately before initiation of all in vivo experiments, cell extracts were analyzed to determine the level of heparanase activity expressed in HPSE cells and NEO controls. As expected, extracts of HPSE cells have high heparanase enzyme activity as measured by the release of immobilized [3H]heparan sulfate (Fig. 1B). The basal heparanase activity of the NEO controls is only slightly above background (buffer), whereas the activity in the HPSE cell extracts is significantly elevated (≈6-fold). The expression of active heparanase does not seem to alter growth properties in vitro as both NEO and HPSE cells exhibit identical growth rates over 72 hours (Fig. 1C).

Next, NEO or HPSE cells were injected into the mammary fat pads of female SCID mice. Despite the identical growth pattern of the HPSE and NEO cells in vitro (Fig. 1C), following fat pad inoculation, HPSE cells formed more rapidly growing tumors compared with the control (Fig. 2A). Similarly, tumor wet weights obtained at sacrifice confirmed that the HPSE tumors have a significant growth advantage over NEO tumors (Fig. 2B). Moreover,
HPSE cell tumors have significantly higher microvessel densities (>2-fold higher) than tumors formed by NEO cells as measured by anti-CD34 antibody staining (Figs. 2C and 3B). The enhanced ability to promote angiogenesis may explain the initial growth advantage that the HPSE cells have over the NEO control cells. These growth- and angiogenesis-promoting abilities of heparanase are consistent with earlier work on heparanase-expressing tumor cells in other systems (14, 15, 26).

To determine if heparanase expression was maintained in vivo, tumors were excised after 2 weeks growth in the mammary fat pad and heparanase expression was analyzed by Western blotting. HPSE cells retain relatively high levels of enzyme expression in vivo and NEO cells maintain low levels of heparanase expression (Fig. 3A). This finding was confirmed by direct immunohistochemistry of the primary tumors using an antibody to heparanase (Fig. 3B).

We have shown previously that the expression of heparanase promotes the spontaneous metastasis of myeloma cells to bone (23). To determine if heparanase has a similar effect on the metastatic potential of human breast cancer cells, we analyzed the skeleton of animals in which tumor cells had been injected into the mammary fat pad. However, even using extensive histology, immunohistochemistry, radiology, microcomputed tomography, and positron emission tomography, we were unable to find convincing evidence of tumor cells in the skeleton (femur, tibia, and spine; data not shown). Despite the lack of evidence for tumors growing in bone, there was consistent evidence of extensive bone resorption as measured by two serum assays. Serum levels of C-telopeptide of type I collagen, a product of osteoclastic bone resorption and an indicator of increased osteoclast activity (33) as well as

Figure 2. Tumors formed by HPSE cells grow faster and have higher microvessel density than tumors formed by NEO cells. A, cells were implanted into the mammary fat pads and the volume was determined for NEO tumors (white columns; n = 24) and HPSE tumors (black columns; n = 27). Columns, mean tumor volume; bars, SD. Ps are given for significant differences on days 14, 17, and 19 as judged by the t test. B, in the primary tumors, cells expressing elevated heparanase (HPSE) produce greater tumor burden per animal compared with primary tumors formed by NEO cells as determined by wet weight at sacrifice. Columns, mean weight of the total primary tumor burden in individual animals. Numbers of primary tumors: experiment 1: NEO, 24; HPSE, 27; experiment 2: NEO, 19; HPSE, 20. Ps are given for significant differences in comparisons as judged by the t test. C, sections of the primary tumor were stained with antibody to mouse CD34 and the vessels were counted. Columns, mean of vessel counts in five microscopic fields per tumor; bars, SE. NEO, n = 7 tumors; HPSE, n = 8 tumors; all from different animals.

HPSE cell tumors have significantly higher microvessel densities (>2-fold higher) than those formed by NEO cells as measured by anti-CD34 antibody staining (Figs. 2C and 3B). The enhanced ability to promote angiogenesis may explain the initial growth advantage that the HPSE cells have over the NEO control cells. These growth- and angiogenesis-promoting abilities of heparanase are consistent with earlier work on heparanase-expressing tumor cells in other systems (14, 15, 26).

To determine if heparanase expression was maintained in vivo, tumors were excised after 2 weeks growth in the mammary fat pad and heparanase expression was analyzed by Western blotting. HPSE cells retain relatively high levels of enzyme expression in vivo and NEO cells maintain low levels of heparanase expression (Fig. 3A). This finding was confirmed by direct immunohistochemistry of the primary tumors using an antibody to heparanase (Fig. 3B).

We have shown previously that the expression of heparanase promotes the spontaneous metastasis of myeloma cells to bone (23). To determine if heparanase has a similar effect on the metastatic potential of human breast cancer cells, we analyzed the skeleton of animals in which tumor cells had been injected into the mammary fat pad. However, even using extensive histology, immunohistochemistry, radiology, microcomputed tomography, and positron emission tomography, we were unable to find convincing evidence of tumor cells in the skeleton (femur, tibia, and spine; data not shown). Despite the lack of evidence for tumors growing in bone, there was consistent evidence of extensive bone resorption as measured by two serum assays. Serum levels of C-telopeptide of type I collagen, a product of osteoclastic bone resorption and an indicator of increased osteoclast activity (33) as well as

Figure 3. Heparanase expression levels are maintained in tumors of NEO and HPSE cells. A, Western blot analysis of heparanase expression in four individual tumors formed from NEO cells and five individual tumors formed from HPSE cells. Each lane was loaded with 30 μg protein. Right, molecular weights (×10^-2). B, immunohistochemistry of tumors demonstrating levels of heparanase (anti-HPSE) and microvessel density (anti-CD34). Tumors of NEO cells are weakly positive for heparanase expression and show a light brown 3,3'-diaminobenzidine (DAB) reaction product as well as the blue hematoxylin counterstain. Tumors of HPSE cells are positive for heparanase as indicated by the dark brown DAB reaction product. Magnification, ×40. The nuclei appear blue and are ~10 μm in diameter. There are significantly fewer blood vessels (anti-CD34 positive; brown) in tissue sections of NEO tumors compared with HPSE tumors. Sections were counterstained with hematoxylin to show all cells. Magnification, ×20 (reduced to fit the entire microscopic fields). The nuclei appear blue and are ~10 μm in diameter.
To test the idea that soluble tumor-derived factors are capable of stimulating osteoclastogenesis in vivo, a well-established in vitro assay using human PBMCs was employed (24). Although conditioned medium from NEO cell cultures stimulates osteoclast formation, HPSE cell conditioned medium stimulates osteoclastogenesis significantly above the level of NEO conditioned medium ($P = 0.037$, Fig. 5B and C). The osteoclasts formed by the conditioned medium of HPSE cells are large and multinucleated and stain positive for TRAP (Fig. 5C). These cells are functional osteoclasts with the capacity to resorb bone (data not shown), as we have described previously (24). The data shown in Fig. 5B and C are representative of two separate experiments using different harvests of conditioned medium that gave reproducible results.

**Discussion**

This work reveals a novel role for heparanase in skeletal complications that accompany breast cancer. We have discovered that breast tumors having elevated levels of heparanase and growing in the mammary fat pads promote bone remodeling before metastases or even microscopic tumor foci can be detected in the bone marrow. In contrast, animals injected with control cells that express low levels of heparanase activity exhibit no systemic increase in bone resorption even when the primary tumor burden is large. The mechanism underlying the heparanase-mediated increase in bone resorption is the stimulation of osteoclastogenesis, because abundant osteoclasts are evident in the long bones of animals bearing tumors expressing high levels of heparanase. Moreover, osteoclastogenesis is stimulated in vitro by conditioned medium from HPSE cells. This effect is not due to a direct action of heparanase on osteoclast precursors because neither the heparanase protein nor the heparanase enzyme activity is detected in the medium of these cells (data not shown). Rather, our data indicate a role for heparanase in mediating the release of osteolytic agents into the circulation. This likely occurs via release of active heparan sulfate fragments and/or release of heparan sulfate–bound growth factors that then travel through the circulation to act on bone. The release of heparan sulfate–bound osteostrogenic factors by tumor-generated heparanase is consistent with recent studies demonstrating the important role of heparan sulfate proteoglycans, such as syndecan-1, in binding to and regulating the activity of effector molecules, such as interleukin (IL)-8, hepatocyte growth factor, FGF, and osteoprotegerin (1, 5, 35–39).

Importantly, a role for active heparan sulfate fragments directly stimulating bone turnover is consistent with the well-established observation that treatment with heparin, a highly sulfated form of heparan sulfate, often causes bone resorption and decreased bone density in humans (40, 41). Heparin has also been shown to induce the loss of trabecular bone by increasing osteoclastogenesis and osteoclast activity in experimental animals (42, 43). These effects are mediated by a synergistic interaction of heparin with IL-11 resulting in Stat3 activation that in turn stimulates osteoclast formation (44). The breast cancer cells used in our model express elevated heparanase before bone metastases.

Osteoclast-derived TRAP-5b, an osteoclast-specific enzyme, are markedly elevated in the serum of animals bearing HPSE tumors (Fig. 4A and B). This was confirmed in a separate experiment in which serum levels of TRAP-5b are shown to be elevated in the serum of animals with HPSE tumors and not in that of animals with NEO tumors (Fig. 4C). The TRAP-5b assay is particularly useful because it measures only the active enzyme and thus provides an accurate assessment of osteoclast activity at the time the serum was harvested (34). Consistent with the elevated bone remodeling observed in animals bearing tumors of HPSE cells, abundant TRAP-positive osteoclasts were identified on the bone surface of these animals (Fig. 5A).
are observed raises the possibility that tumors condition the bone marrow for metastases by first stimulating osteoclastogenesis and bone resorption. This increase in bone resorption releases numerous factors stored in the bone that fuels further tumor growth, thereby leading to continued stimulation of osteoclasts (24, 45). In breast cancer, bone metastases frequently result in lytic lesions with numerous osteoclasts and areas of bone erosion immediately surrounding foci of metastatic tumor cells (45). The progression of osteolytic bone metastases requires the establishment of functional interactions between metastatic cancer cells and bone cells (24, 46), which are presumably mediated by soluble stimulators of osteoclast activity (46, 47). Our results show that established foci of tumor cells are not necessary for the stimulation of bone resorption and support the idea that bone resorption is induced by the release of a systemic osteoclast-stimulating factor(s) from tumor cells expressing high levels of heparanase.

Unlike our findings with myeloma cells, expression of heparanase did not promote metastasis of the MDA-MB-231 subline to bone within the timeframe of these experiments. In the myeloma studies, metastases were apparent in 30 of 31 animals bearing s.c. tumors of myeloma cells expressing elevated heparanase, whereas only 3 of 23 animals bearing tumors of control cells expressing low levels of heparanase had bone metastases (23). There are several important differences between that study and the one reported here that may explain in part why bone metastases were not detected with the breast cancer cells. First, the s.c. tumors of myeloma cells expressing heparanase grow more slowly than their breast cancer cell counterparts, thereby allowing the myeloma experiments to be carried out for 7 and 9 weeks (23). The relatively slow growth may be associated with the fact that myeloma is primarily restricted to the bone marrow microenvironment and only become extramedullary late in disease progression. Thus, myeloma does not grow as well in nonbone locations. In the case of breast cancer cells implanted in the mammary fat pads, their rapid growth forced sacrifice of the animals by 21 days and in some experiments as early as 14 days after implantation of the tumor cells. Therefore, the animals may have been sacrificed before primary tumor was able to successfully seed metastatic cells in the bone. Second, myeloma cells implanted s.c. are at a site distant from their preferred microenvironment and may experience selective pressure to escape the s.c. site and home to bone. In contrast, the mammary fat pad is thought to resemble the microenvironment of the breast and the selective pressure to leave the breast and home to the bone may not have been as great.
In summary, this work reveals that expression of heparanase by a tumor distal to the bone can have a dramatic impact on bone turnover. In addition to affecting skeletal integrity, these events aid in preparing a growth-enriching bone microenvironment that will support metastatic tumor cells once they enter the bone. Thus, it may be beneficial to employ inhibitors of heparanase as an early therapeutic approach to impede progression of breast cancer as well as other bone-homing tumors.

References


XXIII. Bendre MS, Montague DC, Peery T, Akel NS, Gaddy D, Suva LJ. Interleukin-8: stimulation of osteolastogenesis and bone resorption is a mechanism for increased osteolysis of metastatic bone disease. Bone 2003;33:528–35.


Expression of Heparanase by Primary Breast Tumors Promotes Bone Resorption in the Absence of Detectable Bone Metastases

Thomas Kelly, Larry J. Suva, Yan Huang, et al.