Tumorigenesis and Neoplastic Progression

Bone Marrow-Derived Cathepsin K Cleaves SPARC in Bone Metastasis

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Bone metastasis is a hallmark of advanced prostate and breast cancers, yet the critical factors behind attraction of tumors to the skeleton have not been validated. Here, we investigated the involvement of cathepsin K in the progression of prostate tumors in the bone, which occurs both by direct degradation of bone matrix collagen I and by cleavage of other factors in the bone microenvironment. Our results demonstrated that bone marrow-derived cathepsin K is capable of processing and thereby modulating SPARC, a protein implicated in bone metastasis and inflammation. The coincident up-regulation of SPARC and cathepsin K occurred both *in vivo* in experimental prostate bone tumors, and *in vitro* in co-cultures of bone marrow stromal cells with PC3 prostate carcinoma cells. PC3-bone marrow stromal cell interaction increased secretion and processing of SPARC, as did co-cultures of bone marrow stromal cells with two other cancer cell lines. In addition, bone marrow stromal cells that were either deficient in cathepsin K or treated with cathepsin K inhibitors had significantly reduced secretion and cleavage of SPARC. Increases in secretion of pro-inflammatory cytokines (ie, interleukin-6, -8) coincident with overexpression of cathepsin K suggest possible mechanisms by which this enzyme contributes to tumor progression in the bone. This is the first study implicating bone marrow cathepsin K in regulation of biological activity of SPARC in bone metastasis. (Am J Pathol 2009, 175:1255–1269; DOI: 10.2353/ajpath.2009.080906)

Despite recent progress in cancer detection and treatment, it remains unclear which skeletal-specific factors are among the critical determinants in preferential localization of metastatic cells to the bone. Recent clinical and experimental data suggest that accelerated bone remodeling may be responsible for homing of tumor cells to the bone.^{1,2} This is evidenced by the increased metastasis in response to experimental treatment with calciotropic hormone or to androgen ablation^{1,2} and reduced incidence of metastasis with antiresorptive therapies.³ Establishment of tumors in bone requires multidirectional interactions between tumor cells, bone cells, stromal cells, and inflammatory components, as well as extracellular matrix proteins. This complex interplay between tumor cells and the bone microenvironment facilitates increased bone turnover and promotes tumor cell survival.

The key enzyme responsible for osteolysis of bone is the cysteine protease cathepsin K, which is the only known mammalian protease capable of degrading both the helical and non-helical regions of collagen I, the main component of the organic bone matrix.⁴ Within the bone microenvironment, cathepsin K localizes predominantly to osteoclasts and its overexpression results in increased bone turnover.⁵ Accordingly, a deficiency in this potent collagenase results in a bone-sclerosing disorder called pycnodysostosis in man and osteopetrosis in mice.^{6,7} The presence of cathepsin K has been demonstrated in many malignancies including prostate and breast cancers, both of which have a high propensity to metastasize to bone.^{8–10}

A role for cathepsin K in advanced cancers has been attributed mainly to its ability to degrade native collagen I and facilitate the expansion of tumors in the bone. Our recent studies and data by other groups suggest that cathepsin K also cleaves and thereby modulates the biological activity of several important proteins in the bone microenvironment.^{11–15} Of particular importance is

Supported by grants from the DOD PC030325, DOD PC074031, DOD DAMD1702-1-0693, R01 CA 56586, MIRL: U54 RR020843, P30 CA 22453, P30 ES 06639.

Accepted for publication June 4, 2009.

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Prostate and breast cancers commonly metastasize to skeletal sites and locally disrupt normal bone remodeling.

cleavage of secreted protein acid and rich in cysteine (SPARC/osteonectin/BM-40), the most abundant noncollagenous component of the bone matrix and a modulator of cell-matrix interactions.¹⁶ The precise role of SPARC in tumor growth and metastasis is unclear. In the vast majority of human cancers, SPARC is a marker of aggressiveness and poor prognosis; however, SPARC is also anti-tumorigenic, suggesting multiple roles for this protein in tumor growth and progression.^{17–20} Increased levels of SPARC in prostate cancer have been correlated with an invasive phenotype and suggested to facilitate homing of tumor cells to the bone.^{21,22}

Several proteases, including cathepsin K, have been shown to cleave SPARC *in vitro*,^{11,23,24} a process that gives rise to smaller peptides with higher affinity for collagens and presumably basement membrane.²⁴ Proteolytic activation of SPARC not only enhances its binding affinity, which might be essential for matrix storage, but also leads to the release of biologically active cleavage products.²⁵ These bio-peptides regulate several growth factors including vascular endothelial growth factor, platelet-derived growth factor, and fibroblast growth factor-2, and ultimately contribute to the enhancement of tumor-associated angiogenesis.²⁶ Expression of SPARC is often coincident with induction of matrix-degrading enzymes, in particular, matrix metalloproteinases (MMPs) 1, 2, 3, and 9, and MT1-MMP, a process leading to facilitation of extracellular matrix remodeling and induction of inflammatory responses.^{17,27,28} Here, we demonstrated coincident up-regulation of SPARC and the bone-resorbing enzyme cathepsin K both *in vivo* in experimental prostate bone tumors and *in vitro* in co-cultures of bone marrow stromal cells (BMSC) with prostate and breast carcinoma cells. The involvement of cathepsin K in cleavage and processing of SPARC was validated by the use of a selective cathepsin K inhibitor and cathepsin K-deficient bone marrow stromal cells. Overexpression of cathepsin K coincident with changes in pro-inflammatory cytokines was demonstrated by human cytokine antibody arrays and further confirmed by cathepsin K inhibition – a result suggesting possible mechanisms by which this enzyme contributes to tumor progression in the bone.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium, 2-[*N*-morpholino]ethane-sulfonic acid, piperazine-NVNV-bis[2-ethanesulfonic acid], Hanks' salt solution, sodium bicarbonate, antibiotics, dimethyl sulfoxide, paraformaldehyde, the broad spectrum cysteine protease inhibitor E-64, a human monoclonal β -actin antibody, a human monoclonal cytokeratin antibody (clone C-11+PCK-26+CY-90+KS-1A3+M20+A53-B/A2) and other chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO). Fetal bovine serum, trypsin-EDTA and collagenase were obtained from Invitrogen (Carlsbad, CA). Vitrogen-100 collagen type I was from Cohesion Laboratories (Palo Alto, CA). The fluorogenic substrate Z-Gly-Pro-Arg-

NHMec and its cleavage product NH₂Mec were purchased from Bachem (King of Prussia, PA). CA074 was purchased from Peptides International (Louisville, KY). Cathepsin K inhibitor L-873724²⁹ was obtained from (Merck-Frosst, Canada). Mouse anti-human cathepsin K antibodies were purchased from Novocastra (Newcastle, UK). Mouse anti-human SPARC antibodies were purchased from Hematological Technologies (Essex Junction, VT). Mouse anti-human tartrate resistant acid phosphatase (TRAcP) antibodies were from Zymed Laboratories, and mouse anti-vascular endothelial growth factor, rabbit anti- α -smooth muscle actin, and rabbit antivimentin antibodies were from Abcam (Cambridge, MA); rabbit anti-CD68 antibodies were from R&D Systems (Minneapolis, MN) and mouse anti-CD31 antibodies were from Dako (Carpinteria, CA). Mouse anti-human interleukin (IL)-6, goat anti-human IL-8 antibodies, and goat anti-human receptor activator for nuclear factor κ -B ligand (RANKL) neutralizing antibodies (AF626) were from R&D Systems (Minneapolis, MN). Alexa Fluor 488- and Alexa Fluor 564-conjugated donkey anti-mouse and anti-rabbit IgG were from Molecular Probes (Eugene, OR) and normal donkey serum was obtained from Jackson ImmunoResearch (West Grove, PA). Horseradish peroxidase-labeled goat anti-rabbit IgG and Micro BCA protein kits were purchased from Pierce (Rockford, IL). RayBio Human Cytokine Antibody Arrays V were purchased from RayBiotech (Norcross, GA). Western blotting detection kits were from Amersham Pharmacia Biotechnologies (Piscataway, NJ). The Vectastain Elite ABC immunohistochemistry kit and NovaRED kit for peroxidase were purchased from Vector Laboratories, (Burlingame, CA).

Antibodies to Cleavage Fragments of SPARC

Peptides for polyclonal antibody production were designed according to SPARC cleavage fragments reported by Sasaki et al,²⁴ using the antigenic search website <http://mobyte.pasteur.fr/cgi-bin/portal.py?form=antigenic>. last accessed July 31, 2002). The peptides were synthesized at the Federal Drug Administration (Bethesda, MD). Rabbit anti-human antibodies were made at Covance (Denver, PA).

Cell Lines and Human Bone Fragments

PC3, an androgen-independent osteolytic line derived from a bone metastasis of a high-grade adenocarcinoma,³⁰ and DU145, an androgen-independent osteolytic line derived from a brain metastasis³¹ were purchased from American Type Culture Collection (Manassas, VA). The human prostate cancer C4-2B cell line is a derivative of the LNCaP cell line³² and was kindly provided by Dr. Leland W. K. Chung, Emory University, Atlanta, Georgia. The MDA-231BO is a bone-seeking clone derived from MDA MB-231 breast carcinoma cells³³ and was kindly provided by Dr. Toshiyuki Yoneda (University of Texas Health Science Center, San Antonio, TX). All cell lines were cultured in Dulbecco's modified Eagle's medium supple-

mented with 10% fetal bovine serum and maintained in a 37°C humidified incubator ventilated with 5% CO₂.

For derivation of human bone marrow stromal cells (hBMSC) and for *in vivo* tumor implantation, human male fetal femurs (16 to 19 weeks gestation) were purchased from Advanced Bioscience Resources (Alameda, CA) as previously described.³⁴ Primary hBMSCs were isolated from human fetal bones by flushing the marrow first with 0.25 ml of 0.05% trypsin-EDTA, and then twice with 0.5 ml of Dulbecco's modified Eagle's medium. The cell suspension was overlaid on a 10% to 30% serum gradient and centrifuged for 5 minutes at 700 × *g* to remove the majority of hematopoietic cells. The resulting cell pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and seeded in 35 mm cell culture dishes. After 24 hours, nonadherent cells were removed by replacing the medium. Cells were cultured to confluency by replacing the medium every 2 to 3 days and then expanded and used for co-culture experiments with PC3 cells.

Primary mouse bone marrow cells (mBMSC) were isolated from femurs and tibiae of 6- to 8-week-old cathepsin K-null (FVB/n, N5 *CTSK*) and wild-type (FVB/n, N5 *CTSK*) mice that were kindly provided by Dr. Lisa Coussens (University of California at San Francisco, San Francisco, CA).

Tissue Implantation and Establishment of Human Bone Tumors

Five-week-old male homozygous ICRSC-M severe combined immunodeficient (SCID) mice were purchased from Taconic Farms (Germantown, NY) and were allowed to acclimate in their housing for 1 week. Mice were maintained under aseptic conditions according to NIH guidelines as found in the "Guidelines for the Care and Use of Experimental Animals" (<http://grants.nih.gov/grants/olaw/Guidebook.pdf>, last accessed February 28, 2005). All experimental protocols were approved by the Animal Investigation Committee at Wayne State University. Implantation with human bone fragments and tumor cell injections were performed under isoflurane inhalational anesthesia according to previously published procedures.³⁵ Briefly, human fetal bones were cut into 1.5 cm-long cylinders and implanted into the flanks of SCID mice (2 pieces/animal). Following a 4-week engraftment period, PC3 cells (10⁵ cells/20 μl, right flank) or PBS (control, 20 μl, left flank) were injected directly into the marrow of implanted bone fragments using a 27-gauge needle. Bone tumors and their corresponding controls were removed 2, 4, and 6 weeks after injection. Before tissue removal, mice were euthanized by CO₂ inhalation, followed by cervical dislocation.

Immunoblots and Activity Assays in Tissue Extracts

Harvested bone tumors and their corresponding controls were divided into two groups. Half of the samples were

immediately fixed and embedded for sectioning (see *Immunohistochemistry of Human Bone Tumors* section below). The remaining samples were homogenized using an electric tissue grinder in 500 μl of 250 mmol/L sucrose, 25 mmol/L 2-[*N*-morpholino] ethane-sulfonic acid, 1 mmol/L EDTA, pH 6.5, and 0.1% Triton X-100. The resulting extracts were centrifuged at 800 *g* for 10 minutes, and supernatants were collected and frozen at -80°C. Expression of cathepsin K, SPARC, and cleavage products of SPARC was assessed by immunoblotting according to our previously established and published procedures.³⁴ Activity of cathepsin K in tissue extracts was assayed against the fluorogenic substrate Z-Gly-Pro-Arg-NHMe (final concentration, 100 μmol/L). In addition to being a substrate for cathepsin K, Z-Gly-Pro-Arg-NHMe is also effectively cleaved by cathepsin B (B. E. Linebaugh and B. F. Sloane, unpublished observations), therefore we performed the reaction in the presence of the highly selective cathepsin B inhibitor CA074 (5 μmol/L).³⁴ The progress of the reaction was monitored every minute for a period of 30 minutes on a Fluoroskan II microplate reader. Results of activity assays are expressed as relative fluorescence units formed per minute per cell unit. Cell units were calculated as the protein/DNA ratio (mg protein/μg DNA).³⁴ Statistical significance was determined by a two-tailed *t*-test with assumed equal variance and *P* ≤ 0.05 was considered statistically significant.

Immunohistochemistry of Human Bone Tumors

Tumors were fixed overnight in 4% paraformaldehyde, decalcified in 10% EDTA for 2 weeks and embedded in paraffin. Serial sections (4 μm) were cut, deparaffinized and rehydrated. Adjacent sections of each tumor were analyzed by H&E staining for histological changes and by immunofluorescence for expression and localization of proteins of interest [ie, mouse cathepsin K, 1:200; SPARC, 1:400; 28 kDa and 10 kDa SPARC fragments (O28 and O10), 1:200; CD68, 1:200; cytokeratin, 1:300; vimentin, 1:500; α-smooth muscle actin, 1:500; TRAcP, 1:100]. Controls were run in the absence of primary antibody. For immunofluorescent staining, secondary antibodies were Alexa Fluor 488 (green)- and Alexa Fluor 564 (red)-conjugated donkey anti-rabbit IgG and donkey anti-mouse IgG. For immunohistochemical analysis of cathepsin K expression in PC3 bone tumors, biotinylated secondary antibodies conjugated with peroxidase were used along with a NovaRED kit as a substrate for the peroxidase-mediated reaction.

In Vitro Cleavage of SPARC

Recombinant human platelet SPARC (500 ng) was incubated with 50 ng of recombinant human cathepsin K in the absence and presence of 10 μmol/L cysteine protease inhibitor E-64. The reaction was carried in a 50 mmol/L 2-[*N*-morpholino] ethane-sulfonic acid buffer, containing 2 mmol/L EDTA and 4 mmol/L dithiothreitol at pH 6.0 and pH 7.4. Samples were electrophoresed on 10% to 20% acrylamide gels and immunoblotted using

Figure 1. SCID-hu/intrabone model. **A:** Schematic diagram of the model. Human bones are cut into 1.5 cm-long cross sections and implanted into the flanks of SCID mice. Following a 4-week engraftment period, tumor cells are injected into the marrow cavity of the bone. **B–E:** Histological analysis and blood vessel staining of control bone (**B, D**) and PC3 bone tumor (**C, E**). **B–C:** H&E, composites of $\times 10$ images, pink staining indicates bone matrix, purple nuclei indicate tumor cells; (**D–E**) CD-31 immunostaining, **arrows** indicate blood vessels.

antibodies to full length SPARC and antibodies to cleavage fragments of SPARC.

Cell Cultures on Collagen I Gels

Collagen I gel solutions were prepared according to manufacturers instructions. The individual cultures of BMSCs (1×10^6 cells/dish) and tumor cells (1×10^6 cells/dish) were mixed with 4 ml of collagen and seeded on 100-mm² tissue culture dishes. For co-culture experiments BMSCs (9×10^5 cells/dish) and tumor cells (1×10^5 cells/dish) were mixed together and embedded in collagen I as described for individual cultures. In initial experiments, ratios of 2:1, 5:1 and 10:1 (BMSC: PC3) were tested. Based on the most significant changes in expression and activity of cathepsin K, a final ratio of 10:1 (BMSC: PC3) was chosen for all subsequent experiments. To compare the effects of three-dimensional and two-dimensional environments on the interaction of bone marrow stromal cells with prostate cancer cells, all single and mixed BMSC and PC3 cultures were also grown on uncoated dishes. All cells were cultured for 60 hours, and then serum-starved for an additional 12 hours. Cells were harvested from collagen I gels using 0.1% collagenase in PBS at 37°C. Cell pellets were washed in PBS; resuspended in 250 mmol/L sucrose, 25 mmol/L 2-[N-morpholino] ethane-sulfonic acid, 1 mmol/L EDTA, pH 6.5, and 0.1% Triton X-100 buffer, lysed by sonication; and frozen at -80°C until used. DNA concentration was determined by the method of Downs and Wilfinger,³⁶ and protein was determined using a Micro BCA protein assay kit. Conditioned media (unless otherwise specified) were passed through Millipore 100K concentrators (Burlington, MA) at $150 \times g$ to remove large collagen fragments and were concentrated using Millipore 10K concentrators. Samples were analyzed by immunoblotting and activity assays as

described for tissue samples (see *Immunoblots and Activity Assays in Tissue Extracts* section above). To assay the activity of procathepsin K in conditioned media, pepsin activation was performed as previously described³⁷ and cathepsin K activity measured as described above.

Cytokine Antibody Arrays

The RayBio Human Cytokine Antibody Array V was used for the simultaneous detection of 79 cytokines. Experiments were performed according to manufacturer's instructions, using media conditioned by either individual BMSC or PC3 cells or BMSC-PC3 co-cultures in the absence or presence of cathepsin K inhibitor. Briefly, membranes were incubated in a blocking buffer for 30 minutes, followed by 2-hour incubation with conditioned media. Media samples (1 ml) were appropriately diluted based on the ratio of protein/DNA in corresponding cell lysates.³⁴ Following incubation with media, membranes were washed and incubated overnight with biotinylated primary antibodies. Membranes were then washed and incubated for 2 hours with horseradish peroxidase-labeled streptavidin solution (1:1000), followed by detection of secreted cytokines by enhanced chemiluminescence. Cytokines with the highest levels of secretion in BMSC-PC3 cultures, as determined by densitometric analysis relative to individual PC3 and BMSC cultures, were further assessed by immunoblot analysis.

Results

Establishment of a SCID-hu Intrabone Model of Bone Metastasis

In our previous studies, we have used the SCID-hu model of bone metastasis to demonstrate that expression of the

CTSK expression in PC3 Bone Tumors

Figure 2. Cathepsin K is highly expressed in areas of the tumor undergoing intense bone remodeling (**A**) and is highly associated with osteoclasts (**B**). Less intense cathepsin K staining is observed in areas away from the bone (**D**), where cathepsin K localizes to stromal cells (**E**), and areas around tumor cells (**F**). Cathepsin K does not co-localize with the epithelial marker cytokeratin (**G**), the myoepithelial/myofibroblast marker smooth muscle actin (**H**), or the fibroblast marker vimentin (**I**). Stromal cathepsin K co-localizes with the human macrophage marker CD68 (**J–L**). Nova-Red (brownish-red) staining. **Blue arrows**, osteoclasts; **black arrowheads**, stromal cells; and **blue arrowheads**, tumor cells. Magnification: $\times 10$ (**A**), $\times 20$ (**D**), and $\times 40$ (**B**, **C**, **E**, and **F**). Control in the absence of primary antibody (**C**). Double immunolabeling for cathepsin K (red) and cytokeratin (**G**, green), smooth muscle actin (**H**, green), or vimentin (**I**, green). Magnification, $\times 40$. **J–L**: Double-immunolabeling for cathepsin K (**J**, red) and CD68 (**K**, green); **L** is a red, green, and blue (4,6-diamidino-2-phenylindole, nuclei) fluorescent image. Areas of co-localization of cathepsin K and CD68 appear as yellow fluorescence and are highlighted by boxed areas. Magnification, original $\times 40$.

cysteine protease cathepsin B is modulated by the bone microenvironment.³⁴ Here, we modified the SCID-hu model, cutting fetal bones into 1.5-cm-long cross sections and implanting them into the flanks of SCID mice (Figure 1A). Bones implanted in such a way remain viable and become vascularized during the 4-week engraftment period (Figure 1, D–E). Tumor cells were implanted directly into the center of the bone to more closely recapitulate the interactions between tumor cells and the bone microenvironment *in vivo*. PC3 tumors colonize the bone and show signs of osteolysis as early as 3 weeks after implantation (Figure 1B with Figure 1C).

Localization of Cathepsin K in Experimental PC3 Bone Tumors

Cathepsin K has been implicated in many malignancies, including the cancers of breast and prostate, both of

which metastasize to the skeleton.^{8,9} In clinical prostate carcinomas, levels of this potent protease were higher in bone metastases than in the corresponding primary tumors and soft tissues from the same patients.⁸ Here, in experimental prostate bone tumors, intense cathepsin K staining in multiple cell types in the areas around resorbing bone (Figure 2A), and less prominent immunoreactivity in tumor areas away from the bone (Figure 2D) were observed, a result consistent with an involvement of cathepsin K in tumor-mediated osteolysis. The highest cathepsin K expression was associated with osteoclasts (Figure 2B). Strong cathepsin K positivity was detected in the stromal cells (Figure 2E) and around tumor nests and individual tumor cells (Figure 2F); however, double-immunolabeling studies for cathepsin K and cytokeratin did not reveal association of this protease with the epithelial component of the tumor (Figure 2G). In addition, double immunofluorescent staining for cathepsin K and smooth

Figure 3. Expression and activity of cathepsin K in PC3 bone tumors. **A:** Immunoblot analysis of cathepsin K expression (28 kDa) in extracts from 2-, 4-, and 6-week PC3 tumors (T) and corresponding bone control (C). Blots are representative of at least four experiments. India ink is shown as loading control. **B:** Cathepsin K activity assayed against a Z-Gly-Pro-Arg-NHMe substrate, following a preincubation with a highly selective cathepsin B inhibitor, 5 μ mol/L Ca074. Activity is calculated as RFU/min/mg protein/ μ g DNA and represented as % control (tumor versus its respective control from the same mouse); Gray, control bone extracts; black, tumor extracts. Four tumors and four controls per time point were analyzed. Data are represented as mean $\pm$ SD ($n = 4$). * $P < 0.05$ is considered statistically significant. **C:** TRAcP staining of osteoclasts. NovaRED (brownish-red) staining of representative sections from 2-, 4-, and 6-week PC3 bone tumors; **black arrows**, osteoclasts. Magnification, original $\times 40$.

muscle actin or cathepsin K and vimentin did not show immunolocalization of this protease to myoepithelial/myofibroblast cells (Figure 2H) or fibroblast (Figure 2I) components of the tumor. Strong cathepsin K immunoreactivity was detected in a subpopulation of CD68-positive cells (Figure 2, J–L), which is in agreement with previous evidence that cathepsin K is expressed in macrophages.^{38–41} As illustrated by the yellow fluorescence in Figure 2L, cathepsin K immunolocalized to only a population of macrophages within PC3 bone tumors. This suggests that there is heterogeneity in the pool of macrophages present in the bone tumor. Heterogeneity of tumor-associated macrophages has been reported and suggested to affect cell–cell interactions within the tumor microenvironment.⁴²

Expression and Activity of Cathepsin K in PC3 Bone Tumors

To assess changes in cathepsin K expression and activity during expansion of tumors in the bone, levels of this potent collagenase were assessed at 2, 4, and 6 weeks after introducing the PC3 cells into the marrow. Immunoblot analysis of tumor extracts and corresponding bone controls revealed the highest expression of cathepsin K protein in 4-week tumors, and then reduced levels at 6 weeks (Figure 3A). This result is consistent with the disappearance of trabeculae and a corresponding decrease in the number of osteoclasts at 6 weeks (Figure 3C). The 28-kDa active form was the major form of cathepsin K detected in bone tumors and the corresponding controls. In addition, the 37-kDa proenzyme was present at high levels in 4-week tumors. Levels of cathepsin K expression in human bone tumors corresponded

with cathepsin K activity. The highest expression, observed at 4 weeks (Figure 3B), correlated with increases in osteoclast recruitment as evidenced by an abundance of TRAcP-positive cells at this time (Figure 3C).

In Vitro Cleavage of SPARC (Osteonectin) by Cathepsin K

Cathepsin K has been shown to degrade other components of bone matrix, and thus potentially affect their function within the bone microenvironment. One such protein is SPARC, a substrate of several proteases, including MMPs and cathepsin K.^{11,24,43} In the current study, we used antibodies to specific cleavage products of SPARC (Figure 4A) to assess enzymatic processing of this protein by cathepsin K. All four antibodies confirmed cleavage of the full-length protein by cathepsin K (not shown). As demonstrated by immunoblot analysis with antibodies against the 28 kDa fragment, SPARC was processed rapidly, within 60 minutes, at a pH 6.0, optimal for cathepsin K activity (Figure 4B).¹¹ Major fragments of approximately 28 kDa and 18 kDa, and the lesser amounts of 10 kDa peptides were detected, a result consistent with previous findings by Bossard et al.¹¹

In Vivo Expression and Cleavage of SPARC

Up-regulation of SPARC expression has been previously demonstrated in prostate cancer cell lines and tissues associated with bone metastasis.⁴⁴ Here we examined SPARC expression in experimental PC3 bone tumors. Immunohistochemical analysis revealed heterogeneous

A ANTIBODIES RECOGNIZING CLEAVAGE PRODUCTS OF SPARC

B ANTIBODIES AGAINST 28 kDa FRAGMENT OF SPARC

Figure 4. Cathepsin K cleaves full-length SPARC. **A:** Antibodies recognizing cleavage products of SPARC. Domain structure of SPARC indicating binding sites for antibodies to the cleavage products (**top**); expected cleavage fragments (middle: 40 kDa, 28 kDa, 10 kDa, and 5 kDa); and amino acid sequence with the cleavage sites for cathepsin K and MMPs depicted by **arrows** and **arrowheads**, respectively (**bottom**). Sequences of peptides used for antibody production are bolded and underlined; **B:** *In vitro* cleavage of SPARC by cathepsin K. Recombinant human platelet SPARC (500 ng) was incubated with 50 ng of recombinant human cathepsin K in the absence and presence of the cysteine protease inhibitor E-64. Immunoblot of SPARC cleavage using antibodies recognizing a 28-kDa fragment. At pH 6.0, SPARC was degraded within 30 minutes. Peptides of approximately 28, 18, and 10 kDa were detected. Cleavage was not detected within 60 minutes at pH 7.4.

SPARC immunoreactivity throughout the tumor. The full-length protein was detected in the bone matrix, tumor cells, and surrounding stromal cells (Figure 5A). To assess whether enzymatic processing of SPARC can be detected *in vivo*, we performed immunoblot analysis of SPARC cleavage fragments in extracts of PC3 bone tumors and their corresponding bone controls (Figure 5B). The levels of full-length SPARC (43 kDa band, top panel) were significantly reduced in 6-week tumor extracts indicating enzymatic processing. In addition to full length SPARC, high molecular weight species were detected in some, but not all bone and tumor extracts (top panel); their nature is presently not known. The major SPARC cleavage products detected in PC3 tumor extracts were the 28 kDa and 10 kDa species (Figure 5C). A 18-kDa fragment, which may be an intermediate species generated by removal of the 10-kDa peptide was not detected.

The highest levels of cleavage fragments were found in 6-week tumors, yet there were reduced levels of cathepsin K at this time. This suggests that other SPARC-degrading enzymes, such as MMPs, may be contributing to processing and regulation of bioactivity of SPARC in the bone tumor microenvironment.

Up-Regulation of Cathepsin K and its Substrate SPARC upon Interactions of PC3 Cells with hBMSCs

To further examine the role of the bone microenvironment in regulation of cathepsin K and the involvement of the bone microenvironment in prostate cancer growth, we isolated primary hBMSCs from human bone. PC3 cells and BMSC were grown alone or in co-culture (10:1 ratio of BMSC: PC3 cells) on plastic or in collagen I gels and expression of cathepsin K was assessed. Increased cathepsin K activity (Figure 6A) and protein (Figure 6, B and C) were observed upon interaction of BMSC with PC3 cells in 3D collagen I gels. In addition, hBMSC-tumor cell interactions resulted in increased expression of full-length SPARC (Figure 6B). Analysis of overnight conditioned media revealed a slight increase in secretion of cathepsin K at both the activity (Figure 6D) and the protein levels (Figure 6, E and F). Both the 37-kDa proenzyme and 28-kDa active enzyme were secreted from hBMSC-PC3 co-cultures as well as individual BMSC cultures grown on collagen I. Intracellular up-regulation of SPARC (Figure 6B) resulted in increased secretion of this protein into overnight conditioned media (Figure 6E). In addition to higher levels of the full-length protein in the media, a second band around 40 kDa was detected, suggesting enzymatic processing of SPARC. Both secreted cathepsin K and SPARC were only detectable when conditioned media was not passed through 100K concentrators, a procedure that removes collagen fragments from the media. This result is in agreement with the previously reported ability of SPARC to bind collagen I⁴⁵ and also suggests collagen I-binding properties for cathepsin K.

Effect of Cathepsin K Inhibition on Enzymatic Processing of SPARC

To determine whether cathepsin K might play a role in enzymatic processing of SPARC, we treated co-cultures of hBMSCs and PC3 cells with L-873724, a selective cathepsin K inhibitor (Merck-Frosst, Canada).²⁹ Immunoblot analysis using antibodies against full length SPARC showed that inhibition of cathepsin K did not have a significant effect on intracellular levels of this protein (Figure 7A, Lysates, top panel). However, a significant reduction in the levels of secreted SPARC and disappearance of the 40-kDa band, indicative of reduced enzymatic processing, were observed in the presence of the cathepsin K inhibitor (Figure 7A, media, top panel). This result was further validated by immunoblot analysis of cell lysates using antibodies against the 28-kDa fragment of SPARC. Here, the presence of a 43-kDa band in cell lysates suggests that upon inhibition of

A FULL-LENGTH SPARC

B IN VIVO CLEAVAGE FRAGMENTS

C 10 kDa FRAGMENT IN OSTEOCLASTS

Figure 5. *In vivo* expression of SPARC and its cleavage products in PC3 bone tumors. **A:** SPARC expression in PC3 bone tumors; Nova Red staining indicating SPARC localizes to bone matrix (upper left, arrowheads), tumor cells (upper right, black arrows), stromal cells (lower left, blue arrows); lower right panel: no primary antibody control. **B:** Detection of SPARC cleavage products in tumor extracts. Immunoblot analysis using antibodies to full length (top) and cleavage fragments of SPARC: 28 kDa (top middle, O28 antibody) and 10 kDa (bottom middle, O10 antibody). India ink was used as a loading control (bottom). 28-kDa and 10-kDa cleavage products are detected in tumor extracts from 4- and 6-week tumors. **C:** Immunolocalization of 10-kDa SPARC fragment to osteoclasts. **Top:** Double-immunolabeling for cathepsin K (red) and O10 (green). **Bottom:** Double-immunolabeling for osteoclast marker, TRAcP (red) and O10 (green). Yellow fluorescence indicates co-localization of cathepsin K and O10 (top right) or TRAcP and O10 (bottom right) in osteoclasts (white arrows). Blue fluorescence (4,6-diamidino-2-phenylindole) indicates nuclei. Images ($\times 40$) are representative of at least three experiments.

cathepsin K cleavage of full-length SPARC is greatly reduced (Figure 7C, Lysates, top panel). Intracellular levels of the 10-kDa fragment were increased in PC3-BMSC co-cultures, in comparison with individual cultures, and they were moderately reduced upon the inhibition of cathepsin K (Figure 7C, Lysates, lower panel). The presence of 10-kDa and 28-kDa fragments in the cell lysates corresponds well with their detection in PC3 bone tumors *in vivo* (Figure 5B). Analysis of overnight conditioned media with antibodies against the 28-kDa fragment of SPARC confirmed increased secretion and processing of this protein in PC3-BMSC co-cultures (Figure 7C, Media, top panel). The extracellular levels of full-length protein and its cleavage products were reduced in the presence of L-873724, further indicating an involvement of cathepsin K in this process. We were not able to detect the 10-kDa fragment in the media under these experimental conditions.

Effect of Bone Marrow-Derived Cathepsin K on Secretion and Processing of SPARC

The major sources of cathepsin K in the bone microenvironment are the osteoclasts,⁵ macrophages (Figure 2), and bone marrow stromal cells (Figure 6B). PC3 cells do not express detectable levels of cathepsin K protein under *in vitro* conditions (Figure 6B and 8) and cathepsin K does not associate with epithelial component of experimental prostate bone tumors *in vivo* (Figure 2G). Thus, we speculate that it is the bone marrow-derived cathepsin K that plays a role in SPARC secretion and cleavage in the bone microenvironment. To validate the role of stromal cathepsin K in enzymatic processing of SPARC, we used mBMSCs from wild-type and cathepsin K-null mice. Co-cultures of PC3 cells with mBMSC deficient in cathepsin K revealed re-

Figure 6. Interaction of PC3 cells with hBMSC cells leads to upregulation of cathepsin K and SPARC. PC3 cells and hBMSC were grown alone or in co-culture (10:1 ratio of BMSC: PC3 cells) on plastic or in collagen I gels. **A:** Cathepsin K activity in the cell lysates was assayed against the fluorogenic substrate Z-Gly-Pro-Arg-NHMeC in the presence of a highly selective cathepsin B inhibitor CA074 (5 μ mol/L). Data are represented as mean $\pm$ SD ($n = 3$). * P value ≤ 0.05 is considered statistically significant. **B:** Immunoblot analysis of cathepsin K (top) and SPARC (middle) in cell lysates. Antibodies to full-length SPARC were used. Samples were loaded based on the ratio of mg protein/ μ g DNA (an index of cell size). Actin was used as a loading control. Data are representative of three experiments. **C:** Densitometric analysis of cathepsin K bands (37kDa and 28kDa; top) and SPARC bands (bottom) normalized to actin. Data (expressed as ratios of AU/mm²) are representative of at least three experiments. * P value ≤ 0.05 is considered statistically significant. **D:** Cathepsin K activity in overnight conditioned media was assayed after pepsin activation using the fluorogenic substrate Z-Gly-Pro-Arg-NHMeC in the presence of the selective cathepsin B inhibitor CA074 (5 μ mol/L). Data are represented as mean $\pm$ SD ($n = 3$). **E:** Immunoblot analysis of cathepsin K and SPARC in overnight conditioned media from individual cultures and co-cultures of PC3 and BMSC cells in collagen I gels. Antibodies to full-length SPARC were used. Both forms of cathepsin K (37 kDa proenzyme and 28 kDa active form) are detected in conditioned media from individual BMSC cells and PC3-BMSC co-cultures (top); increased levels and enzymatic processing of SPARC are observed in PC3-BMSC co-cultures (middle). Samples were loaded based on the ratio of mg protein/ μ g DNA in corresponding cell lysates. Coomassie stain of the gel is shown to demonstrate equal loading (bottom). **F:** Densitometric analysis of cathepsin K bands (37kDa and 28kDa; top) and SPARC bands (bottom) normalized to actin in corresponding cell lysates. Data (expressed as ratios of AU/mm²) are representative of at least three experiments. * P value ≤ 0.05 is considered statistically significant.

duced intracellular and extracellular levels of SPARC (Figure 7B), a result consistent with literature evidence that SPARC expression is regulated by proteases that cleave it.¹⁷ The appearance of the 40-kDa band was less intense and thus indicative of reduced enzymatic processing, although not completely eliminated as observed in the presence of the cathepsin K inhibitor. This result, further validated using antibodies to cleavage fragments of SPARC (Figure 7D), suggests that some degree of compensation exists by other SPARC-degrading proteases, eg, MMP-9

and MT1-MMP, which are overexpressed by cathepsin K-null mice.⁴⁶

SPARC Secretion and Processing upon BMSC Interaction with Other Prostate and Breast Tumor Cell Lines

To determine the relevance of the results obtained with PC3 cells to prostate cancer and bone metastasis, we

Figure 7. Enzymatic processing of SPARC is reduced by inhibition or absence of cathepsin K. **A:** Immunoblot analysis of cell lysates (**top** and **middle**) and conditioned media (**bottom**) from co-cultures of PC3 and hBMSC cells grown in collagen I gels in the absence and presence of a 100 nmol/L concentration of a cathepsin K inhibitor (L-873724). Cathepsin K inhibition results in slightly increased levels of full length SPARC in cell lysates (lysates, **top**) and significantly decreased secretion and processing of SPARC in conditioned media (media, **top**). **B:** Immunoblot analysis of lysates and conditioned media from PC3 cells co-cultured with wild-type and cathepsin K-null mBMSC. Co-culture with cathepsin K-null cells leads to reduced expression, secretion, and processing of SPARC. **C:** Immunoblot analysis of intracellular levels of 28 kDa (lysates, **top**) and 10 kDa (lysates, **bottom**) cleavage fragments of SPARC in PC3-hBMSC co-cultures. Increased intracellular levels of full length SPARC are detected in co-cultures incubated with the cathepsin K inhibitor. Levels of the 10 kDa fragment increased in co-culture and are moderately reduced by the cathepsin K inhibitor. Reduced secretion and extracellular processing of SPARC in the presence of the cathepsin K inhibitor are validated by immunoblotting with antibodies against the 28 kDa fragment (media, **top**). **D:** Intracellular (lysates, **top**) and extracellular (media, **top**) levels of cleavage fragments of SPARC in co-cultures of PC3 cells with wild-type and cathepsin K-null mBMSC. Reduced secretion and processing of SPARC are observed in co-cultures with cathepsin K-null cells. **Arrowheads**, SPARC fragment affected by cathepsin K inhibition. Samples (lysates and media) were loaded based on the ratio of mg protein/ μ g DNA in cell lysates and are representative of at least three experiments. Glyceraldehyde-3-phosphate dehydrogenase (lysates) and Coomassie stain (media) are shown as loading controls.

examined SPARC secretion and processing in co-cultures of hBMSC with two other prostate carcinoma cell lines (DU145 and C4-2B) and one breast cancer line (MDA-231BO). DU145 cells, originating from a brain metastasis,³¹ express cathepsin K message,⁸ but do not express SPARC.⁴⁴ Our results demonstrated that SPARC secretion was induced in DU145-hBMSC co-cultures, but there was no evidence of its enzymatic processing in the absence or presence of the cathepsin K inhibitor (Figure 8A, top panels). Increased secretion and cleavage of SPARC were detected in hBMSC co-cultures with C4-2B, a bone-metastasis derived LNCaP subline³²; however, the effect of the cathepsin K inhibitor was very modest (Figure 8A, middle panels). In contrast, a dramatic effect on SPARC secretion and cleavage was observed when hBMSC interacted with the bone-seeking MDA-231BO cells.³³ As observed in the PC3-hBMSC co-cultures (Figure 7A), a significant reduction in the levels of secreted SPARC and disappearance of the 40-kDa band, indicative of reduced enzymatic processing, were observed in the presence of the cathepsin K inhibitor (Figure 8A, bottom panels). Further investigation using cathepsin K-deficient mBMSCs revealed impaired expression, secretion, and processing of SPARC in the absence of bone marrow-derived cathepsin K (Figure 8B).

Secretion of Pro-Inflammatory Cytokines

The coincident up-regulation of cathepsin K and SPARC observed in this study and the literature suggesting an involvement of these proteins in inflammation and bone resorption^{17,18,47,48} prompted us to examine the secretion of pro-inflammatory cytokines by co-cultures of PC3 and hBMSC cells. Interaction of hBMSC and PC3 cells led to increased secretion of several molecules implicated in advanced prostate cancer and bone matrix destruction (Figure 9A-B). We observed increased levels of monocyte chemoattractant protein 1, one of the key regulators of tumor-induced osteoclast differentiation and fusion.⁴⁹ Levels of several cytokines implicated in diseases like rheumatoid arthritis, osteoporosis and periodontitis, which are known to involve cathepsin K, were also increased, including: epithelial neutrophil activating protein-78, neutrophil chemotactic factor (GRO), GRO- α , IL-1 α , IL-1 β , IL-6, and IL-8.⁵⁰⁻⁵² The involvement of cathepsin K in regulation of the above mentioned chemokines was demonstrated by their reduced secretion in the presence of the cathepsin K inhibitor L-873724 (Figure 9B, right panel).

The two secreted factors most significantly affected by cathepsin K inhibition were IL-6 and IL-8, cytokines pre-

A Co-cultures with hBMSC cells (+/- CTSK inhibitor) **B** MDA-231-BO/mBMSC co-cultures

Figure 8. Effect of cathepsin K inhibition on secretion and processing of SPARC in co-cultures of BMSC with prostate and breast carcinoma cells. **A:** Immunoblot analysis of overnight conditioned media (**top**) from individual cultures of tumor cells (TC), hBMSC, and co-cultures of hBMSC with DU145, C4-2B, and MDA-231-BO cells in the absence and presence of 100 nmol/L L-873724. There is increased secretion but no evidence of extracellular processing of SPARC by DU145-hBMSC co-cultures (**top**). Secretion of SPARC is increased in C4-2B-hBMSC co-cultures but processing is not affected by cathepsin K inhibition (**middle**). Significantly reduced processing of SPARC is observed in MDA-231-BO-hBMSC co-cultures in the presence of cathepsin K inhibitor (**bottom**). **B:** Immunoblot analysis of intracellular (**top**, lysates) and extracellular (**bottom**, media) SPARC in co-cultures of MDA-231-BO cells with wild-type and cathepsin K-null mBMSC. Expression, secretion, and processing of SPARC are reduced in co-cultures with cathepsin K-null mBMSC. Samples were loaded based on the ratio of mg protein/ μ g DNA in corresponding cell lysates. Glyceraldehyde-3-phosphate dehydrogenase (lysates) and Coomassie stain (media) are shown as loading controls.

viously implicated in prostate cancer-induced bone resorption.⁵³ Immunoblot analysis of PC3-BMSC conditioned media confirmed reduced levels of both cytokines in the presence of the cathepsin K inhibitor (Figure 9C, top panel). The apparent difference in IL-6 and IL-8 levels on inhibitor treatment as determined by the immunoblot analysis was much higher than the difference observed on the cytokine array. This is most likely due to differ-

ences in the sample preparation and the sources of antibodies for the two techniques. Cathepsin K expression and activity have been previously shown to be regulated by RANKL.^{13,54} Therefore, we also assessed secretion of IL-6 and IL-8 from PC3-BMSC co-cultures in the presence and absence of RANKL-blocking antibody and observed significantly reduced secretion of IL-8, but not IL-6 (Figure 9C, lower panel). This suggests that interac-

Figure 9. Effect of cathepsin K on secretion of pro-inflammatory cytokines. Antibody array analysis of media conditioned by individual cultures of hBMSC and PC3 cells (**A**), and BMSC-PC3 co-cultures grown in collagen I gels in the absence and presence of a cathepsin K inhibitor (**B**). Factors up-regulated by BMSC-PC3 interaction are indicated by black open boxes (**B, left**), and factors decreased in the presence of inhibitor are marked by white open boxes (**B, right**). **C:** Immunoblot analysis of IL-8 and IL-6 secretion by hBMSC-PC3 co-cultures in the absence and presence of the cathepsin K inhibitor (**top**) and RANKL blocking antibody AF626, R&D systems (**bottom**). Secretion of IL-8 and IL-6 are significantly reduced by treatment with 100 nmol/L L-873724. Secretion of IL-8 is also reduced on treatment with 50 and 100 ng/ml of RANKL Ab. Samples were loaded based ratio of mg protein/ μ g DNA in corresponding lysates and are representative of at least three experiments. Glyceraldehyde-3-phosphate dehydrogenase (lysates) and Coomassie stain (media) are shown as loading controls. **D:** Cathepsin K activity in overnight conditioned media (after pepsin activation) in PC3-hBMSC co-cultures in the absence and presence of RANKL blocking antibody (100 ng/ml). Data (expressed as relative fluorescence units/min/mg protein/ μ g DNA) are representative of at least three experiments. **P* value ≤ 0.05 is considered statistically significant. Cathepsin K activity is significantly reduced in the presence of antibody.

tion of cathepsin K with IL-8 may be RANKL-dependent, as was further validated by the dramatically reduced cathepsin K activity on treatment with RANKL-neutralizing antibody (Figure 9D).

Discussion

Although bone metastasis is the most common complication associated with advanced prostate and breast cancers, the reasons behind the attraction of tumor cells to the skeleton are not well understood. Once the tumor cells arrive in the bone, their interaction with multiple bone-derived factors and bone resident cells leads to the development of skeletal lesions. Nonetheless, the critical players responsible for this organ-specific predisposition have not been validated. Skeletal metastases are often identified in the areas of the skeleton exhibiting increased marrow cellularity, in particular red marrow-rich regions of the bone, which are also known to undergo active bone turnover.² Cathepsin K is a potent collagenase that has been implicated in bone resorption due to its ability to degrade collagen I, the main component of the bone matrix. In addition, cathepsin K has been shown to be involved in the proteolytic processing and intracellular trafficking of TRAcP, an enzyme highly expressed in osteoclasts, and one that has been implicated in osteoclast-mediated bone turnover.⁵⁵ Cathepsin K is also important for differentiation of osteoclasts as evidenced by a reduction in the number of TRAcP-positive multinucleated cells on treatment with anti-sense cathepsin K oligonucleotides.⁵⁶

In this study, we investigated both the direct and indirect involvement of stromal-derived cathepsin K in establishment of tumor cells within the bone marrow. We have shown that cathepsin K is expressed in the PC3 bone tumor microenvironment and that cathepsin K levels correlate with loss of trabecular bone in an experimental model for prostate bone metastasis. The strongest cathepsin K expression was exhibited by osteoclasts and the stromal components of the tumor. Although there is an extensive literature indicating the importance of stroma-derived proteases^{57–60} in malignant progression, cathepsin K has been studied mainly with respect to osteoclastic bone resorption, and the functions of stroma-associated cathepsin K in malignant progression have been under-investigated. A limited literature shows an association of this potent collagenase with the host stroma in lung cancer xenografts⁶¹ and a specific localization to macrophages and fibroblasts in the stroma of invasive lung adenocarcinomas.⁴¹ In breast cancer clinical samples, cathepsin K has been shown to localize to fibroblasts⁶² and myoepithelial/myofibroblast cells.⁶³ In the present study, we demonstrated that macrophages are the major cathepsin K-expressing stromal cells in experimental PC3 bone tumors, yet only a subpopulation of these inflammatory cells expressed cathepsin K. A growing body of evidence suggests that subpopulations of macrophages and fibroblasts with distinct functions exist and affect cell–cell interactions within the tumor microenvironment.^{64,65} Clusters of tumor-associated macrophages that have been reported in many malig-

nancies correlate positively with poor prognosis.^{64,66} Whether cathepsin K-positive macrophages play a distinct function in the bone microenvironment remains to be determined.

Major targets of protease activity in the bone microenvironment are matricellular proteins, such as SPARC, biological mediators with multiple physiological and pathological functions. The activity of SPARC is modulated by its cleavage²⁶ and its pleiotropic nature is attributed to the broad range of activities of different regions of this protein.^{18,25} Proteolytic degradation of SPARC leads to unmasking of biological functions that are distinct from those observed for the native protein. In the present study, enzymatic cleavage of SPARC was demonstrated *in vitro*, using recombinant proteins, and *in vivo* in PC3 bone tumor extracts. SPARC peptides were detected in 4-week tumors, coincident with high expression and activity of cathepsin K, and their levels further increased with a declining activity of cathepsin K in 6-week tumors. This indicates that multiple enzymes may participate in processing and regulation of SPARC bioactivity. MMP-2 and MMP-9, which have been shown to cleave SPARC,²⁴ increase in expression in parallel with prostate cancer progression, in the experimental PC3 tumors generated in the SCID-hu model⁶⁷ and in clinical samples.^{68,69} Both the cathepsin K- and MMP-mediated cleavage of SPARC were previously suggested to increase the affinity of SPARC for collagens, a process ultimately affecting cell–cell and cell–extracellular matrix interactions within the tumor microenvironment.²⁴ SPARC internalization by alternatively activated macrophages has been suggested to regulate extracellular levels of SPARC, as well as levels of enzymes that are capable of cleaving SPARC (eg, MMPs).⁷⁰ Bone marrow macrophage-derived SPARC was recently shown to mediate tumor metastasis through interaction with $\alpha v \beta 5$ integrin and regulation of tumor cell adhesion to extracellular matrix.⁷¹ Here, we presented evidence of internalization of SPARC peptides by the osteoclasts and mononuclear TRAcP-positive cells (Figure 5C), which occurred coincident with the highest levels of osteoclast recruitment and activity. Osteoclasts are derived from the same myeloid precursors as macrophages, they are regulated in a similar way, and have recently been named an immune cell of the bone.⁷² Both the osteoclasts⁷³ and the macrophages³⁸ secrete active cathepsin K pericellularly, where this protease retains its enzymatic activity. Coincident with this secretion there is an increase in vacuolar H⁺-type ATPase, which allows cells to create a pericellular acidic environment optimal for cathepsin K activity and enzymatic processing of SPARC. Whether there is a link between localization of the 10-kDa fragment to osteoclasts and the levels of cathepsin K and osteoclast activity remains to be determined. Both cathepsin K^{6,7,46} and SPARC⁷⁴ play important roles in bone remodeling. Proteolytically derived SPARC bioactive peptides regulate growth factors, such as vascular endothelial growth factor.²⁶ In the bone tumor microenvironment increased SPARC-induced vascular endothelial growth factor production and engagement and activation of $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins by SPARC

mediates osteoclast recruitment and tumor cell migration to the bone matrix.²¹

The involvement of cathepsin K in proteolytic processing and potentially in the modulation of the biological activity of SPARC was validated in our *in vitro* studies with bone marrow stromal cells. Here, we demonstrated that SPARC secretion and cleavage were reduced in the presence of a selective cathepsin K inhibitor (Figure 7, A and C). The short-term inhibitor treatment appeared to affect processing and not the expression of SPARC. In contrast, cathepsin K deficiency in mBMSCs resulted in both reduced processing, which is consistent with bone marrow-derived cathepsin K cleaving SPARC, and reduced intracellular and extracellular levels of this protein (Figure 7, B and D). Our observation that levels of SPARC are reduced in cells deficient in cathepsin K is consistent with literature evidence that SPARC expression is regulated by proteases that cleave it.¹⁷ SPARC cleavage was observed in three tumor cell lines that are derived from either clinical (ie, PC3) or experimental (ie, C4-2B, MDA-231BO) bone metastases (Figure 8). In two of these lines (ie, PC3 and MDA-231BO), enzymatic processing of SPARC was reduced by inhibition of cathepsin K. This suggests that SPARC cleavage by cathepsin K may be important for establishment and progression of tumors in the bone.

One way by which cathepsin K-SPARC interaction may contribute to metastatic spread is through regulation of inflammatory processes in the bone tumor microenvironment. In a recent study by Asagiri et al, a selective cathepsin K inhibitor NC-2300 abrogated TLR9-induced secretion of pro-inflammatory cytokines by bone marrow-derived dendritic cells, suggesting a role for this protease in autoimmune and inflammatory diseases.⁴⁷ Production of SPARC by tumor cells and their surrounding stromal cells was proposed to regulate infiltration of inflammatory cells into the tumor microenvironment.¹⁸ Many cells within the tumor microenvironment, including prostate cancer cells and bone marrow cells, have been shown to produce and secrete chemokines.^{49,75–77} A role for proteases in the secretion and regulation of inflammatory factors has also been suggested.^{78,79} In BMSC-PC3 co-cultures, we identified several cytokines implicated in bone remodeling, inflammation and prostate-cancer induced bone resorption. Specifically, we observed increased secretion of the inflammatory stress molecules, IL-6 and IL-8. The 5'-untranslated region of cathepsin K contains a response element for IL-6,⁸⁰ indicative of an interaction between the two proteins. A clinical correlation between this cytokine and cystatin C, an endogenous inhibitor of cysteine cathepsins such as cathepsin K, as well as between cystatin C and cathepsin K, has already been demonstrated in patients with prostate cancer-induced metastatic bone disease.^{81,82} Both IL-6 and IL-8 are known to attract and activate osteoclasts and to play a role in prostate cancer.^{49,53,83,84} Osteoclast formation by PC3-derived IL-6 and IL-8 was recently shown to be RANKL-independent.⁵³ Our results suggest a RANKL-dependent interaction between IL-8 and cathepsin K, but not between IL-6 and cathepsin K. Given the importance of cathepsin K for osteoclast differentiation

and function,^{55,56} further studies will be needed to validate the contribution of cathepsin K-mediated secretion of pro-inflammatory factors to osteolysis and progression of prostate tumors in the bone. It is intriguing, however, that pro-inflammatory and chemotactic factors, specifically IL-8 and GRO, have recently been shown to be regulated by SPARC and that this regulation is associated with tumor-promoting effects of SPARC in melanoma.⁸⁵ Interestingly, in the present study, secretion of GRO, similar to IL-6 and IL-8, was induced upon the interaction of prostate carcinoma cells with bone marrow stromal cells, coincided with an increase in expression and secretion of SPARC and cathepsin K and was reduced in the presence of a cathepsin K inhibitor. Regulation of pro-inflammatory factors may represent the mechanism by which SPARC and proteases that cleave SPARC influence tumor-host interactions.

Stromal-derived cathepsin K may be an important factor in colonization and growth of tumors in the skeleton. Our results suggest that the role of cathepsin K in the bone microenvironment likely extends beyond its obvious functions in osteoclast-mediated collagen degradation to involve other substrates (ie, SPARC), cell types (ie, macrophages and bone marrow stromal cells), and inflammatory factors within the bone marrow milieu, all of which are important contributors to malignant progression. To our knowledge, this is the first study implicating bone marrow-derived cathepsin K in regulation of the biological activity of SPARC in prostate cancer bone metastasis.

Acknowledgments

We gratefully acknowledge Dr. David Percival (Merck Frosst, Canada) for providing the cathepsin K inhibitor L-873724. We also thank Dr. Kamiar Moin and the Microscopy & Imaging Resources Laboratory for assistance with confocal and conventional light microscopy analyses.

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