Cathepsin K Is a Critical Protease in Synovial Fibroblast-Mediated Collagen Degradation

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Synovial fibroblasts (SFs) play a critical role in the pathogenesis of rheumatoid arthritis (RA) and are directly involved in joint destruction. Both SF-resident matrix metalloproteinases and cathepsins have been implicated in cartilage degradation although their identities and individual contributions remain unclear. The aims of this study were to investigate the expression of cathepsin K in SFs, the correlation between cathepsin K expression and disease severity, and the contribution of cathepsin K to fibroblast-mediated collagen degradation. Immunostaining of joint specimens of 21 patients revealed high expression of cathepsin K in SFs in the synovial lining and the stroma of synovial villi, and to a lesser extent in CD68-positive cells of the synovial lining. Cathepsin K-positive SFs were consistently observed at sites of cartilage and bone degradation. Expression levels of cathepsin K in the sublining and vascularized areas of inflamed synovia showed a highly significant negative correlation with results derived from the Hannover Functional Capacity Questionnaire ($r = 0.78$, $P = 0.003$; and $r = 0.70$, $P = 0.012$, respectively) as a measure of the severity of RA in individual patients. For comparison, there was no correlation between Hannover Functional Capacity Questionnaire and cathepsin S whose expression is limited to CD-68-positive macrophage-like synoviocytes. The expression of cathepsin K was also demonstrated in primary cell cultures of RA-SFs. Co-cultures of SFs on cartilage disks revealed the ability of fibroblast-like cells to phagocytose collagen fibrils whose intralysosomal hydrolysis was prevented in the presence of a potent cathepsin K inhibitor but not by an inhibitor effective against cathepsins L, B, and S. The selective and critical role of cathepsin K in articular cartilage and subchondral bone erosion was further corroborated by the finding that cathepsin K has a potent

aggrecan-degrading activity and that cathepsin K-generated aggrecan cleavage products specifically potentiate the collagenolytic activity of cathepsin K toward type I and II collagens. This study demonstrates for the first time a critical role of cathepsin K in cartilage degradation by SFs in RA that is comparable to its well-known activity in osteoclasts. (Am J Pathol 2001, 159:2167–2177)

Rheumatoid arthritis (RA) is an inflammatory joint disorder characterized by a progressive destruction of articular cartilage and subchondral bone that eventually leads to loss of joint function. RA cartilage erosion is a three-directional process caused by activated chondrocytes, a cartilage-overlying synovial pannus, and a subchondral cellular infiltration of the hyaline cartilage. Subchondral cartilage is primarily degraded by osteo/chondroclasts, multinucleated giant cells, and mononuclear precursor cells, although invading synovial fibroblasts (SFs) are considered the main culprit cells in pannus-mediated joint destruction.^{1–4}

Although the degradation of the extracellular matrix in joints is clearly mediated by proteolytic activities, the nature of the individual proteases remains unknown in most cases. To date, two protease families have been implicated in cartilage degradation: metalloproteinases of the MMP and ADAMs families, and cysteine proteases.^{5–7} Traditionally, metalloproteinases have been favored as potential culprit enzymes over cysteine proteases, although inhibitors of both protease classes have proved to be equally effective in reducing inflammation and cartilage erosion in animal models of RA.^{8,9} Recent advances in the identification and characterization of novel cysteine proteases have directed increased attention to the cathepsins as potential drug targets to treat tissue degenerative and inflammatory processes. Current interest is focused on the roles of cathepsin K in bone resorption and cathepsin S in antigen presentation.¹⁰ Cathepsin K has been identified as the predominant osteoclastic protease with a unique and potent collagenolytic activity.^{11–14} The critical involvement of cathepsin K in bone remodeling is best supported by the finding that

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cathepsin K deficiency causes the bone-sclerosing disorder pycnodysostosis,¹⁵ that, on the molecular level, is characterized by insufficient degradation of type I collagen during bone remodeling.¹⁶ In contrast, the nature of the proteases responsible for the cartilage erosion by SFs remains elusive although several matrix metalloproteinases and cathepsins L and B have been prime suspects.^{6,17} Here, we report the specific expression of cathepsin K in the inflamed RA synovium and discuss the potential involvement of this protease in SF-mediated bone and cartilage degradation and synovial remodeling.

Materials and Methods

Patient Characteristics

All 21 patients fulfilled the American College of Rheumatology-revised criteria for the classification of RA.¹⁸ All were positive for rheumatoid factor and had radiographic erosions. Parameters of disease activity were recorded as follows: 1) the swollen joint count evaluated 28 joints (shoulders, elbows, wrists, metacarpophalangeal-, interphalangeal-, proximal interphalangeal-, and knee-joints) as described in Fuchs and Pincus.¹⁹ 2) The modified Lansbury index reflects the number of swollen joints adjusted for their relative size. It is comparable to the area weighted swollen joint index described in van Leeuwen and colleagues.²⁰ This parameter is distinct from the Lansbury index in that it does not refer to "pain on motion." In our opinion, the latter can also be influenced by degenerative changes without inflammation. 3) The Keitel functional index²¹ is based on the scoring of limitations of joint movements observed by the physician. This test evaluates the function of hands, wrists, shoulders, and lower limbs by grading defined joint movements. 4) The Hannover Functional Capacity Questionnaire (HFCQ) was performed on 12 of 21 patients. This questionnaire is the most widely used questionnaire in Germany. It has been validated by several studies and shows good correlation ($r = 0.87$) with the health assessment questionnaire (HAQ).²² Severe impairment of joint function is defined as a reduction of the HFCQ below 50%²²) Visual analog scale of joint pain was assessed using a scale ranging from 0 (no pain) to 10 (most severe pain). 6) The laboratory parameters included the erythrocyte sedimentation rate that was determined by the Westergren method. C-reactive protein was measured by nephelometry (monarch 2000; Instrumentation Laboratory GmbH, Kirchheim Heimstetten, Germany) using specific anti-serum (Biokit GmbH; Kirchheim Heimstetten, Germany). Patient characteristics are given in Table 1.

Synovial Specimen

Synovial tissue samples were obtained at the time of synovectomy ($n = 10$), or reconstructive surgery of joints, mostly resection of metatarsal heads ($n = 11$). All 21 tissue samples were obtained from the Department of Rheumatology, Rostock, Germany. The respective joints were metatarsophalangeal ($n = 13$), wrist ($n = 5$), meta-

Table 1. Patient Characteristics

Number of patients	21*
Age	51 (43–61)
Female patients	15
Rheumatoid nodules present	13
Number of failed DMARD	4 (2–6)
Patients on MTX at the time of surgery	13
Number of swollen joints	4 (3–11)
Modified Lansbury index	13 (5.2–38.5)
Joint function test (KFI)	77 (66–87)
HFCQ (percent)	81 (57–87.9)
Erythrocyte sedimentation rate (mm/h)	22 (11–46)
C-reactive protein (mg/L)	10 (8.7–14.8)

*Figures are given either as total number or as median and interquartile range.

carpophalangeal ($n = 2$), and knee joint ($n = 1$). The samples were fixed in 5% buffered formalin and embedded in paraffin wax. The paraffin blocks were labeled with a numerical code only and the staining and the evaluation of the slides was performed without knowledge of any activity parameters of the patients.

Immunohistochemistry

Paraffin sections (5 μ mol/L) were mounted onto Vectabond slides (Vector Laboratories, Burlingame, CA), dried overnight, and dewaxed with xylene. Sections were incubated with mouse monoclonal anti-human cathepsin K antibody at a dilution of 1:200 as previously described²³ for 1 hour at room temperature in a humidified chamber, followed by incubation with biotinylated goat anti-mouse secondary antibody for 20 minutes. Peroxidase-labeled avidin was used to localize the secondary antibody with oxidized diaminobenzidine as chromogen (Biogenix, San Ramon, CA). Counterstaining was performed with Meyer's hematoxylin and eosin. All slides were evaluated using a departmental Nikon Eclipse E800 microscope.

For double-immunofluorescence staining, we used a rabbit polyclonal anti-human cathepsin K antibody as previously described.²³ Secondary antibodies were replaced by anti-mouse antibody conjugated with tetramethylrhodamine isothiocyanate and anti-rabbit antibody conjugated with fluorescein isothiocyanate (Sigma, St. Louis, MO).

Monoclonal anti cathepsin K antibodies were generated by immunization of A/J mice with purified bacterially-expressed cathepsin K. After an initial intraperitoneal boost of 50 μ g of cathepsin K in complete Freund's adjuvant, the mice were allowed to rest for 8 weeks. They were then boosted intraperitoneally at 2-week intervals; first with 25 μ g of cathepsin K in incomplete Freund's adjuvant; then with 10 μ g of cathepsin K in 10 mmol/L phosphate, 150 mmol/L sodium chloride, pH 7.2; and finally with 5 μ g of cathepsin K in 10 mmol/L phosphate, 150 mmol/L sodium chloride, pH 7.2, intravenously. The spleens of two mice were then fused in accordance with standard procedures. Supernatants were screened with a solution-phased enzyme-linked immunosorbent assay in which yeast-expressed (activated) and bacterially expressed cathepsin K bound to a microplate well was

incubated with supernatant to be tested. Sequential incubations with anti-mouse IgG-horseradish peroxidase conjugate and then substrate allowed detection of anti-cathepsin K antibodies. Clone M21 was selected for further use and purified from hybridomas grown in HB Pro serum-free media (Irvine Scientific, Santa Ana, CA). Terminal culture supernatants were harvested by centrifugation when cell viability fell to <10 to 20%. Supernatants were purified over fast-flow Protein A-Sepharose (Amersham/Pharmacia, Piscataway, NJ) after 0.2- μ m (pore size) filtration and addition of saturated sodium borate and sodium chloride to final concentration of 100 g/L and 3 mol/L, respectively. Bound antibody was eluted with 0.1 mol/L of glycine, pH 3.0, and fractions were neutralized by the addition of 10% (v/v) 1.2 mol/L of Tris, pH 8.5. Antibody-containing fractions were pooled and dialyzed into 10 mmol/L of phosphate and 150 mmol/L of sodium chloride, pH 7.2.

For control staining experiments, the antibody was preincubated for 1 hour with nitrocellulose paper strips containing recombinant human cathepsin K. Briefly, 100 μ g of recombinant cathepsin K was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4 to 20% Tris/glycine gels and the protein was electroblotted onto nitrocellulose (Fisher Scientific, Pittsburgh, PA). The cathepsin K band with an apparent molecular weight of 29 was excised after Ponceau staining and the nitrocellulose strip was blocked with 9% nonfat milk powder in phosphate-buffered saline and subsequently used for affinity binding of anti-cathepsin K antibodies. Anti-cathepsin K antibody-depleted antibody solution did not reveal any detection of cathepsin K-positive material in tissue sections.

Semiquantitative Analysis

Tissue sections were coded and randomly analyzed by an investigator blinded to the origin and history of the samples. Tissue sections were scored separately for the presence of cathepsin K in the intimal lining layer, the interstitial stromal regions, and vascular-rich areas. Scores were assigned semiquantitatively on a 0 to 4 scale: 0, no staining; 1, rare positive staining or trace staining (1 to 5%); 2, scattered clusters of positive cells (5 to 20%); 3, moderate staining in a specific region (21 to 50%); and 4, extensive staining throughout a region (51 to 100%). Three sections per tissue sample were analyzed.²⁴

Statistical Analysis

Statistical analysis was performed by means of the SPSS software (SPSS Inc.). Because most of the data in the groups did not follow a Gaussian distribution, data are given as the median value and the interquartile range. The latter represents the range between the 25th and the 75th percentile. Correlation analysis was performed by Spearman's rank correlation.

Primary Cell Culture of SFs

Two surgical specimens from joint replacement surgery were obtained from the Department of Orthopaedics, Mount Sinai School of Medicine. The patients fulfilled the American College of Rheumatology-revised criteria for the classification of RA.¹⁸ Tissue samples were minced into $\sim$ 1-mm³ pieces and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml of penicillin and 100 mg/ml of streptomycin (Mediatech, Herndon, VA) was added. The cells were cultured at 37°C in a 5% CO₂ incubator with medium exchanges twice a week. At cell confluence, cells were passaged and then analyzed between passages 3 and 8. Cartilage degradation by SFs was assessed by culturing SFs on pieces of bovine cartilage (4 $\times$ 4 $\times$ 1 mm) under the conditions described above. After 3 days, the culture media were supplemented by 10 μ mol/L of the cysteine protease inhibitors Mu-Leu-hPh-VS-Ph or Mu-Np-hPh-VS-Np, respectively (both compounds were kindly provided by Axys Pharmaceuticals, South San Francisco, CA). The inhibitors were added to each media exchange for 10 days. Mu-Leu-hPh-VS-Ph is a potent cathepsin K inhibitor whereas Mu-Np-hPh-VS-Np is a very poor cathepsin K but a very potent inhibitor for cathepsins B, L, and S.²⁵ Primary SF cultures were cyto-stained for the fibroblast marker, 5B5 (proline-4-hydroxylase) and the macrophage marker, CD68 (Chemicon, Temecula, CA), and for cathepsin K using the monoclonal antibody M21 as described above. Fluorescent assays for intracellular cathepsin K activity were performed as previously described by Xia and colleagues²⁶ using Z-Gly-Pro-Arg-4-methoxy- β -naphthylamide (M β NA) and Z-Arg-Arg-M β NA as control for cathepsin B activity (Bachem Inc., Bubendorf, Switzerland).

Electron Microscopy

The cells cultured on the sliced cartilage specimens were immediately immersed in a solution containing 3% glutaraldehyde with 0.2 mol/L of sodium cacodylate at pH 7.4. After overnight fixation the fixative solution was removed and replaced with phosphate buffer followed by 1% osmium tetroxide buffered with sodium cacodylate. After 1 hour the osmium was replaced with increasing concentrations of ethanol through propylene oxide and flat-embedded in Embed 812 (EMS, Fort Washington, PA). One μ m plastic sections were cut perpendicular to the plane of the cells grown on the cartilage surface, stained with methyl blue and azure II, and observed by light microscopy. Representative areas were chosen for ultrathin sectioning (50 nm) and observed with a JEM 100CX transmission electron microscope (JOEL, Ltd., Tokyo, Japan).

In Vitro Digestion of Aggrecan and Collagens

Bovine aggrecan was prepared as described previously²³ and type I (calf skin) and II (calf articular joints) collagens were purchased from United States Biochemical (Cleveland, OH). Recombinant human cathepsin K was pre-

pared as described in Linnevers and colleagues.²⁷ Bovine aggrecan (200 μ g/ml final concentration) was incubated in 100 mmol/L sodium acetate buffer, pH 5.5, or in 100 mmol/L Tris/HCl, pH 7.2, containing each 2.5 mmol/L of ethylenediaminetetraacetic acid and 2.5 mmol/L of dithiothreitol with 800 nmol/L of cathepsin K for 1 hour at 28°C. The digestion reactions were stopped by the addition of 10 μ mol/L of E-64. The digestion samples were subjected to 0.6% agarose/1.2% polyacrylamide gel electrophoresis and the gels were stained with 0.1% toluidine blue (Sigma, St. Louis, MO). Collagen digests were performed in 100 mmol/L of sodium acetate buffer, pH 5.5, containing 2.5 mmol/L ethylenediaminetetraacetic acid and 2.5 mmol/L dithiothreitol with 800 nmol/L cathepsin K at 28°C. Aliquots were taken from the digest mixtures at 0, 2.5, 8, and 24 hours, inhibited with 10 μ mol/L of E-64 and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4 to 20% Tris/glycine gels (Novex/Invitrogen, Carlsbad, CA). Gels were stained with Coomassie Blue. Collagen digests were performed in the absence and the presence of cathepsin K-predigested bovine aggrecan. The concentration of aggrecan in the digest mixtures was 0.1% based on the initial concentration of aggrecan in the predigest. Predigests of aggrecan were performed with 800 nmol/L of cathepsin K for 16 hours at 37°C. Remaining residual activity of cathepsin K in the predigest mixture was heat-inactivated at 65°C for 20 minutes. No Z-LR-MCA hydrolyzing activity (standard substrate for cathepsin K) was observed after the heat inactivation.

Results

Characterization of Anti-Human Cathepsin K Antibodies

Purified monoclonal antibodies against human cathepsin K were shown by Western blotting to recognize cathepsin K specifically among a panel of six other human papain-like cathepsins (recombinant cathepsins B, L, S, F, V, and W) and as two protein bands in a cell extract of RA fibroblasts. The antibody recognized both the mature and the precursor form of cathepsin K (Figure 1). Moreover, immunohistochemical staining of cathepsin K in synovial tissue sections could be quenched by the preincubation of the antibody with nitrocellulose membrane-immobilized recombinant cathepsin K (Figure 2b).

Expression of Cathepsin K in RA Synovial Tissue

Archived paraffin-embedded specimens from 21 RA patients were analyzed by immunohistochemistry. Expression of cathepsin K was observed in all rheumatoid arthritic specimens examined. Five cell types were identified to express cathepsin K protein: fibroblast-like and macrophage-like synoviocytes, chondroclasts/osteoclasts, and giant multinucleated cells. The most dominant expression of cathepsin K was observed in the lining

layer and sublining area of synovial villi. Low-power magnification ($\times 40$ to $\times 100$) revealed either a characteristic ribbon-like distribution that extended from the stroma to the lining surface of synovial villi (Figure 2a) or a more uniform expression pattern of cathepsin K-positive cells (Figure 2c). In contrast, normal synovium obtained from a cancer patient revealed only a very few isolated, individual spindle-shaped cells in the synovial lining (Figure 2d). Examination with high-power magnification suggested that the majority of cathepsin K-positive cells in these areas are fibroblast-like synoviocytes (Figure 2f). The intracellular staining was mostly vesicular implying a lysosomal localization (Figure 2, f and h). It should be noted that cathepsin K-containing fibroblast-like cells are frequently restricted to well-defined areas with cathepsin K-negative cells in their immediate vicinity. Figure 2f shows cathepsin K-positive cells at the edges of a ribbon-like distribution of SFs adjacent to cathepsin K-negative spindle-like cells. Within the synovial stroma, cathepsin K expression is generally restricted to CD68-negative cells (not shown) whereas within the synovial lining CD68-positive cells also displayed immunostaining against cathepsin K (Figure 2e).

Cathepsin K-positive SFs were observed in large quantities in areas of high proliferation and vascularization within the RA synovium (Figure 2; g to i). Cathepsin K-positive cells seemed to invade highly proliferating tissue areas and frequently formed ring-like cellular arrangements around developing blood vessels (Figure 2, g and i). Lateral sections of a necrotic blood vessel are characterized by surrounding cathepsin K-containing fibroblast-like cells. (Figure 2h). Around well-established blood vessels, cathepsin K-positive cells are located beyond the smooth muscle layer (Figure 2i). Endothelial cells are cathepsin K-negative and appear swollen and project into the lumen of the vessels.

Fibroblast-like cells were consistently observed at sites of synovium-mediated bone and cartilage destruction. Figure 2, j to l, shows cathepsin K-positive cells in areas of synovial infiltration of bone and cartilage matrices where they seem to form a scalloped surface on the remaining cartilage (Figure 2k). These cells were CD68-

Figure 1. Western blot analysis of the specificity of monoclonal anti-human cathepsin K antibody. The specificity of monoclonal human cathepsin K antibody was tested by Western blot analysis with human cathepsins B, L, S, K, V, F, and W. Eight pmol of each enzyme were loaded onto 12% sodium dodecyl sulfate Tris-Glycine gels and MS21 dilution of 1:2000 was used. Different cathepsins are indicated at the **top** and molecular mass standards are at the **left**. The protein amount of the SF extract subjected to PAGE was 10 μ g.

Figure 2. Immunohistochemical analysis of cathepsin K in synovial RA specimens. A mouse monoclonal antibody against human cathepsin K was used to localize cathepsin K in the synovium from the patients with RA. For fluorescence double staining, a rabbit polyclonal anti-human cathepsin K antibody was used. **a:** Cathepsin K expression in inflamed synovium showing a banding-like distribution. **b:** Consecutive tissue section to **a** used as control; anti-cathepsin K antibody was removed by immunoabsorption with immobilized recombinant cathepsin K. **c:** Cathepsin K expression in inflamed synovium showing a more uniform distribution in the deeper synovium and at the surface of synovial villi. **d:** Minor cathepsin K expression in normal synovium. **e:** Cathepsin K expression in FLS and CD68⁺ synovial macrophages in synovial lining of RA synovium. CD68⁺ cells are rhodamine stained (red) and contain cathepsin K (yellow). Green staining (fluorescein) represents cathepsin K expression in CD68⁻ FLS. **f:** Cathepsin K-positive FLS at the edge of a fibroblast band suggesting an expansion or invasion of cathepsin K-containing FLS within the inflamed synovium. **g-i:** Cathepsin K expression in FLS in the vascular areas of the inflamed synovium. FLS adjacent to the endothelial and smooth muscle cells. **j-l:** Cathepsin K-positive FLS and/or mononuclear cells invading and eroding bone matrix (**j** and **k**) and cartilage and bone (**l**). **m:** Cathepsin K-positive FLS in the vicinity of bone debris in the deeper synovium. **n** and **o:** Fibrotic cyst within the deeper synovium with an accumulation of cathepsin K-positive multinucleated giant cells and FLS. Areas of cathepsin K-expressing cells reveal the lack or a decreased presence of collagenous fibers (**o**). Of note, amyloid-like deposits are attacked by multinucleated giant cells as indicated by arrows in **o**. Original magnifications: $\times 40$ (**a**, **c**); $\times 200$ (**d**, **e**, **g**, **j**, **l**, **n**, **o**); $\times 600$ (**f**, **h**, **i**, **k**, **m**). Tissue sections were counterstained with H&E.

Figure 3. Cathepsin K expression in subsynovial lining and in vascular areas in correlation to the HFCQ. Cathepsin K expression was quantified using a 0 to 4 scale, with 0 indicating no staining to 4 indicating extensive staining throughout a region (51 to 100%) as described in Materials and Method and plotted against HFCQ, an indicator for the clinical severity of RA (0% complete incapacitation of joints, 100% full functionality).

negative (not shown) and in contrast to the spindle-like SFs displayed a rounder shape. Interestingly, SFs also seem to specifically attack bone debris particles deep in the synovial stroma (Figure 2m). Of note is the high accumulation of cathepsin K-positive multinucleated giant cells and mononuclear cells, including fibroblast-like cells at sites of deposits resembling amyloids (Figure 2, n and o). At the sites of cathepsin K-positive cells, the fibrous extracellular matrix is absent suggesting its complete degradation (Figure 2o).

Correlation between Cathepsin K Expression and Clinical RA Assessment

The staining intensity for cathepsin K did not correlate with the number of swollen joints, the modified Landbury index, or the Keitel functional index. On the other hand, there was a highly significant negative correlation of the results of the HFCQ index with the cathepsin K staining in the subsynovial ($r = 0.78$, $P = 0.003$) and perivascular regions ($r = 0.70$, $P = 0.012$) (Figure 3). No correlation was found for the erythrocyte sedimentation rate or the C-reactive protein. Cathepsin S expression did not reveal any correlation with any of the tested parameters of disease activity (data not shown). The immunohistochemical analyses of the tissue specimens was performed at the Mount Sinai School of Medicine in New York City, NY (by W-SH and DB) without the knowledge of the clinical patient parameters. The correlation between the immunostaining and the HFCQ data were performed at the Martin Luther-University-Halle, Germany (by GK).

Characterization of Primary RA-Synovial Fibroblast Cultures

Synovial fibroblast-like cells were derived from two patients undergoing joint replacement surgery. Expression of cathepsin K in primary SF cell cultures was demonstrated at the mRNA and protein level by Northern and Western blot analyses (data not shown). To determine the intracellular localization of cathepsin K, cells were cultured on tissue culture slides and stained with M21. Figure 4a shows the perinuclear and vesicular distribution of

Figure 4. Expression of cathepsin K in primary cultures of RA-derived FLS. All fibroblast-like cells derived from RA synovial specimens stained positive for cathepsin K protein and showed intracellular hydrolysis of a cathepsin K-specific substrate (a). Primary cultures of FLS stained positive with 5B5, a monoclonal antibody against the fibroblast marker proline 4-hydroxylase (b) but were negative against the macrophage marker CD68 (c). d: The hydrolysis of Z-Gly-Pro-Arg-MβNA, which is preferentially cleaved by cathepsin K and to a lesser extent by cathepsin B. In the presence of ibunH-EPS-Leu-Pro, a selective cathepsin B inhibitor, the Z-Gly-Pro-Arg-MβNA cleaving activity of FLS was only slightly reduced indicating that it solely represents cathepsin K activity (e). No hydrolysis of the substrate was observed in the presence of a potent cathepsin K inhibitor, Mu-Leu-Hph-VS-Ph (f). Mu-Np-Hph-VS-Np, a potent, cathepsin B, L, and S but very poor cathepsin K inhibitor only partially inhibited the Z-Gly-Pro-Arg-MβNA-hydrolyzing activity within FLS (g). The inhibition by this compound was comparable to ibunH-EPS-Leu-Pro indicating that papain-like cysteine proteases such as cathepsin L are not involved in the cleavage of Z-Gly-Pro-Arg-MβNA.

cathepsin K polypeptide indicating a lysosomal localization of the protease. The fibroblast identity of the primary cell culture was verified by the positive staining against the fibroblast marker, proline 4-hydroxylase (5B5), and the negative staining against the macrophage marker CD68 (Figure 4, b and c). Using Z-Gly-Pro-Arg-MβNA for *in situ* substrate staining, fluorescent signals revealed the same distribution as the protein staining suggesting that cathepsin K is processed and fully active in lysosomes (Figure 4d). Z-Gly-Pro-Arg-MβNA is an efficient substrate for cathepsin K and to a lesser degree for cathepsin B.²⁶ Other cathepsins such as cathepsins L, S, H, F, and V do not hydrolyze this compound (D. Brömme, unpublished results). To exclude cathepsin B as a Z-Gly-Pro-Arg-MβNA-hydrolyzing activity, cells were incubated with the specific cathepsin B inhibitor, ibunH-EPS-Leu-Pro (kindly provided by Dr. Gour, Adherex Technologies, Ottawa, Ontario, Canada).²⁶ The remaining Z-Gly-Pro-Arg-MβNA-hydrolyzing activity was concluded to be cathepsin K-specific (Figure 4e). Cathepsin K activity could be abolished by the addition of 1 $\mu\text{mol/L}$ of Mu-Leu-hPh-VS-Ph, a potent and selective cysteine protease inhibitor (Figure 4f). In contrast, Mu-Np-hPh-VS-Np, a potent cathepsin B, L, and S inhibitor but an extremely poor cathepsin K inhibitor showed only a weak inhibition of the Z-Gly-Pro-Arg-MβNA-hydrolyzing activity comparable to that observed for the cathepsin B-specific ibunH-EPS-Leu-Pro compound (Figure 4g).²⁵ The lysosomal uptake and intracellular activity of the selective cathepsin B inhibitor and Mu-Np-hPh-VS-Np were demonstrated by the inhibition of the hydrolysis of the cathepsin B substrate, Z-Arg-Arg-MβNA (not shown).

Figure 5. Electron microscopy images of RA-derived SFs growing on bovine cartilage disks. **a:** SFs were grown on bovine cartilage disks in the absence of cysteine protease inhibitors. The **inset** displays the engulfment of type II collagen fibrils by the SFs. No intracellular accumulation of collagen fibrils was observed. **Arrows** indicates the uptake of collagen fibrils. **b:** SFs were grown on bovine cartilage disks in the presence of 10 $\mu\text{mol/L}$ of Mu-Np-hPh-VS-Np (weak cathepsin K inhibitor but potent against other cysteine cathepsins; $^{25} k_2/K_i \ll 300 \text{ mol/L}^{-1} \text{ s}^{-1}$). No intracellular accumulation of collagen fibrils was observed. **c:** SFs were grown on bovine cartilage disks in the presence of 1 $\mu\text{mol/L}$ of Mu-Leu-hPh-VS-Ph (potent cathepsin K inhibitor; $^{25} k_2/K_i = 773,000 \text{ mol/L}^{-1} \text{ s}^{-1}$). SFs accumulate large amounts of collagen fibrils within the cells. Original magnifications, $\times 10,000$.

In Vivo Cartilage Degradation

To determine whether SFs degrade cartilage, cells were cultured for 10 days on bovine cartilage pieces in the presence or absence of synthetic cysteine protease inhibitors. Cartilage-attached cells were analyzed by light

and electron microscopy. Immunohistochemical analysis of cartilage attached SFs showed a vesicular distribution of cathepsin K (not shown). Although SFs seem to invade the cartilage matrix, the highest optical magnification achieved by light microscopy was not adequate for visualizing the cellular uptake of cartilage matrix compo-

Figure 6. *In vitro* activities of recombinant cathepsin K. **A:** Degradation of bovine aggrecan by cathepsins K, L, and S at pH 5.5 and 7.2. Separation of aggrecan cleavage products using agarose gel electrophoresis and detection of glycosaminoglycan (GAG) products by toluidine blue staining. All three cathepsins are able to release GAG fragments that are showing the same gel mobility as free chondroitin sulfate. **B:** Type I and II collagen degradation by cathepsin K. Both collagens were incubated with cathepsin K alone or with GAGs generated from the hydrolysis of bovine aggrecan by cathepsin K. In the presence of aggrecan-derived GAGs, cathepsin K reveals a strong increase in its collagenolytic activity.

nents. Using electron microscopy, the engulfment and internalization of individual type II collagen fibers became apparent (Figure 5a). Interestingly, there was no significant difference in the ability of RA-SFs in the absence or the presence of inhibitors to ingest collagen fibers. In the absence of inhibitors or in the presence of a non-cathepsin K but cathepsin B, L, and S inhibitor (Mu-Np-hPh-VS-Np), no or only a very slight accumulation of undigested collagen fibrils was observed in lysosomes (Figure 5, a and b). This suggested a rapid degradation of collagen fibers once they arrived in the lysosomal/endosomal compartment. In contrast, a potent cathepsin K inhibitor (Mu-Leu-hPh-VS-Np; this inhibitor has in addition a similar potency toward cathepsins L, B, and S as the Mu-Np-hPh-VS-Np compound) prevented degradation of intracellular collagen leading to an accumulation of undigested fibers within the cells. The comparison of the effects of both inhibitors strongly suggested that cathepsin K was the primary lysosomal activity responsible for the hydrolysis of type II collagen. The detection of very few undigested fibers in cells treated with 10 μ mol/L of Mu-Np-hPh-VS-Ph suggested a partial inhibition of cathepsin K activity rather than the involvement of other redundant cysteine proteases. Because both compounds are irreversible inhibitors it can be anticipated that a long-term incubation with Mu-Np-hPh-VS-Ph leads

to a partial inhibition of cathepsin K despite its extremely weak potency toward cathepsin K (second-order rate constant of inhibition of cathepsin K by Mu-Np-hPh-VS-Ph is more than 6 orders of magnitude lower when compared with Mu-Leu-hPh-VS-Np²⁵). The cellular uptake of both inhibitors by rat osteoclasts has been previously demonstrated by the inhibition of lysosomal cathepsin B activity using a cathepsin B-specific *in situ* substrate, Z-Arg-Arg- β MNA²⁶ (D. Brömme, unpublished results). The uptake of both compounds by human SFs is shown in Figure 4 and discussed above.

In Vitro Aggrecan and Collagen Degradation by Cathepsins K, L, and S

In vivo collagen degradation experiments indicated that endocytosed collagen fibers in SFs are rapidly degraded by a lysosomal cysteine protease activity that is specifically inhibited by a cathepsin K inhibitor. In addition, the presence of SFs at sites of bone erosion (Figure 2; j to m) suggested an active role of cathepsin K in bone matrix degradation as discussed for osteoclasts.²⁸ To demonstrate the ability of cathepsin K to degrade both major components of cartilage, aggrecan, and type II collagen as well as the major bone component, type I collagen, *in vitro* degradation experiments were performed. For comparison, digestion experiments with recombinant human cathepsins L and S were performed. Digestion experiments clearly showed the ability of the tested recombinant human cathepsins to degrade bovine aggrecan under acidic conditions typical for lysosomes (pH 5.0) and to a lesser degree at pH 7.2 reflecting the extracellular pH. Only cathepsin S had an equally potent activity at pH 7.2 and pH 5.0. Glycosaminoglycan staining with toluidine blue revealed a complete degradation of aggrecan to the size of free chondroitin sulfate polymers (Figure 6A) at pH 5.0. This may indicate that cathepsins K, L, and S are able to cleave at multiple sites between the G2 and G3 domain of aggrecan that harbors the glycosaminoglycan side chains. Based on the recently identified potentiating effect of free chondroitin sulfate on the activity of cathepsin K toward collagens,²³ predigested aggrecan was added to the collagen degradation assays. The addition of predigested aggrecan increased the efficiency of triple helical collagen degradation by cathepsin K comparable to the addition of free chondroitin sulfate (Figure 6B).²³ Similarly to free chondroitin sulfate, the activity-enhancing effect of the aggrecan predigest was restricted to acidic pH conditions. No significant degradation of collagen was observed at neutral pH (data not shown).

Discussion

Cathepsin K is a papain-like cysteine protease that constitutes the main proteolytic activity in bone-resorbing osteoclasts.^{12,29} Whereas early studies suggested that cathepsin K is exclusively expressed in osteoclasts, recent research has revealed a wider distribution pattern.

Besides its expression in osteoclasts and related chondroclasts and multinucleated giant cells, cathepsin K has also been described in mononuclear cells that may act as precursor cells for osteoclasts,³⁰ in macrophages,³¹ and various epithelial cells.^{32–35} *In situ* hybridization experiments have demonstrated cathepsin K expression in synoviocytes of RA patients.³⁶ However, no qualitative, quantitative, or functional data were available to characterize cathepsin K expression in SFs on the protein level. Here, we demonstrated high levels of cathepsin K expression in spindle-shaped fibroblast-like cells throughout the inflamed RA synovium. Although the expression of cathepsin K in SFs is widespread, it is not uniform. The finding that cathepsin K is only expressed in a subset of SFs within the inflamed synovium supports the hypothesis that the SF population in RA tissue is heterogeneous.³⁷ Considering the potent collagenolytic-, elastinolytic-, and proteoglycan-degrading activity of cathepsin K, it is tempting to speculate that cathepsin K-positive SFs in the deeper stroma are involved in synovial remodeling, whereas cells attached or in close vicinity to cartilage or bone surfaces directly contribute to joint erosion. These cells have been described as highly proliferating mesenchymal cells within the synovial stroma that may extend to the surface of synovial villi where they seem to replace the original lining cells.³⁸ Two types of cathepsin K-containing cells appear to be present in the synovial lining: CD68⁺ and CD68[−] cells. The data presently available do not allow us to classify the CD68⁺ cells as macrophage-like synoviocytes because the CD68 marker has also been described on activated SFs.³⁹ However, the dense population of cathepsin K-positive cells in the lining of the inflamed synovium suggests a role of the enzyme in cartilage- and bone-invasive processes. This role is further supported by the observation that cathepsin K-positive SFs accumulate at sites of bone and cartilage erosion.

Increased synovial remodeling including angiogenesis and vascular recession are common features in RA.⁴⁰ Cathepsin K-positive SFs were highly enriched at sites of vascularization and angiogenesis but also around necrotic vessels. It can be hypothesized that the protease plays a role in the loosening of the surrounding matrix to facilitate angiogenic growth on one side and contributes to the degradation of expired vessels on the other side. Similar to stromal cells in tumor tissues, stromal cells such as SFs could support the turnover of the synovial extracellular matrix in response to angiogenic signals. It is known that SFs are directly involved in angiogenesis by the expression and secretion of the angiogenic vascular endothelial growth factor whose secretion is in particular augmented under conditions of hypoxia.⁴¹ Thus, the simultaneous activation/recruitment of endothelial cells by fibroblast-derived vascular endothelial growth factor and the degradation of the extracellular matrix by fibroblast-derived cathepsin K would be beneficial for the generation of new blood vessels.

Another hallmark of rheumatoid synovitis is the enormous influx of lymphocytes. Lymphocyte infiltration and migration through the synovium requires local matrix degradation. As described above, cathepsin K protein-

containing SFs are concentrated in areas of lymphocytic infiltration. It can be speculated again, that cathepsin K activity facilitates the migration of these cells.

Synovial fibroblasts have also been directly linked to the degradation of articular cartilage and bone. Bone and cartilage erosions are considered an irreversible degenerative process leading to the loss of joint function. Various functional indices have been developed to address the clinical status and progression of joint destruction and the responsiveness to therapeutic treatments. The Health Assessment Questionnaire Disability Index (HAQ) is considered one of the most comprehensive tests presently available. In this study, we used the HFCQ, a modified version of this test applied to the German speaking population. The questionnaire comprises 19 questions about daily activities of life.²² A value of 0% reflects maximal incapacitation whereas 100% indicates full functionality. HFCQ data were available for 12 RA patients and they correlated with the quantitative assessment of cathepsin K expression in perivascular and subsynovial areas of these patients. Although the number of patients available was rather small in this study, the *r* and *P* values of 0.70 (*P* = 0.012) and 0.78 (*P* = 0.003) were highly correlative and significant for the degree of the impairments. Interestingly, there was no correlation between HFCQ and the expression of cathepsin S that has been implicated in the inflammatory component of RA⁴² (D Brömme, unpublished results).

It is well accepted that cathepsin K is the major osteoclastic proteolytic enzyme responsible for bulk collagen degradation during bone remodeling. This function of the protease is best documented in the phenotype of cathepsin K deficiency, which causes the autosomal-recessive bone-sclerosing dysplasia, pycnodysostosis. On the cellular level, the enzyme deficiency is characterized by an accumulation of undigested collagen fibrils in affected osteoclasts that was reminiscent of the collagen fiber accumulation first seen in E-64-treated osteoclast cultures.⁴³ Analogous to cathepsin K-deficient osteoclasts, SFs treated with a cathepsin K inhibitor displayed high amounts of collagen fibrils stored in intracellular vesicles. Neither one of the tested cysteine protease inhibitors prevented the phagocytosis of collagen fibers by the SFs that suggested that the collagen degradation primarily occurs intracellularly. This conclusion is supported by the previous observation that cells at the pannus-articular cartilage junction contained membrane-bound collagen fibrils, apparently in various stages of digestion.⁴⁴ It is possible, however, that cathepsin K is also involved in the extracellular degradation of matrix molecules. It has been demonstrated that cathepsin K is present in the extracellular resorption lacunae of osteoclasts and that the depth of the osteoclastic excavation pits on the bone surface depends on cathepsin K activity.^{26,28} In contrast to the well-characterized polarized morphology of osteoclasts allowing the formation of an extracellular acidified resorption lacuna underneath the cell, SFs are not known to form such an extracellular matrix resorption compartment. In general, it is argued that because of the pH-neutral extracellular matrix secreted cysteine proteases, with the exception of the neutral pH stable cathepsin S are unlikely to be

proteolytically active. Parak and colleagues,⁴⁵ however, demonstrated that SFs from RA patients showed an enhanced secretion of acidic components that would acidify their pericellular microenvironment. Under these conditions, an extracellular activity of cathepsin K would be likely because primary cultures of SFs secrete mature cathepsin K as well as its precursor molecule that would be autoactivated at acidic pH (D. Brömme, unpublished results).⁴⁶

The inhibition of intracellular collagen fibril degradation by cathepsin-specific inhibitors in SFs raises the question about the involvement of matrix metalloproteinase in type II collagen degradation. Collagen-degrading matrix metalloproteinases have a neutral pH activity optimum and are expressed on the outer cell membrane or are secreted. In general they are regarded as the main collagenolytic activities in mammalian tissues and it is thought that collagen degradation is primarily an extracellular event. However, the finding reported here and previous observations about the intracellular accumulation of type I collagen fibrils in cathepsin K-deficient osteoclasts⁴² suggests that 1) the bulk degradation of collagen fibrils occurs intralysosomally and 2) cathepsin K is a key protease for the intracellular collagen degradation in osteoclasts and SFs. Matrix metalloproteinase activities are likely to be involved in the extracellular predigestion of collagen fibrils or their loosening from the extracellular matrix structure thus allowing their phagocytosis.

It is of particular interest that the collagenolytic activity of cathepsin K is specifically activated by degradation products of aggrecan. Concentrations of chondroitin sulfate similar to those found in synovial fluids lead to an optimal stabilization of cathepsin K activity and subsequently to an increase of its collagenolytic activity.²³ In this report, we could demonstrate that chondroitin sulfate necessary for the increase of the collagenolytic activity can be generated by cathepsin K-catalyzed aggrecan degradation. *In vitro* aggrecan digestion assays with cathepsin K revealed a complete hydrolysis of the proteoglycan at acidic pH and at least partial degradation at physiological neutral pH. It is very likely that in SFs endocytosed aggrecan molecules are degraded intralysosomally by resident cathepsins including cathepsin K that will generate a chondroitin sulfate-rich microenvironment supportive to the collagenolytic activity of cathepsin K.

In conclusion, this report describes the expression of one of the most potent extracellular matrix-degrading mammalian activities, cathepsin K in SFs that are regarded as the pivotal cell type in RA-associated cartilage and bone erosion. The expression of cathepsin K in the subsynovium and in perivascular synovial areas correlates with the severity of the disease based on the HFCQ. The inhibition of cathepsin K in RA-derived SFs results in a lysosomal accumulation of undigested type II collagen fibrils comparable to that seen in cathepsin K-deficient osteoclasts in pycnodysostosis whereas the inhibition of cathepsins L, B, and S has no effect. The specific activation of the protease activity toward types I and II collagen by cathepsin K-digested aggrecan, corroborates the potential role of cathepsin K in RA-associated joint destruction.

Taken as a whole, these findings identified cathepsin K as a potential target for anti-resorptive drugs in RA.

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