Cathepsin K mRNA and Protein Expression in Prostate Cancer Progression*

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ABSTRACT

Prostate cancer (CaP) is the most commonly diagnosed malignancy in men and is often associated with bone metastases, which cause much of the morbidity associated with CaP. Lesions associated with CaP generally exhibit increased bone formation and resorption. Increased bone resorption may release factors from the extracellular matrix that contribute to tumor growth. Cathepsin K (cat K) is a cysteine protease that exhibits strong degradative activity against the extracellular matrix and is involved in osteoclast-mediated bone destruction. In this study, we analyzed the expression of cat K in CaP cell lines and patient samples. Cat K message was detected in CaP cell lines by reverse transcription-polymerase chain reaction (RT-PCR) and in primary CaP and metastases by in situ hybridization. Immunohistochemistry revealed variable expression of cat K in primary CaP samples, as well as nonosseous metastases, whereas expression in bone metastases was significantly higher than in primary CaP, and normal prostate tissues were negative. Cat K protein was detected in CaP cell lines by Western blotting after immunoprecipitation. Cat K enzymatic activity was also detected in CaP cell lines by a fluorogenic assay and by an assay for degradation of collagen type I. Increased levels of NTx, a marker of bone matrix degradation mediated primarily by cat K, were also detected in sera of patients with CaP bone metastases. We hypothesize that CaP-expressed cat K may contribute to the invasive potential of CaP, while increased expression in bone metastases is consistent with a role in matrix degradation. (J Bone Miner Res 2003;18:222–230)

Key words: cathepsin K, prostate cancer, bone metastases, cysteine proteases

INTRODUCTION

Bone is the second most common site of metastasis (after lymph nodes) for prostate cancer (CaP). Although most CaP bone metastases are considered osteoblastic, based on patterns observed on radiographs, there is evidence that markers of bone resorption are elevated in patients with CaP bone lesions.1–3 Detailed studies of bone metastases using histomorphometry have also demonstrated that radiographically sclerotic lesions exhibit both bone formation and resorption.4–7 Recently it has been established that various cancers, including breast and prostate, support osteoclastogenesis through expression of the receptor activator of NF-κB ligand (RANKL),8–10 providing one possible mechanism to explain the increased bone resorption observed with bone metastases. In addition to supporting osteoclast formation, it has been demonstrated that CaP expresses proteases that degrade matrix proteins, representing another potential mechanism of increased bone matrix degradation associated with bone metastases. In 1998, Sanchez-Sweatman et al.11 reported that a CaP cell line, PC-3, degrades bone extracellular matrix and mineralized bone in vitro by secreting matrix metalloproteinases (MMPs). Nemeth et al.12 demonstrated that MMP inhibitors prevented bone degradation in a PC-3-human bone-SCID mouse model. They observed that PC-3 tumors expressed MMPs in vivo, raising the possibility that the tumor cells participate in the process of bone turnover through expression of proteases.

Cathepsin K (cat K) is a cysteine protease secreted by osteoclasts that degrades extracellular matrix during bone resorption. Its significance in bone remodeling is demonstrated by the osteopetrotic phenotype observed in knockout studies.13 Cat K has one of the highest matrix degradation

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activities known, cleaving type I collagen with higher efficiency than other cathepsins and MMPs, and its elastolytic activity is higher than that of cat L and pancreatic elastase.\textsuperscript{14,15} Because of its ability to destroy matrix components efficiently, cat K and some of its family members have been implicated in diseases involving bone and cartilage destruction, including tumor invasion\textsuperscript{16–19} and rheumatoid arthritis.\textsuperscript{20,21}

While its proteolytic activity is strongest at low pHs as in the lacunae of osteoclasts,\textsuperscript{22} cat K can also degrade extracellular matrix components at neutral pHs, suggesting a general role in tissue destruction and remodeling.\textsuperscript{23} In addition to the strong expression observed in osteoclasts, cat K protein has been detected in breast cancer,\textsuperscript{24} sites of granulomatous inflammation,\textsuperscript{25} human lung,\textsuperscript{26} thyroid epithelial cells,\textsuperscript{27} human endometrium,\textsuperscript{28} and mouse ovary.\textsuperscript{29}

In this study, we report detection of cat K message and protein in samples representing various stages of CaP progression and show that cat K expressed by CaP cells possesses enzymatic activity. Our data suggest that cat K derived from CaP cells may play a role in tumor invasiveness and extracellular bone matrix degradation.

\section*{MATERIALS AND METHODS}

\subsection*{Tissue culture}

CaP cell lines, DU 145, PC-3, B-PC-3, N-PC-3, LNCaP, C4, and C4-2 were cultured in RPMI-1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FBS (Life Technologies) at 37°C under standard conditions. Murine osteoclasts were generated from bone marrow of Balb/c mice (Charles River, Hollister, CA, USA) as follows. Femur marrow cavities were flushed five to six times with phenol red-free MEM (Life Technologies), and cells were centrifuged at 500 g and plated at a density of 1 x 10^6 cells per 9.6 cm² well. Cells were cultured in MEM supplemented with 2% FBS, 30 ng/ml colony-stimulating factor-1 (CSF-1; R&D Systems, Minneapolis, MN, USA), and 50 ng/ml RANKL (Chemicon, Temecula, CA, USA) for 5–6 days. Media was replaced every third day of culture. Osteoclast formation was confirmed by detection of TRACP+ multinucleated cells (TRACP assay, Sigma, St. Louis, MO, USA) after 5–6 days in culture, and the appearance of resorptive activity on dentine wafers (Immunodiagnostic Systems Ltd., Boldon, UK).

\subsection*{Tissue samples}

Human prostate tissue samples used in this study were obtained from organ donors (n = 2), radical prostatectomies (n = 16), or rapid autopsies (n = 14). Tissues were fixed in 10% buffered formalin and embedded in paraffin. Bone samples were fixed in formalin and decalcified in 5% formic acid before embedding. Five-micrometer sections were used for immunohistochemistry (IHC) and in situ hybridization (ISH). Slides were baked at 60°C overnight before deparaffinization in xylene (x3) and rehydration in a series of 100%, 95%, and 70% ethanol (EtOH) rinses. Tissue integrity was assessed by hematoxylin and eosin staining, and the presence of CaP cells was assessed by prostate specific antigen (PSA) staining.

\subsection*{RT-PCR}

Total RNA was isolated from CaP cell lines using STAT 60 (Tel-Test, Friendswood, TX, USA) according to the manufacturer’s instructions. cDNA was generated from 1 μg total RNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Clontech, Palo Alto, CA, USA) with random hexamers. A 399-bp fragment of cat K (bps 906-1305, accession no. X82153) was amplified using primers: 5’-CAGCAGGGTTGTTATATATGAGAAAGC and 3’-ATGGTTGAGAGAAGCGAAATAGGAGG. Beta-tubulin cDNA was amplified as a control for RNA quality, and polymerase chain reaction (PCR) conditions were as previously reported\textsuperscript{30}: 1 cycle at 80°C for 3 minutes, and then 30–35 cycles of 94°C for 10 s, 69°C for 1 minute, and a final extension period at 72°C for 7 minutes.

\subsection*{ISH}

The 399-bp amplicon of cat K was cloned into the pGEM-T vector (Promega, Madison, WI, USA). DNA sequencing was used to confirm the identity and determine the orientation of the insert. Purified plasmid was linearized using either NcoI or NotI and digoxigenin-labeled anti-sense and sense riboprobes were generated using a T7 and SP6 in vitro transcription kit (Roche, Indianapolis, IN, USA). The probes were separated on a 1.2% agarose gel, and relative amounts of RNA were quantified using a digital image analyzer (Alpha Innotech Corp., San Leandro, CA, USA). Hybridization was performed using the Ventana genII automated ISH system (Ventana Medical Systems, Tucson, AZ, USA). Hybridization was performed using 25 ng/slide sense or anti-sense riboprobes at 45°C for 5 h in hybridization buffer (8 mM Tris, pH 8.0, 50% deionized formamide, 10% dextran sulfate, 1 x Denhardt’s solution, 0.3 M NaCl, 0.8 mM EDTA, 2 mg/ml yeast tRNA, 10.0 mM DTT), with subsequent rinses of 2x, 1x, SSC buffer at 45°C. An anti-digoxigenin monoclonal antibody (1:2000; Sigma) was used with biotinylated rabbit anti-mouse antibodies, streptavidin-horseradish peroxidase (HRP), and diaminobenzidine (DAB) as the substrate. Sections were counterstained with Harris’ hematoxylin before mounting.

\subsection*{IHC}

Endogenous peroxidase was quenched by incubation of tissues in 0.3% hydrogen peroxide in PBS for 10 minutes. Nonspecific binding was blocked with serum block (5% goat, 5% horse, 5% rabbit sera) in PBS for 1 h at room temperature. Endogenous biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). An affinity purified chicken antibody against human cat K (Immunodiagnostic Systems Ltd.) was applied at 40 ng/ml, and the sections were incubated overnight at 4°C in a humidified chamber. Negative control sections were incubated with isotype-matched chicken IgY (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Sections were incubated with biotin-conjugated rabbit anti-chicken antibodies (1:250, Chemicon) for 30 minutes at room temperature and processed with an ABC kit (Vector Laboratories) with DAB. Sections were counterstained in Harris hematoxylin and blue in ammonia water.
before mounting. Giant cell tumor tissues were used as a positive control for cat K staining.

Statistics for IHC samples

The percentage of cat K-positive cells in tissues was assessed as 0%, 1–25%, 25–50%, 50–75%, or 75–100% by a pathologist, who was blinded to the samples, and assigned values of 0, 1, 2, 3, or 4, respectively. Immunoreactivity in primary CaP and nonosseous and osseous metastases were compared using a one-way ANOVA with a Bonferroni’s comparison posttest.

Immunoprecipitation of cat K

Approximately 500 μg CaP tissue or 5 × 10⁶ LNCaP or PC-3 cells were homogenized in 1.0 ml lysis buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 0.25% Triton X-100, 0.25% sodium deoxycholate, 0.5% CHAPS, and Complete protease inhibitors) and centrifuged for 20 minutes at 14,000 rpm before immunoprecipitation. Cat K protein was immunoprecipitated overnight at 4°C using a mixture of mouse monoclonal anti-cat K antibodies CP1A240.2 and CP1K338.4 (0.25 mg each, a gift from Beckman Coulter Inc., Fullerton, CA, USA), which were bound to protein G Sepharose beads (Pierce, Rockford, IL, USA). After extensive washes to release unbound proteins, cat K was eluted from the column as recommended by the manufacturer. Lysates from PC-3, LNCaP, DU 145, and osteoclasts (200 μl total volume, 150 μg protein; 10 μg for Oc), prepared in the same assay buffer used in the Z-GPR-MCA experiments, were added to wells in a 96-well plate coated with 1.2 μg/ml purified rat type I N-[propionate-2,3-³H]propionylated collagen (NEN Life Science Products, Boston, MA, USA). After a 6-h incubation at 37°C, 75 μl of the reaction was collected, 3 ml CytoScint scintillation cocktail (ICN, Costa Mesa, CA, USA) was added, and release of degradation products of collagen was measured using a Beckman LS-3801 beta spectrometer (Beckman Coulter Inc.). Lysates were also treated with E64 (1 μM) to inhibit cysteine protease activity. Samples were run in triplicate in three separate experiments. The experimental data are presented as the mean ± SEM of the three sets, which have been normalized to the maximum signal strength. Statistical significance between untreated and 1-μM E64 samples was determined by unpaired t-tests.

Bone resorption pit assay

LNCaP and PC-3 (50,000 cells/well) were cultured on dentine wafers (Immunodiagnostic Systems Ltd.) in 24-well plates with 1 ml RPMI 1640 + 10% FBS. Osteoclasts, generated as described in the tissue culture section, were used as a positive control for resorption. After 5–6 days of culture, the media was removed and replaced with 1 M NH₄OH for 30 minutes. The dentine wafers were then cleaned by ultrasonication for 30 minutes and stained with hematoxylin (Zymed, South San Francisco, CA, USA) for 1 minute and washed with distilled water. Resorption pits were visualized under transmitted light.

N-telopeptides of type I collagen ELISA

Cross linked N-telopeptides of type I collagen (NTx) were measured in serum samples from CaP patients using the Osteomark assay kit (Ostex International Inc., Seattle, WA, USA). Serum samples were obtained from 10 donors (mean PSA = 1.09 ± 0.16); 10 patients with organ-confined CaP, stages T2a–c or T1c (mean PSA = 7.43 ± 1.16); 5 patients with bone metastases who had not received any treatment (mean PSA = 557.6 ± 173.3); 8 patients with bone metastases who had received one or more treatments for CaP, including androgen ablation, chemotherapy or radiation (mean PSA = 1087 ± 464); and 6 patients who had received bone resorption inhibitors in addition to other treatments listed above (mean PSA = 4097 ± 1729). All patients gave informed consent. The majority of the patients used for this assay were the same patients used in the ISH and IHC studies. NTx levels were compared for statistical significance using a one-way ANOVA with a Bonferroni’s comparison posttest.
RESULTS

Expression of cat K mRNA

Cat K mRNA was detected in all CaP cell lines tested, which include the androgen-independent cell lines DU 145 and PC-3, the PC-3 sublines, B-PC-3 and N-PC-3, the androgen-sensitive cell line LNCaP, and its androgen-independent sublines C4 and C4–2 by RT-PCR (Fig. 1). In addition, the presence of cat K mRNA was assessed in 2 samples of normal prostate from organ donors, 7 localized CaP tumors, and 25 metastatic samples from 7 patients by ISH. ISH demonstrated the presence of cat K mRNA in epithelial cells of primary CaP in 5 of 7 samples (Fig. 2A), while a moderate signal was observed in basal and luminal cells of normal glands (3/7, Fig. 2A, arrow) adjacent to the cancer. Samples of normal prostate from organ donors were negative. Cat K mRNA was detected in nonosseous metastases including liver (2/2), lymph node (2/3, Fig. 2B), and lung (1/1), and 15/19 osseous CaP metastases (Fig. 2C). When present, osteoclasts were intensely positive for cat K message. Cat K mRNA was not observed in stromal fibroblasts, stromal smooth muscle, or vascular smooth muscle. Staining with the sense probe was negative (Figs. 2D–2F).

Cat K protein expression in CaP

The presence of cat K protein was assessed in 14 samples of localized CaP and in 52 metastases from 14 patients. Immunohistochemistry revealed the presence of cat K protein in the epithelial cells of cancerous glands in 6/14 primary CaP samples (Fig. 3A). Normal glands adjacent to CaP were negative. Cat K protein was also detected in metastases to liver (3/4), lung (3/3), adrenal (1/1), and lymph node (4/10, Fig. 3B); however the immunoreactivity in these samples was variable, ranging from no staining to 100% positivity. Osseous metastases were positive for cat K protein expression in 30/34 samples (Fig. 3C). Intense immunoreactivity for cat K was observed in osteoclasts in CaP bone metastases (Fig. 3C, arrow). A giant cell tumor was
used as the positive control for cat K staining (Fig. 3D).

Table 1 summarizes IHC results for metastases from nine patients in which multiple metastatic sites were evaluated. The percentage of tumor cells exhibiting cat K immunoreactivity in osseous metastases was significantly higher than in primary CaP (p < 0.05) and nonosseous (p < 0.001) samples. The percentages of tumor cells exhibiting cat K immunoreactivity in primary CaP versus nonosseous metastases were not significantly different (p > 0.05).

To confirm the presence of cat K protein in CaP, we performed Western blot analysis of cell lysates from CaP cell lines LNCaP and PC-3, and CaP tissues that were positive by IHC. We were unable to detect the cat K protein under these conditions. Therefore, we immunoprecipitated cat K from CaP cell lines and CaP tissues to obtain more concentrated samples. After this, a 40-kDa band was labeled in blots from CaP tissues (Fig. 4A, arrow) and LNCaP and PC-3 cell lines (Fig. 4B, arrow). The 40-kDa protein is most likely the proform of cat K. The light chain band in Fig. 4B would be expected to mask the active form of cat K band at 28 kDa, but when non-denaturing gels were run as in Fig. 4A, we still did not observe a band at 28 kDa.

**Cat K enzymatic activity**

The presence of cat K enzymatic activity in CaP cells was evaluated using Z-GPR-MCA, a substrate that is highly selective for cat K versus cathepsins L and S. Z-GPR-MCA cleavage activity was detected in LNCaP, PC-3, and DU 145 cell lines (Table 2). Because cathepsin B (cat B) also cleaves Z-GPR-MCA, but at a much lower rate, the portion of the activity caused by cat B was determined using the cat B inhibitor CA 074. CA 074 blocked approximately 75% of the activity in LNCaP and DU 145, and 50% of the activity in PC-3. The remaining activity was inhibited by a general cysteine protease inhibitor, E64, suggesting that it was caused by cat K. For inhibition of cat B activity, we used 50 nM CA 074, a concentration determined from dose-response experiments. Fifty nanomolar CA 074 inhibited purified human cat B activity with the Z-GPR-MCA substrate and a specific cat B substrate, Z-RR-MCA (data not shown) as effectively as E64. LNCaP and DU 145 exhibited 2.5- to 3-fold more Z-GPR-MCA cleavage activity than PC-3 after inhibition of cat B. Activity of cat K in osteoclast lysates, which were used as the positive control, was approximately 50-fold higher (normalized to protein) than in the LNCaP and DU 145 CaP cell lines. Because our osteoclast purity was 30–50% as determined by TRAP staining, the cat B activity in this preparation is most likely caused by contaminating mononuclear cells, because osteoclasts express low levels of cat B.

We have also evaluated whether CaP cells exhibit pitting activity using dentine wafers. We were unable to detect pitting capabilities of CaP cells; osteoclasts were used as a positive control (data not shown). Because cat K can degrade type I collagen, we also performed experiments to determine whether CaP cells can degrade collagen. We observed type I collagen-degradation activity in LNCaP, DU 145, and osteoclast lysates under the same assay conditions used in the Z-GPR-MCA assay (Fig. 5). PC-3 lysates were unable to degrade collagen under the conditions used. The general cysteine protease inhibitor E64 blocked approximately 50% of the cleavage activity in LNCaP, DU 145, and osteoclast lysates, confirming that the activity is attributable to cysteine proteases.

**NTX levels in sera from CaP patients**

Increased type I collagen degradation associated with CaP bone metastases was demonstrated by measuring NTx levels in the sera of patients with either organ-confined CaP or CaP bone metastases (Fig. 6). The levels in patients with bone metastases who received treatment for CaP, including androgen ablation, which has been reported to increase bone resorption, were statistically significant from the normal donors (p < 0.001) and primary CaP (p < 0.01; Table 2).
3). NTx levels in patients with bone metastases who were not treated for CaP were statistically significant from the normal controls \( (p < 0.05) \).

**DISCUSSION**

In this study, we present data suggesting that cat K, a cysteine protease normally associated with osteoclastic bone resorption, is associated with CaP progression. The presence of cat K in primary CaP is consistent with a possible role in matrix degradation via digestion of type I collagen, before dissociation and dissemination. However, the same enzymatic activity at metastatic sites in the bone may promote establishment of micrometastases and may also lead to release of factors supporting tumor growth.

We observed cat K protein in cancerous glands of approximately 40% of the primary CaP tissues tested, exhibiting no correlation with Gleason score or cancer grade. Although prostate basement membrane consists primarily of type IV collagen\(^{36,37}\) and laminin,\(^{38,39}\) Burns-Cox et al.\(^{40}\) observed an increase in type I collagen synthesis and degradation at the cancer foci in biopsy specimens from patients with primary CaP. Enzymes that cleave type I collagen, such as the cathepsins and MMPs, are reported to be upregulated in CaP\(^ {17,41-46}\) Therefore, cat K may be an important factor at the local level in CaP dissemination. However, we did not detect a significant increase in NTx levels in CaP patients with organ-confined disease versus control samples. A possible explanation is that the levels of
activity of cat K and type I collagen in organ-confined CaP are insufficient to cause detectable systemic increases in the NTx levels. In the absence of long-term follow-up information on these patients, it is not possible to determine whether the presence of cat K in the primary tumor is strongly correlated with subsequent metastasis, but it is at least conceivable that this enzyme is important or even essential for dissemination of CaP cells from the primary tumor. This hypothesis is supported by the observation that metastases in general exhibited equal or higher positivity than the primary tumors (41/52).

We also observed higher cat K immunoreactivity in osseous metastases versus nonosseous and primary CaP. This is consistent with the NTx assay results, which demonstrated increased matrix degradation in patients with bone metastases versus normal controls and patients with primary CaP. Type I collagen is the major extracellular matrix component of bone, and there is an extensive body of evidence that type I collagen breakdown products, including NTx, are present at elevated levels in the serum and urine of patients with CaP bone metastases. Increased resorption associated with bone metastases, including CaP bone metastases, is linked to stimulation of osteoclastogenesis through RANKL expression. Increased NTx levels are generally associated with increased osteoclastic bone resorption activity because of cat K expressed by these cells, yet we observed osteoclasts in samples from only 3/14 patients with primary CaP (p < 0.01).
sample 20 bone sites per patient, it is possible that we missed areas with extensive bone resorption and osteoclasts. Alternatively, the increased NTx levels may be in part because of the breakdown of type I collagen by cells other than osteoclasts, such as the CaP cells expressing cat K.

Detection of cat K mRNA and protein in CaP tissues and cell lines led us to determine whether the enzyme was catalytically active. We observed that LNCaP and DU 145 had 2.5- to 3-fold more activity than PC-3, whereas osteoclasts exhibited 50-fold more activity than LNCaP and DU 145 CaP cells in assays of Z-GPR-MCA cleavage. We also determined that LNCaP and DU 145 lysates exhibit type I collagen-degradation activity, whereas PC-3 do not. When CaP cells were plated on labeled collagen or dentine wafers, cleavage activity was not detected, although osteoclasts exhibited activity. Our activity data suggest that CaP cell lines in vitro express low levels of cat K, which seem not to be active in culture but can be activated at a low pH in lysates. The low levels of cat K activity in CaP cells correlated with our Western blotting/immunoprecipitation results: we detected a faint 40-kDa protein, corresponding to the proform of the enzyme. We were unable to detect the 28-kDa active form of cat K, possibly because the quantity immunoprecipitated was below the level of detection. In addition, the presence of cystatins, natural inhibitors of the cathepsins, in CaP cell lines may also contribute to the low activity observed or difficulty in immunoprecipitation.

It is intriguing that PC-3 cells had lower levels of cat K activity than LNCaP, because PC-3 metastases in bone are osteolytic, whereas LNCaP lesions exhibit both bone formation and resorption. —PC-3 express MMPs, —urokinase plasminogen activator, —RANKL, —endothelin-1, and —PTHrP —factors involved in metastases to bone and bone remodeling. The formation of PC-3 osteolytic bone metastases seems to involve a complex set of mechanisms, and cat K may play a very minor role in this case. Moreover, the low cat K activity in CaP cell lines may not be representative of the expression or activity levels in CaP in vivo.

In this study we report our observations of the expression of cat K mRNA and protein in CaP cell lines and tissues, as well as cat K activity in CaP cell lines. The presence of cat K in primary CaP may promote the process of dissemination, whereas in osseous metastases, which exhibit higher levels of markers of bone formation and resorption, cat K is most likely involved in degradation of the extracellular matrix. This process may not only provide sites for CaP cell anchorage, but also cause the release of growth factors essential for tumor growth. Inhibition of cat K activity in vivo using xenograft models may permit a more precise definition of the role of CaP-expressed cat K and yield insight regarding novel therapeutic strategies.

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