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Acidic Cysteine Endoproteinase Cathepsin K in the Degeneration of the Superficial Articular Hyaline Cartilage in Osteoarthritis

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Objective. **To measure cartilage pH in patients with osteoarthritis (OA) and to analyze the presence of cathepsin K, the recently discovered acidic endoproteinase, in phenotypically altered chondrocytes.**

Methods. **Intraoperative measurements of the pH of clinically normal, fibrillated, superficially fissured, and deeply fissured cartilage surfaces (grades 0–3, respectively) in OA patients undergoing primary hip replacement surgery were performed with the use of a sting electrode sterilized with microbicidic plasma. Fluorescent pH probes were used for in situ assessment of cartilage matrix pH. Cathepsin K was assessed using quantitative reverse transcriptase–polymerase chain reaction and immunohistochemistry methods.**

Results. **The pH of grade 0 cartilage surfaces was 7.1 ± 0.4 (mean ± SD), compared with 6.2 ± 0.9 ($P < 0.05$), 5.7 ± 1.0 ($P < 0.001$), and 5.5 ± 1.0 ($P < 0.001$) for grades 1–3 cartilage surfaces, respectively. Fluorescent pH probes and acid-dependent autocatalytic conversion of cathepsin K into its active, low molecular weight form in cartilage confirmed these findings. Cathepsin K messenger RNA levels increased in relation to the severity of OA, and the number of cathepsin K–containing chondrocytes increased from a mean ± SD of 12 ± 3 in grade 0 cartilage surfaces to 47 ± 7, 50 ± 6, and 100 ± 12 in grades 1–3 cartilage surfaces, respectively ($P < 0.001$ for all comparisons).**

Conclusion. **Acid-activated, but pharmacologically inhibitable, cathepsin K is induced in phenotypically altered chondrocytes in OA. The findings suggest that cathepsin K, rather than neutral matrix metalloproteinases, degrades the superficial gliding surfaces of the articular hyaline cartilage in OA.**

Osteoarthritis (OA) is a common and crippling degenerative joint disease that affects the elderly population. In adult cartilage, chondrocytes occupy <5% of the total tissue volume. They reside in an avascular, anoxic environment and depend on anaerobic metabolism. Articular hyaline cartilage consists of a hydrated, proteoglycan-rich matrix in which a network of type II collagen–rich fibers is embedded (1,2). The hydrated proteoglycan matrix has a high swelling pressure, which is counteracted by the stiff collagen network (2). This gives unique biomechanical properties to the articular hyaline cartilage.

While depletion of proteoglycans alone is considered to be reversible, once the collagen network is destroyed, the damage is permanent, because the articular hyaline cartilage has an almost nonexistent capacity to regenerate (3–5). Therefore, degradation of type II collagen is the crucial event in the pathogenesis of OA.

OA has been considered to be strictly a wear-and-tear disease, in which the destruction of the collagen fiber network is mechanically induced. However, it is now widely accepted that OA is a biochemically medi-

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ated, active process (1,6,7). This idea has greatly stimulated research in this field, as well as a search for chondroprotective drugs. The rationale is that recognition of the key pathogenic event will provide targets for future drug development.

Interstitial mammalian collagenases and other matrix metalloproteinases (MMPs) have been considered to be the key players in cartilage destruction (1,6,7). MMPs are neutral endoproteinases, which usually require neutral or slightly alkaline pH for optimum function (8). However, they can also act at relatively low pH. For example, MMP-3 (stromelysin 1), which is highly expressed in chondrocytes, has an optimum pH of 5.5 (9). The major collagenases, MMP-1 (collagenase 1), MMP-8 (collagenase 2), and MMP-13 (collagenase 3), have rather broad pH optima, between 6.5 and 8. Although these collagenases are less active at pH 5.5, they are nevertheless active (10).

In 1994, a new and effective collagenolytic enzyme, cathepsin K, was discovered (11). Cathepsin K is a bacterial collagenase-like enzyme that cleaves the collagen superhelix at several sites. It effectively cleaves type II collagen. However, it is an acidic cysteine endoproteinase, which is not able to act at neutral pH (12–15). We hypothesized that avascular cartilage, which is dependent on oxygen supplied by the synovial fluid, is acidic to such an extent that cartilage-destroying MMPs are not able to function, and that, instead, collagenolytic cathepsin K is produced by phenotypically altered chondrocytes. The results of our studies are reported here.

PATIENTS AND METHODS

Patients. Twelve patients with primary OA of the hip joint underwent total hip replacement surgery. The average age of the study patients was 72 years (range 48–87 years), and all patients gave their informed consent. The Declaration of Helsinki was followed throughout the study, and the study was approved by the local ethics committee.

Cartilage samples for staining and messenger RNA (mRNA) isolation (see below) were cut from the femoral heads under direct visual control using a surgical knife. It was necessary to ascertain that the cartilage samples did not contain even traces of contaminating subchondral bone, which is rich in cathepsin K–containing osteoclasts and mononuclear precursor cells. Therefore, a large safety margin was left between the lower margin of the cartilage chip sample and the subchondral bone. It was also visually and microscopically ascertained that the sample did not contain any subchondral bone.

To put the cathepsin K gene copy numbers in OA cartilage into quantitative perspective, 10 samples of human synovial membrane–like interface tissues from loosened total hip replacement implants were collected at the time of revision operations, and 2 samples were collected from operatively treated osteoclastomas. Both of these tissues contain many cathepsin K–positive multinuclear and mononuclear cells (ref. 16 and Hukkanen M, et al: unpublished observations).

Tissue extraction. To test the specificity of the antibodies we used, osteoclastoma and liver tissue samples were minced into small pieces and homogenized with Ultra-Turrax T25 (IKA Laborteknik, Staufen, Germany) in an ice bath after addition of lysis buffer (50 or 10 m*M* Tris HCl, 150 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 0.5% Triton X-100; 1 ml of buffer per 0.75 mg of tissue), with or without 1 tablet of Complete Protease Inhibitor (Roche Diagnostics, Mannheim, Germany). After mechanical homogenization, the samples were further treated by sonication with a Vibra-Cell 501 Ultrasonicator (Sonics & Materials, Danbury, CT). Homogenate fluid was centrifuged at 15,000*g* for 40 minutes at 4°C and clarified with the use of a 0.45-μm filter (Millipore, Bedford, MA).

To assess the eventual presence and molecular forms of cathepsin K in articular hyaline cartilage, grade 0 (normal) and grade 3 (superficially fissured) samples were pulverized with a mortar while being cooled with liquid nitrogen. The resulting powder was suspended in lysis buffer (4*M* guanidine hydrochloride, 10 m*M* Tris HCl, and 150 m*M* NaCl; 1 ml of buffer per 0.75 mg of tissue) with 1 tablet of Complete Protease Inhibitor (Roche Diagnostics). The resulting mixture was incubated for 24 hours at 4°C, with gentle mixing. The samples were then clarified by centrifugation at 15,000*g* for 40 minutes at 4°C.

Measurement of pH. Measurements of pH were performed using the Sentron 1001 pH system with a sting electrode (Sentron Europe, Roden, The Netherlands). The equipment was calibrated, and sterilization was performed in a vacuum chamber injected with hydrogen peroxide, which upon evaporation produced microbicidic plasma in an electrical field generated by radiowave frequencies in a Sterrad 100S sterilizer (Advanced Sterilization Products, Irvine, CA). The cartilage of the whole femoral head was graded visually by the orthopedic surgeon who performed the operations. Cartilage was graded on a scale of 0–3, where 0 = clinically normal, 1 = fibrillated, 2 = superficially fissured, and 3 = deeply fissured.

The pH of all grades of cartilage from a luxated intact femoral head was measured. After use, the electrodes were recalibrated with standard pH solutions, and corrections were made for the ambient temperature. The Sentron pH system has an automatic temperature compensation between 0°C and 60°C with a high resolution (pH ±0.01, mV ±1, temperature ±0.5°C).

Acridine orange staining. After pH measurements, the specimens were snap-frozen in liquid nitrogen and stored at −70°C until further analyzed. For in situ pH measurement with an acridine orange fluorescent probe, sections were treated with ferriammonium sulfate for 15 minutes, rinsed in distilled water, and stained with 1 mg/ml of acridine orange (catalog no. A-6014; Sigma, St. Louis, MO) for 1.5 minutes (17). For purposes of comparison, some sections from grade 0 samples were balanced to different pH values by a 3-day incubation at pH 4.0–8.0 (acetate buffer for pH 4.0 and 5.0, phosphate buffer for pH 6.0, 7.0, and 8.0, and citrate buffer for pH 4.0, 5.0, 6.0, 7.0, and 8.0). Samples were analyzed using an epifluorescence microscope at 160× magnification.

Quantitative reverse transcriptase—polymerase chain reaction (RT-PCR). Samples (n = 5, 4, 6, and 6 for grades 0–3, respectively) were pulverized in liquid nitrogen and homogenized in guanidinium isothiocyanate followed by ultracentrifugation through a CsCl gradient (18). Messenger RNA was isolated using magnetic $(dT)_{25}$ polystyrene beads (Dynal, Oslo, Norway) and a magnetic collector. Then, 100 ng of mRNA was used to prepare primary complementary DNA using $(dT)_{12\text{-}18}$ primers and SuperScript enzyme (Invitrogen, Carlsbad, CA), followed by RNase H treatment.

PCR for cathepsin K and β-actin was run on 10 ng of complementary DNA using 0.2 μ*M* primers as follows: sense probes were 5′-CAGTGAAGAGGTGGTTCAGA-3′ and 5′-TCACCCACACTGTGCCCATCTACGA-3′, respectively; antisense probes were 5′-AGAGTCTGGGGCTCTACCTT-3′ and 5′-CAGCGGAACCGCTCATTGCCAATGG-3′, respectively. Amplification with a LightCycler PCR machine (Roche Molecular Biochemicals, Mannheim, Germany) resulted in products that were 109 and 295 bases long, respectively. Cathepsin K and β-actin were quantified against genomic DNA with TaqMan probes containing a reporter dye (FAM) at the 5′ end and a quencher dye (TAMRA) at the last T for cathepsin K and at the second T for β-actin. The 3′ ends were phosphorylated (19).

Immunohistochemistry. Cryostat sections were cut from cartilage samples (n = 8, 10, 10, and 10 for grades 0–3, respectively) onto chromium potassium sulfate–gelatin coated slides. After hyaluronidase treatment (240 units/ml) for 30 minutes at 22°C and fixation in acetone at −20°C for 20 minutes, sections were incubated with a polyclonal goat antiserum to human cathepsin K (2 μg/ml in phosphate buffered saline [PBS] containing 1.25% bovine serum albumin [BSA]; Santa Cruz Biotechnology, CA) for 60 minutes, followed by tetramethylrhodamine isothiocyanate–conjugated donkey anti-goat IgG for 45 minutes. After washes with PBS, the slides were air-dried and mounted.

As a negative control, normal goat IgG was used instead of the primary antibody and at the same concentration. In addition, we used an antigen absorption test (see below) to ascertain the specificity of staining.

Quantitative morphometric assessment was done with a low light–charge screen-mounted 12-bit PC digital image camera (SensiCam, Kelheim, Germany) on a Leitz Diaplan light microscope (Leitz, Wetzlar, Germany). The camera was further linked to a semiautomatic Analysis Pro 3.0 image analysis and processing system (Soft Analysis System, Münster, Germany). The images were visualized under 200× magnification.

Cell counts were performed by 2 independent researchers who were unaware of the source or grade of the tissue sample. The whole sections were used for analysis, and every section was counted for 5 times. The sections were then stained with hematoxylin, and the total number of chondrocytes in each sample was counted. The final results are reported as the mean ± SD number of cathepsin K–positive chondrocytes per 1,000 total cells. One-way analysis of variance and post hoc *t*-tests were used for statistical comparisons.

Antigen preabsorption. Purified cathepsin K (Axys Pharmaceuticals, South San Francisco, CA) was coated onto Dynabead M-270 Epoxy superparamagnetic beads (Dynal Biotech, Oslo, Norway). Briefly, 2×10^8 beads were suspended and washed twice in 0.1*M* sodium phosphate buffer, pH 7.4. The beads were mixed with 60 μg of cathepsin K at a concentration of 1×10^9 beads/ml in 0.1*M* sodium phosphate buffer, pH 7.4, containing 1*M* ammonium sulfate. Covalent binding of cathepsin K to the solid support was then allowed to proceed for 24 hours at 4°C, with gentle mixing. After binding, the beads were washed 4 times with 10 m*M* phosphate buffered 0.9% saline, pH 7.4.

Cathepsin K–coated beads were incubated overnight at 4°C with 100 μl of affinity-purified polyclonal goat anti-human cathepsin K (N-terminal) antibody (200 μg/ml dilution with PBS containing 0.1% sodium azide and 0.2% gelatin; Santa Cruz Biotechnology), with gentle mixing. The superparamagnetic beads with bound cathepsin K–anti–cathepsin K antibody complex were then removed with a magnet. Antigen-preabsorbed antibodies were used to control for the specificity of cathepsin K staining.

Gel electrophoresis and immunoblot analysis. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed according to a modification of the Laemmli method (20) with a 10% polyacrylamide slab gel. Tissue extracts were adjusted to 50 or 100 μg of total protein per sample (as measured by a modification of the Bradford method [21]) (Bio-Rad Laboratories, Richmond, CA) and mixed with SDS sample buffer (187.5 m*M* Tris HCl, pH 6.8, 6% SDS, 30% glycerol, 0.03% phenol red, and 125 m*M* dithiothreitol; at a 1:3 dilution) (New England Biolabs, Beverly, MA).

To avoid precipitation of guanidine hydrochloride with SDS, samples were subjected to trichloroacetic acid (TCA) precipitation. Briefly, the appropriate amount of extract was mixed with an equal amount of 10% TCA, left on ice for 20 minutes, and the precipitate was then centrifuged at 15,000*g* for 15 minutes at 4°C. The resulting pellet was washed with ice-cold ethanol, centrifuged again, dried, and then resuspended in an appropriate amount of SDS sample buffer. The samples were boiled for 5 minutes and applied to the gels. Protein standards (Precision Protein Standards; Bio-Rad) were used as molecular weight markers. After electrophoresis, the gels were blotted onto nitrocellulose membrane (Bio-Rad), as described by Towbin et al (22).

After blocking overnight with a blocking solution (3% BSA in Tris buffered saline) and a 10-minute incubation in a washing solution (0.1% Tween 20, 50 m*M* Tris HCl, and 0.5*M* NaCl, pH 7.5), blotted membranes were incubated for 1 hour at 22°C with affinity-purified polyclonal goat anti-human cathepsin K (N-terminal) antibody (0.4 μg/ml dilution with washing buffer containing 2% BSA; Santa Cruz Biotechnology). The membranes were washed with washing buffer for 30 minutes, with at least 3 changes of buffer, and then incubated for 1 hour at 22°C with alkaline phosphatase (AP)–conjugated rabbit anti-goat Ig (1:5,000 dilution with washing buffer containing 2% BSA; Jackson ImmunoResearch, West Grove, PA). The membranes were then washed with washing buffer for 30 minutes, with at least 3 changes of buffer, and a final wash in Tris buffered saline (50 m*M* Tris HCl, 0.9% NaCl, pH 7.5).

Color development solution (10 ml of Alkaline Phosphatase Conjugate Substrate kit; Bio-Rad) was applied, and the AP binding sites were revealed, based on the colorimetric detection of AP, with a mixture of BCIP and nitroblue tetrazolium. After ~30 minutes, the color reaction was stopped by washing the membranes in distilled water for 10 minutes.

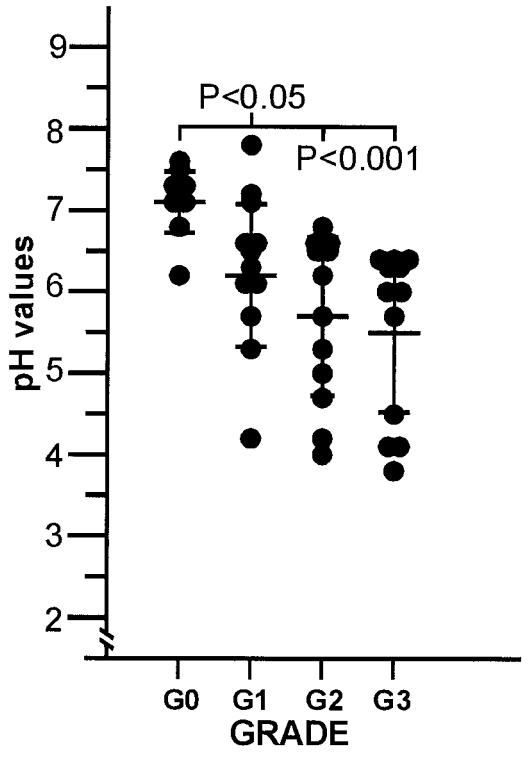

Figure 1. Intraoperative in situ pH values (mean ± SD) in normal and osteoarthritic cartilage, as measured by a Sentron 1001 pH system with a sting electrode. The pH values were measured at sites graded 0–3 (G0–G3) according to the macroscopic appearance of the cartilage, using a standard assessment system, where grade 0 = clinically normal, 1 = fibrillated, 2 = superficially fissured, and 3 = deeply fissured cartilage.

RESULTS

OA cartilage pH values. A sting electrode was used in the measurement of cartilage pH in 12 patients with primary OA of the hip who had undergone total hip replacement surgery. The pH measurement sites in the cartilage samples were graded from 0 to 3 according to their macroscopic appearance. The pH of grade 0 cartilage surface was 7.1 ± 0.4 (mean ± SD), compared with 6.2 ± 0.9 ($P < 0.05$), 5.7 ± 1.0 ($P < 0.001$), and 5.5 ± 1.0 ($P < 0.001$) for grades 1–3 cartilage, respectively (Figure 1). This shows that the degenerating superficial zone of articular hyaline cartilage is acidic.

Results of acridine orange staining. Because the acidity of the superficial articular hyaline cartilage is central to our hypothesis about type II collagen cleavage and the function of cathepsin K (as well as the inhibition of the function of MMPs), we used a supplementary approach to the assessment of the pH. A polycationic acridine orange fluorescent pH probe stained the superficial native grade 0 hyaline cartilage green. It was shown that at low pH, the dye-binding properties of the superficial cartilage were changed, such that the cartilage stained as a gradient from yellow to orange (Figures 2A–D). This pH-dependent change may be irreversible, since the middle/deep zones of the cartilage always stained as a gradient from yellow to orange. Thus, hyaline cartilage exposed to low pH changes its capacity to bind acridine orange in a pH-dependent manner.

We next showed that all OA grade 2 and grade 3 cartilage samples stained as a gradient from yellow to orange (Figures 2E–H). This finding showed that the superficial zone of the degenerating OA cartilage is acidic and confirmed the results obtained with direct pH measurements.

Results of RT-PCR. Neutral endoproteinases such as MMPs have an optimum pH that is slightly alkaline, but cathepsin K has an acidic pH optimum. Cathepsin K efficiently degrades collagen at pH 4.5 and pH 6.0 (23). Furthermore, the collagenolytic activity of cathepsin K is unique among mammalian proteinases; the enzyme is able to cleave outside the helical region of the collagen molecule as well as at various sites inside the helical region (14).

To investigate the eventual presence of cathepsin K mRNA in cartilage, we assessed cathepsin K copy numbers per 1,000 β-actin copies using quantitative RT-PCR. Grade 0 cartilage contained a mean ± SD of 310 ± 145 cathepsin K copies compared with 467 ± 272, 839 ± 430, and 954 ± 316 for grades 1–3 cartilage, respectively ($P < 0.05$ for grade 0 versus grades 2 and 3) (Figure 3A).

To put the cathepsin K gene copy numbers into a quantitative perspective, the same sample processing and RT-PCR protocols were used to assess 10 samples of human synovial membrane–like interface tissues and 2 samples of human osteoclastomas; both tissues contain many cathepsin K–positive multinuclear and mononuclear cells (ref. 16 and Hukkanen M, et al: unpublished observations). The mean ± SEM number of cathepsin K copies in the interface tissues was 1,875 ± 302, compared with a mean of 6,226 and 7,941 in the 2 osteoclastoma samples.

Results of immunohistochemistry. To localize and calculate the numbers of cathepsin K enzyme–containing cells per 1,000 chondrocytes, we performed immunohistochemical analysis (Figure 4). Grade 0 cartilage contained a mean ± SD of 12 ± 3 cathepsin K–positive chondrocytes, compared with 47 ± 7, 50 ± 6, and 100 ± 12 for grades 1–3 cartilage, respectively ($P <$ 0.001 for grade 0 versus all other grades). In grade 0

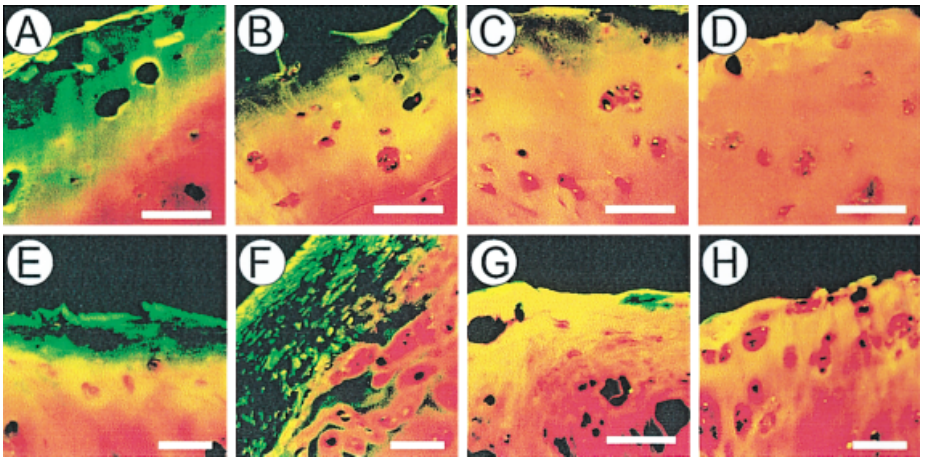

Figure 2. Assessment of in situ pH in normal and osteoarthritic (OA) cartilage with the use of an acridine orange fluorescent probe. Grade 0 cartilage (pH 7.6) was balanced to **A,** pH 7.0, **B,** pH 6.0, **C,** pH 5.0, and **D,** pH 4.0 before incubation with acridine orange. Note that at pH 7.0, the surface layer of the articular hyaline cartilage is green, but sections balanced to lower pH stain as a gradient from yellow to orange. **E,** Similarly, apparently native normal grade 0 cartilage has a green surface layer. Samples of OA cartilage with **F,** grade 1, **G,** grade 2, and **H,** grade 3 changes display progressively more yellow-to-orange–staining surface layer. Fluorescein isothiocyanate filters; bar = 100 μm.

cartilage, cathepsin K–positive cells were found only in the deep zone near subchondral bone, while in grade 3 samples, cathepsin K–positive cells were scattered in all layers. Cathepsin K–positive cells were commonly seen near the fissures of cartilage.

In addition to chondrocyte localization, immunoreactivity for cathepsin K was also seen in the perichondrocyte/territorial matrix. This was particularly clear when the chondrocytes had undergone apoptosis or had been stripped away from their lacunae. This

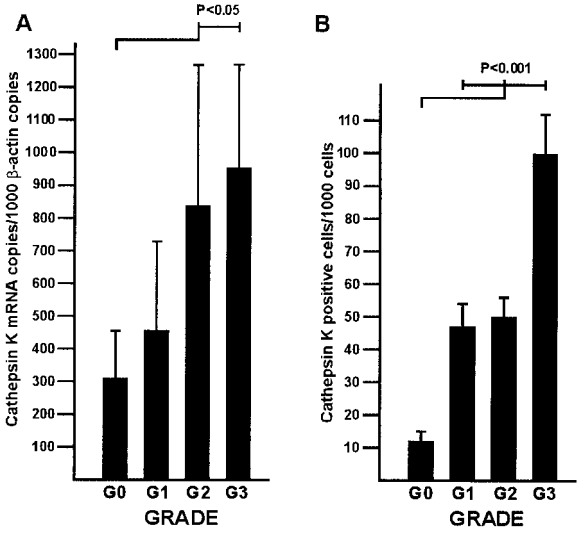

Figure 3. Increases in cathepsin K mRNA expression and the number of cathepsin K–containing cells with progression of osteoarthritis (OA). **A,** Cathepsin K mRNA expression (normalized against 1,000 β-actin mRNA copies) was found to increase as cartilage damage increased. **B,** Similarly, the number of cathepsin K enzyme protein–containing cells increased with progression of OA. Values are the mean and SD.

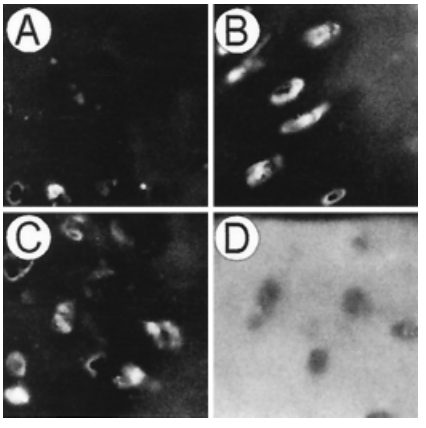

Figure 4. Detection of cathepsin K expression in normal and osteoarthritic cartilage by immunohistochemistry. **A,** In grade 0 cartilage, cathepsin K–positive chondrocytes were relatively few. **B,** In grade 3 samples, cathepsin K–positive chondrocytes were more frequent. **C,** Cathepsin K–positive cells were commonly seen around the fissures penetrating the cartilage. **D,** Negative IgG control, confirming the specificity of the method.

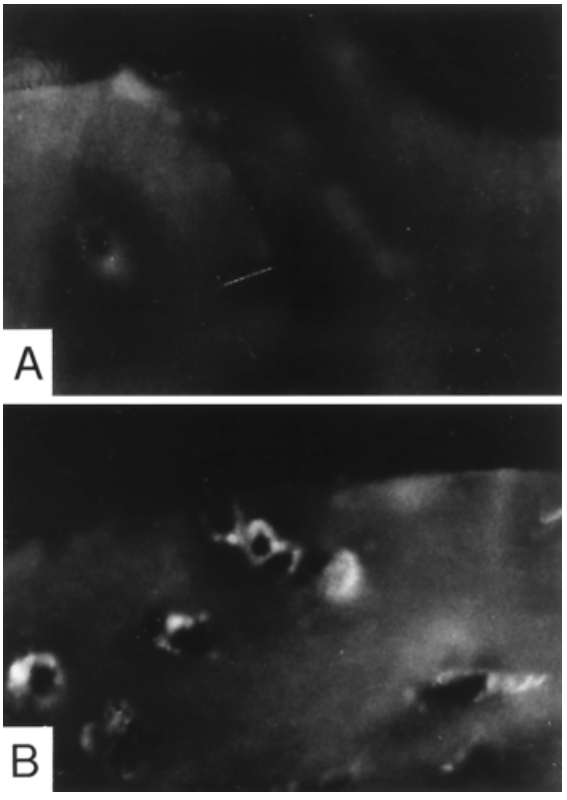

Figure 5. Confirmation of the specificity of the immunohistochemical cathepsin K staining by use of an antigen preabsorption staining control. To test for the specificity of the antibody staining, an antigen preabsorption test was performed. Polyclonal goat anti-human cathepsin K (N-terminal) antibodies were preincubated with cathepsin K–coated Dynabead M-270 Epoxy–activated superparamagnetic particles. Staining with **A,** cathepsin K–preabsorbed antibodies was negative, whereas staining with **B,** untreated antibodies was positive.

observation indicates that chondrocyte-produced cathepsin K is also released into the extracellular cartilage matrix.

The specificity of staining was confirmed with the negative controls, which had been stained with normal goat IgG instead of the primary antibody and at the same concentration. The specificity of staining was also confirmed by immunohistochemical staining using antibodies that had been preabsorbed with purified human cathepsin K (Figure 5).

Results of Western blotting. To assess the reactivity of the antibody we used, a tissue extract was prepared from samples of osteoclastoma, a rich source of cathepsin K–positive cells, and liver, which has very low levels of cathepsin K (23). Together with purified human cathepsin K, these samples were analyzed by Western blotting. The osteoclastoma sample contained

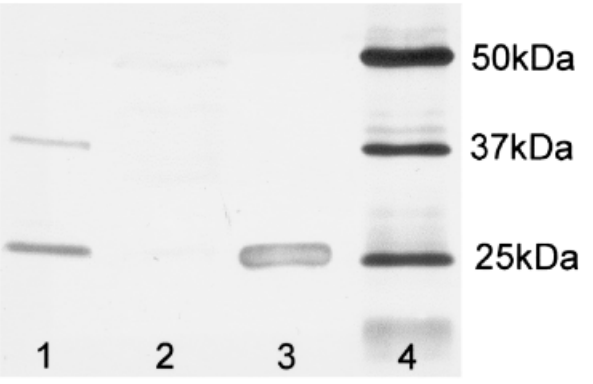

Figure 6. Confirmation of the specificity of the antibodies used for the detection of cathepsin K by use of Western blotting of cathepsin K. Lane 1, Osteoclastoma tissue extract (50 μg total protein). Note the presence of the 42-kd pro form and the 27-kd activated form of cathepsin K. Lane 2, Liver tissue extract (50 μg total protein). Lane 3, Purified and activated 27-kd human cathepsin K (100 ng). Lane 4, Molecular weight standards.

clear cathepsin K bands: a 42-kd pro form and a stronger 27-kd activated form of cathepsin K. The band for the 27-kd activated form moved identically with the purified activated 27-kd human cathepsin K. The cathepsin K band in the liver sample was very weak/negative. The antibodies we used did not disclose any nonspecific labeling/bands (Figure 6).

To assess the eventual presence and molecular forms of cathepsin K in normal-appearing grade 0 and OA grade 3 articular hyaline cartilage, extraction into guanidine hydrochloride extraction buffer was performed. The results showed proteolytic conversion of the latent high molecular weight pro form of cathepsin K into the corresponding active low molecular weight form in vivo (Figure 7).

DISCUSSION

The gliding surfaces of diarthrodial joints are lubricated in a mixed mode manner, which denotes a combination of squeeze-film lubrication and boundary (or solid-to-solid) lubrication. This gives a low coefficient of friction. In OA, the surface of the cartilage is altered, such that the normally smooth (grade 0) gliding surface becomes fibrillated (grade 1). As the disease progresses, superficial fissures (grade 2) and deep fissures that penetrate down to the subchondral bone (grade 3) develop (6). This indicates destruction of the type II collagen network in the superficial layer of the OA cartilage. Indeed, collagenase 1 (MMP-1), collagenase 2 (MMP-8), and in particular, collagenase 3 (MMP-

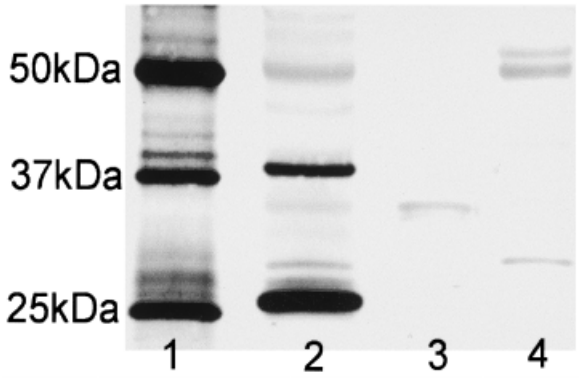

Figure 7. Western blots of normal-appearing grade 0 articular hyaline cartilage compared with grade 3 osteoarthritic (OA) cartilage, showing proteolytic activation of the latent, high molecular weight pro form of cathepsin K into the corresponding active, low molecular weight form in vivo. See Results for further details on the apparent molecular weights. Lane 1, Precision Protein Standard (from Bio-Rad). Lane 2, Osteoclastoma sample. Lane 3, Normal cartilage sample (grade 0). Lane 4, OA cartilage sample (grade 3).

13) are able to cleave the type II collagen superhelix at the initial ^{775}Gly–^{776}Ile cleavage site. After this initial and regulated cleavage, the three-quarter–length and one-quarter–length fragments spontaneously undergo helix-to-coil transition, and the denatured collagen is rapidly cleaved by gelatinases and many nonspecific proteinases. In addition, the solubilizing effect of stromelysin 1 (MMP-3) on the collagen matrix has been considered to be an important event in OA (1,6,7).

However, these neutral endoproteinases usually require neutral or slightly alkaline pH for optimum function. For example, the assay for the traditional interstitial "fibroblast" collagenase (collagenase 1, or MMP-1) activity uses radiolabeled collagen as substrate at pH 7.6, which is the pH optimum of the enzyme (24). However, the neutral endoproteinases can also act at relatively low pH. For example, stromelysin 1 (MMP-3), which is highly expressed in chondrocytes, has optimum activity at pH 5.5. Cathepsin K, the acidic cysteine endoproteinase, is activated (12) and efficiently degrades collagen at pH 4.5 and pH 6.0 (23). Accordingly, cathepsin K extracted from normal-appearing (grade 0) articular hyaline cartilage with neutral pH was in the latent, high molecular weight pro form, whereas cathepsin K in OA grade 3 cartilage with low pH was in the active, proteolytically processed, low molecular weight form. Acidic endoproteinases are inactivated at neutral pH.

Although the difference in the optimum pH for the neutral and acidic endoproteinases seems low, it should be remembered that the pH scale is logarithmic, and a wide range of concentrations are represented on a conveniently compressed exponential scale. In fact, our direct intraoperative pH measurements demonstrated that the H^+ concentration in the superficial layer of the articular hyaline cartilage progressively increased 40-fold, from $10^{-7.1}M$ to $10^{-5.5}M$, as the degree of degeneration increased from grade 0 to grade 3. The results of direct pH measurements were confirmed by the results obtained with acridine orange, a fluorescent pH probe (17). Although our study was a cross-sectional study, it suggests that the progression of the OA disorganization of the superficial articular hyaline cartilage is associated with progressively increasing H^+ concentrations. This indicates that while collagenases and other MMPs are decreasingly active, cathepsin K is increasingly active, as the pH falls and OA progresses.

Demonstration of cathepsin K in articular hyaline cartilage was possible only after some technical difficulties were overcome. Separation of RNA from proteoglycan-rich matrix can be difficult. In the present study, we pulverized cartilage samples in liquid nitrogen. Furthermore, following homogenization in guanidinium isothiocyanate, the samples should, preferably, be ultracentrifuged through a CsCl gradient followed by proteinase K treatment, which is the procedure we used. A high-speed, real-time thermocycler–fluorometer method was used for reproducible and reliable quantitative RT-PCR to assess differences in the mRNA levels between different sample groups.

Similarly, loss of cartilage sections from slide objectives during immunostaining was a problem and was solved either by the use of a free-floating staining method (data not shown) or a strong chromium potassium sulfate–gelatin slide adhesive, which was selected after extensive testing. It was also found that a pretreatment with hyaluronidase was necessary to reveal the masked immunoreactive cathepsin K epitopes in cartilage sections before the blinded morphometric evaluation. These technical difficulties may explain why similar results have not been previously published by other groups of investigators. Quantitative RT-PCR and immunohistochemical staining gave similar results and confirmed the main conclusion concerning the induction of cathepsin K in advancing OA.

Cathepsin K is autoactivated under acidic conditions (12). We found that the extracellular, matrix-bound cathepsin K, exposed to low pH, is activated. Western blots of extracts of articular hyaline cartilage of grade 0 and grade 3 disclosed cathepsin K, and we found that cathepsin K in healthy-appearing articular hyaline

cartilage is in the latent, high molecular weight pro form, whereas cathepsin K in OA grade 3 cartilage is in the proteolytically processed, low molecular weight active form. This confirms that the pH is very low in degenerating articular hyaline cartilage.

The molecular weights of the 2 forms of cathepsin extracted from cartilage did not precisely correspond to those seen in the positive osteoclastoma control samples. This is probably because of the methods used. First, the osteoclastoma samples were denatured just before the SDS–polyacrylamide gel electrophoresis, whereas articular hyaline cartilage samples were already denatured during the extraction procedure. Second, guanidine hydrochloride was required for the extraction of the cartilage samples, and it was therefore necessary to perform TCA precipitation to avoid precipitating the guanidine hydrochloride with the SDS. This experiment was done 3 times, and the same results were obtained each time. Western blots of the articular hyaline cartilage extracts were very clean, i.e., they did not have any unexplained nonspecific bands.

The cumulative collagenolytic activity of cathepsin K has recently been shown to increase 200-fold in the presence of cartilage-resident chondroitin 4-sulfate (15). In this context, it is interesting that cathepsin K inhibitors are being developed for the treatment of osteoporosis (ref. 25 and the World Wide Web site for Axys Pharmaceuticals at http://www.axyspharm.com/pipeline/meosteo.html). According to the present findings, such drugs could also have the potential for use as chondroprotective agents in the prevention and treatment of OA.

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