EXTENDED REPORT

Up regulation of cathepsin K expression in articular chondrocytes in a transgenic mouse model for osteoarthritis

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Three additional figures W1–W3 are available at <http://www.annrheumdis.com/supplemental>

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The structural properties of articular cartilage are largely ascribed to the network of collagen fibrils and to the aggregating proteoglycans.¹ This supramolecular assembly provides cartilage with its unique shock absorbing function.² During osteoarthritic cartilage degeneration, alterations have been seen both in the collagen network and in aggrecan molecules.³ The role of proteolytic enzymes in this process remains a controversial issue.⁴ Despite intensive research it has been difficult to determine whether the increased activity of proteolytic enzymes seen in osteoarthritic joints precedes and causes articular cartilage degeneration, or is a consequence of the shedding of debris from damaged articular cartilage.

We have recently performed systematic analyses on the molecular pathogenesis of articular cartilage degeneration using a transgenic Del1 mouse model for osteoarthritis, where a small deletion mutation in the transgene coding for type II collagen makes the mice susceptible to early onset degeneration of articular cartilage in the knee joints.⁵ These analyses include studies on matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase,⁶ because it is generally believed that MMPs take part in the degradation of cartilaginous extracellular matrix.⁷ However, only a transient increase was seen in MMP-13 expression at the onset of articular cartilage degeneration.⁶ The enzyme was predominantly localised in subchondral bone and synovial tissue of the knee joints of Del1 mice, but not at sites of cartilage destruction. None of the other MMPs studied showed any clear association with the pathogenesis of articular surface fibrillation.

In search for other proteolytic enzymes which might have a role in the degradation of cartilage matrix, we focused on the cysteine cathepsins B, H, K, L, and S, which can degrade native collagens and other matrix components.^{8–14} The

Objectives: To study the expression of cysteine proteinases, particularly cathepsin K, and their extracellular inhibitor cystatin C in articular cartilage of transgenic Del1 mice which harbour a short deletion mutation in a type II collagen transgene and are predisposed to early onset osteoarthritis.

Methods: Northern analysis was used to measure mRNA levels of cathepsins B, H, K, L, and S, and cystatin C in total RNA extracted from knee joints of Del1 mice, using their non-transgenic litter mates as controls. Immunohistochemistry and morphometry was used to study the distribution of cathepsin K and cystatin C in the knee joints.

Results: Up regulation of cathepsin K mRNA expression was seen in the knee joints of transgenic Del1 mice at the onset of cartilage degeneration. Cathepsin K was found near sites of matrix destruction in articular chondrocytes, particularly in clusters of proliferating cells, and in calcified cartilaginous matrix. In intact articular cartilage of control animals, cathepsin K was only seen in a small number of chondrocytes. Upon aging, control animals also developed osteoarthritis, which was accompanied by increased cathepsin K expression. Cystatin C was mostly localised in and around chondrocytes located in calcified cartilage, with no obvious association with the onset of cartilage degeneration.

Conclusion: The temporospatial distribution of cathepsin K in osteoarthritic cartilage suggests a role for this enzyme in the pathogenesis of osteoarthritis. Because cathepsin K can digest cartilage matrix components it may contribute to the development of osteoarthritic lesions. These data may provide new clues for the development of treatments aimed at preventing cartilage degeneration.

activity of cysteine proteinases is generally dependent on pH values below 7, as found in lysosomes and specific extracellular locations.¹⁵ Over the years, several different cysteine cathepsins have been suggested to participate in osteoarthritic cartilage destruction.^{16–22} However, interpretation of early work on cysteine cathepsins in osteoarthritic joints is difficult, as much of it was performed before the discovery of cathepsin K, the predominant cysteine cathepsin in the skeleton, where it is produced by osteoclasts and hypertrophic chondrocytes.^{23–24}

Under physiological conditions, the activity of cysteine proteinases is regulated by their endogenous inhibitors, cystatins.^{25–26} Cystatin C, particularly, an extracellular inhibitor exhibiting a wide expression pattern, has been linked to arthritis,^{27–28} and its activity has been studied mainly in synovial tissue.^{18–29–31} In osteoarthritic cartilage with severe lesions, decreased inhibitory activity has been detected, which suggests that down regulation of cystatin C contributes to articular cartilage damage.¹⁸

This study was started to perform a systematic analysis on the expression of mRNAs for cathepsins B, H, K, L, and S, and for cystatin C in the transgenic Del1 mouse model for osteoarthritis. As the results indicated that up regulation of cathepsin K expression coincides with onset of articular cartilage damage, immunohistochemistry was used to localise cathepsin K and the endogenous inhibitor cystatin C in the knee joints during development of osteoarthritic lesions.

Abbreviations: MMPs, matrix metalloproteinases; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; SSC, saline-sodium citrate

MATERIALS AND METHODS

Experimental animals

This study was conducted on samples collected from 100 transgenic male Del1 mice and from their 100 non-transgenic male litter mates, which served as controls. The Del1 mice harbour six copies of an engineered 39 kb type II collagen transgene with a deletion mutation of exon 7 and intron 7.³² All mice shared the same C57bl×DBA background. The litters were genotyped by polymerase chain reaction amplification of tail genomic DNA using two oligonucleotide primers flanking the deletion as described earlier.³² Transgenic and non-transgenic siblings from each litter were housed in the same cages and were allowed to move freely about the cages. The animals were killed at birth and at the ages of 1, 2, 4, 6, 9, and 15 months. The samples for total RNA and protein extractions were taken from one hind limb, consisting of proximal tibial and distal femoral ends, with articular cartilage and subchondral bone; they were cut at the level of the growth plates, and thus also contained patella, menisci, ligaments, and joint capsule. The other hind limb was cut into serial histological sections, which were used for grading of osteoarthritic changes as described earlier^{9, 33, 34} and for immunohistochemistry and morphometric analysis. The study protocol was approved by the institutional committee for animal welfare.

Northern analysis

The levels of specific mRNAs were determined by northern hybridisation. Samples for total RNA extractions were pulverised under liquid nitrogen, homogenised into 4 M guanidine isothiocyanate, and subjected to centrifugation through a caesium chloride density gradient.³⁵ Aliquots (10 µg) of total RNA were denatured with formaldehyde, electrophoresed on 1% (w/v) agarose gels, and transferred to Pall Biotryne nylon membranes (Pall Europe Ltd, Portsmouth, UK). Hybridisations were performed using a standard protocol as suggested by the supplier. The membranes were hybridised with the following complementary DNA (cDNA) probes: pMCatB-1, pMCatH-1, pMCatL-1, and pMCatS-1 for mouse cathepsins B, H, L, and S, respectively,²⁴ pMCatK-1 for mouse cathepsin K,²³ Cst3 for mouse cystatin C,³⁶ and with a probe for 28S ribosomal RNA (rRNA).³⁷ For the preparation of probes, cDNA inserts were freed from the plasmid backbones with appropriate restriction enzymes and labelled with [α -³²P]dCTP using the random priming method (Roche, Mannheim, Germany). The filters were prehybridised and hybridised in 5×saline-sodium citrate (SSC) (0.75 M NaCl, 0.075 M Na₂SO₄, pH 7.0), 1×Denhardt's solution, 50% (v/v) formamide, 0.1% (w/v) sodium dodecyl sulphate (SDS), and 250 µg/ml denatured calf thymus DNA at 42°C overnight, and washed at high stringency (twice for 10 minutes in 2×SSC and 0.1% SDS at room temperature, and twice for 20 minutes in 0.1×SSC and 0.1% SDS at 55°C). Bound probes were detected with the Fuji Bas 5000 phosphorimager (Fuji, Tokyo, Japan), and the signals were quantified using Tina 2.0 software package (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany). All hybridisation results were normalised against the 28S rRNA signal to eliminate possible loading variation between different samples.

Immunohistochemistry

The tissue distribution of cathepsin K and cystatin C was studied using polyclonal antibodies raised against mouse cathepsin K³⁸ and mouse cystatin C,³⁹ respectively. The specificity of the affinity purified antibodies against mouse proteins in knee joint homogenates was confirmed by western blotting (data not shown). Immunohistochemistry was performed using the biotin-streptavidin complex method

(Histostain-Plus kit, Zymed, South San Francisco, CA, USA). Dissected hind limbs were fixed overnight in fresh 4% (w/v) paraformaldehyde prepared in phosphate buffered saline (PBS). Samples were decalcified in 10% (w/v) EDTA in PBS (pH 7.4) for 5–20 days, dehydrated, and embedded in paraffin for sectioning in sagittal and frontal planes into 5 µm thickness through the entire sample using an LKB 2218 Histo-Range microtome (LKB-Producter AB, Bromma, Sweden). Histological sections of the knee joints were deparaffinised and rehydrated in a series of descending ethanol concentrations, and digested for 1 hour with bovine testicular hyaluronidase (2000 U/ml) in PBS (pH 5). After rinses with PBS, an endogenous peroxidase activity was removed with hydrogen peroxide, and non-specific binding of primary antibodies was blocked. Appropriate dilutions of primary antibodies were applied in PBS containing 1% (w/v) bovine serum albumin, and the sections were incubated overnight at 4°C. After rinses with PBS, a biotin conjugated secondary antibody was applied and incubated for 10 minutes at room temperature. The slides were washed twice with PBS, and incubated with streptavidin conjugated horseradish peroxidase for 10 minutes. Colour was developed with diaminobenzidine, and the sections were counterstained with haematoxylin. The specificity of the reactions was controlled by omitting the primary antibody or by incubating sections with normal mouse serum.

Morphometric analysis

The immunohistological slides were captured as digital images using Leica's imaging workstation (Leica DMRBE Research Microscope, Leica DC300 F digital camera and Leica TWIN module), and the images were analysed with quantitative microscopy software, Leica QWin Professional (Leica Microsystems, Wetzlar, Germany). Analysis was performed in the frontal plane on uncalcified articular cartilage of tibial lateral condyle. From each condyle three separate sections in the vicinity of articular cartilage defects were evaluated. The results are reported as the number of positive chondrocytes as a fraction of the total number of chondrocytes.

Statistical analyses

All data are expressed as mean (SD). Statistical analyses were performed with statistical software, SPSS 9.0 for Windows (SPSS Inc, Chicago, IL, USA). Because normal distribution of the data could not be assumed for the small sample size, evaluation of the data was based on non-parametric Mann-Whitney U test. Values of $p < 0.05$ were considered significant.

RESULTS

Temporal expression patterns of cysteine cathepsins and cystatin C mRNAs in the knee joints of Del1 and control mice

Northern analysis showed different expression patterns for cathepsins B, H, K, L, and S, and cystatin C mRNAs in the knee joints during growth, aging, and osteoarthritic degeneration at the ages of 1, 2, 4, 6, 9, and 15 months. Because early cartilage degeneration has been seen in transgenic Del1 mice at 4 months of age and in control mice at the age of 9 months (table 1, web extra fig W1, available at <http://www.annrheumdis.com/supplemental>),⁵ we focused our attention on these times. When probes of equivalent specific activity and identical exposure times were used, the strongest hybridisation signal was detected for cathepsin K mRNA (web extra fig W2, available at <http://www.annrheumdis.com/supplemental>). Quantification of the transcript levels showed that statistically significant differences in cathepsin K mRNA levels coincided with the onset and early stages of

Figure 1 Results of northern analyses of mouse knee joints for cathepsins B, H, K, L, and S, and for cystatin C mRNA levels. Total RNAs isolated from the knee joints of transgenic Del1 mice and non-transgenic controls at different ages shown below the columns were analysed by northern hybridisation. After hybridisation with cDNA probes detecting specific mRNAs of various cysteine cathepsins and cystatin C, the hybridisation intensities were quantified by phosphor imaging and normalised as a constant amount of 28S rRNA determined by hybridisation. The results are shown as relative units, and the values present the mean and SD. Significance of the difference in mRNA levels between the control and Del1 mice is shown above the columns (Mann-Whitney U test; $n = 6$ at each time; * $p < 0.05$).

articular cartilage degeneration both in Del1 and control mice (fig 1). At the age of 4 months, cathepsin K mRNA levels were up regulated in Del1 mice. In control mice, cathepsin K mRNA exhibited a gradual increase in parallel with the progression of articular cartilage damage, and reached its highest level at the age of 9 months.

Different expression profiles were seen for the other cysteine cathepsins and for cystatin C (fig 1, web extra fig W2, available at <http://www.annrheumdis.com/supplemental>). Other statistically significant differences between Del1 and control mice were seen in cathepsin K mRNA levels at 1 month of age, in cathepsin S mRNA levels at 9 months of age, and in cathepsins B, L, and cystatin C mRNA levels at the age of 15 months. However, these changes cannot explain the development of early onset degenerative lesions in Del1 mice.

Immunohistochemical detection of cathepsin K in the knee joints of Del1 and control mice at the ages of 4 and 9 months

In control knee joints at 4 months of age, many of the chondrocytes throughout uncalcified articular cartilage exhibited a pericellular and intracellular staining for cathepsin K (fig 2A). In the knee joints of Del1 mice, more intense staining of uncalcified cartilage chondrocytes with cathepsin K antibodies was seen, together with mainly territorial staining of the cartilaginous matrix in the calcified cartilage (fig 2B). Quantitative morphometric analysis confirmed that the number of chondrocytes containing cathepsin K was increased in uncalcified cartilage of Del1 mice (table 1).

In control mice at the age of 9 months, mild cartilage degeneration was seen in the weightbearing areas of knee cartilage, and a large number of cathepsin K positive chondrocytes were concentrated in these areas as well as in the menisci (fig 2D). In Del1 mice, weightbearing articular surfaces were severely eroded. Cathepsin K staining was seen on the eroded surface and in chondrocyte clusters of the remaining articular cartilage in non-weightbearing areas as well as in the menisci (fig 2E). At this time, weak cathepsin K staining of the calcified cartilage matrix was seen also in control animals.

In the joint compartment used for RNA extraction, cathepsin K was also detected in osteoclasts of subchondral bone and in the matrix of epiphyseal growth plates as shown in figs 2F and G for control and Del1 mice, respectively, and in synovial tissue, as described below. There was no immunostaining when primary antibodies were omitted or replaced by normal mouse serum (fig 2C).

Table 1 Quantification of cathepsin K expression during early cartilage degeneration.

	Control	Del1
Cartilage erosion grade	0.37 (0.49)†, $n = 30$ ‡	1.82 (0.80), $n = 22$ ***
Relative cathepsin K mRNA levels	0.16 (0.08), $n = 6$	0.35 (0.06), $n = 6$ *
Relative number of chondrocytes expressing cathepsin K	0.34 (0.06), $n = 6$	0.46 (0.04), $n = 6$ **

Significance of the results was analysed by non-parametric Mann-Whitney U test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

†Results are shown as mean (SD) at the age of 4 months; ‡n, denotes the number of samples used.

Figure 2 Immunohistochemical detection of cathepsin K in the mouse knee joints at 4 and 9 months of age. Tissue distribution is shown in sagittal sections of knee joints obtained from control (A, D, F) and Del1 mice (B, C, E, G). Immunostaining for cathepsin K at the age of 4 (A, B) and 9 (D, E, F, G) months is identified as a brown precipitate. There was no staining when primary antibodies were omitted or replaced by normal mouse serum (C). The bar in panel A corresponds to 25 μ m for panels A–E and the bar in panel F to 150 μ m for panels F and G. Paraffin sections: biotin-streptavidin complex with horseradish peroxidase label, and diaminobenzidine detection: counterstaining with haematoxylin.

Immunohistochemical detection of cystatin C in the knee joints of Del1 and control mice at the ages of 4 and 9 months

The localisation of cystatin C in the articular cartilage did not show systematic differences between Del1 and control knee joints. At 4 months of age in both genotypes, intracellular and pericellular staining of chondrocytes for cystatin C was seen in the calcified cartilage (figs 3A and B). At the age of 9 months, cystatin C staining remained predominantly in and around chondrocytes located in calcified zone. The staining was more intense in control animals (fig 3D) than in Del1 mice (fig 3E).

In the joint compartment used for RNA extraction, cystatin C was also present in menisci and growth plates as shown in figs 3F and G for control and Del1 mice, respectively, and in synovial tissue, as described below. There was no immunostaining when primary antibodies were omitted or replaced by normal mouse serum (fig 3C).

Localisation of cathepsin K and cystatin C in the synovial tissue

Both in control and Del1 mice, a fraction of cells located in the synovium was also found to stain positive for cathepsin K, as shown for 4 month old mice (web extra figs W3A and B, available at <http://www.annrheumdis.com/supplemental>). As the synovial membrane was thickened in Del1 mice, the number of cathepsin K positive cells was greater than in control mice. However, a majority of synovial lining cells remained negative for cathepsin K in both genotypes. Diffuse staining of synovial membrane for cystatin C was seen in control and Del1 mice (web extra figs W3D and E, available at <http://www.annrheumdis.com/supplemental>). The situation remained similar at 9 months of age. In the synovial tissue of Del1 and control mice, there was no immunostaining for cathepsin K or cystatin C when primary antibodies were omitted or replaced by normal mouse serum.

DISCUSSION

The most interesting finding of this study was the increased expression level of cathepsin K mRNA in the knee joints during early cartilage degeneration. In transgenic Del1 mice this occurred at 4 months of age, and in non-transgenic controls at the age of 9 months. As the total RNA analysed in this study included RNA from all joint structures—for example, cartilage, bone, synovium, and ligaments, immunohistochemistry was used to localise cathepsin K at cell and tissue level. In addition to intense immunostaining of matrix in calcified articular cartilage and epiphyseal growth plates, an increased number of cathepsin K positive cells were detected in uncalcified articular cartilage, particularly in the vicinity of articular cartilage defects in early osteoarthritic lesions (table 1). This and the staining of defective cartilage surface and synovial tissue with cathepsin K antibodies suggest that cathepsin K has a role in the pathogenesis of osteoarthritis in the Del1 mouse model. Whether the role is associated with degradation of interterritorial matrix and/or with reorganisation of pericellular and territorial matrix during alterations in chondrocyte phenotype remains to be determined.

The present observations are largely in agreement with those of Kontinen and coworkers, who observed increased expression of cathepsin K in human osteoarthritic articular cartilage.²² A relationship was found between the mRNA levels and the severity of osteoarthritis. Furthermore, increased cathepsin K levels have been found in synovial tissue of joints affected with osteoarthritis or rheumatoid arthritis,^{14 20 21 40} particularly at sites of cartilage and bone degradation.

Additional proof for a potential role of cathepsin K in the pathogenesis of osteoarthritis comes from pH measurements on degenerating articular cartilage, which have demonstrated pH values between 6.2 and 5.5 on affected cartilage surfaces.²² This indirectly suggests that enzymes active at

Figure 3 Immunohistochemical detection of cystatin C in the mouse knee joints at 4 and 9 months of age. Tissue distribution is shown in sagittal sections of knee joints obtained from control (A, C, D, F) and Del1 mice (B, E, G). Immunostaining for cystatin C at the age of 4 (A, B) and 9 (D, E, F, G) months is identified as a brown precipitate. There was no staining when primary antibodies were omitted or replaced by normal mouse serum (C). The bar in panel A corresponds to 25 µm for panels A–E and the bar in panel F to 150 µm for panels F and G. Paraffin sections: biotin-streptavidin complex with horseradish peroxidase label, and diaminobenzidine detection; counterstaining with haematoxylin.

acidic pH, such as cathepsin K, are more likely to participate in cartilage degradation than those active at neutral or even at slightly alkaline pH, such as most MMPs.⁴¹ The ability of cathepsin K to degrade major components of cartilage, such as type II collagen and aggrecan, has been established.^{13–14} Recently, it has been shown that the collagenolytic activity of cathepsin K is enhanced by chondroitin sulphate, particularly by chondroitin-4-sulphate molecules, which oligomerise with cathepsin K molecules.^{42–43} Because the ratio of chondroitin-6-sulphate and chondroitin-4-sulphate in cartilage varies significantly during skeletal growth and aging,^{44–45} this has been suggested to participate in the regulation of matrix degradation.⁴²

In earlier studies, it has been suggested that other cysteine cathepsins, particularly cathepsin B, participate in cartilage degeneration in osteoarthritis.^{16–18, 29–46–50} At the onset of cartilage degeneration, the expression of cathepsin B has been shown to increase, and later on to decline as osteoarthritis advances to more degenerative stages.^{18–46–48} More recently, it has been suggested that alternative splicing of cathepsin B transcripts contributes to this activation.⁵⁰ In the Del1 mouse model, no evidence was found for the up regulation of cathepsin B, H, L, or S expression during the development of degenerative lesions of articular cartilage. However, in control animals the mRNA levels for cathepsins L and S exhibited similar changes to those of cathepsin K at the age of 9 and 15 months. Thus we cannot completely rule out their role in osteoarthritic cartilage degeneration.

Having discovered the association of increased cathepsin K expression with early stages of osteoarthritis in Del1 mice, we turned to the endogenous inhibitor of cysteine cathepsins, cystatin C, which has also been linked to arthritis.^{18–29–31} Although a gradual age dependent increase was seen in cystatin C mRNA levels, no compensatory increases were observed in these levels at the times when cathepsin K mRNA

levels were increased in Del1 knee joints. Furthermore, immunolocalisation showed that cystatin C was predominantly expressed by chondrocytes in calcified cartilage, and weakly by cells in synovial tissue, menisci, and growth plates, but not by articular chondrocytes, which expressed increased levels of cathepsin K. Insufficient cystatin C expression, particularly in uncalcified cartilage and on cartilage surface, may contribute to articular cartilage damage in the Del1 mouse model.

The calcified articular cartilage below the tidemark is becoming a site of particular interest. We have earlier reported on increased MMP-13 expression in this area and in subchondral bone during the onset of articular cartilage degeneration.⁶ In the present study cathepsin K was localised in interterritorial matrix during early cartilage degeneration, and cystatin C exhibited intracellular and pericellular staining during aging. Moreover, the expression of both cathepsin K and cystatin C declined as the osteoarthritis advanced to more degenerative stages. The possible association of these observations with the deep horizontal tears seen in articular cartilage of Del1 mice and with remodelling of subchondral bone in human osteoarthritis⁵¹ remains to be determined.

In summary, the present data suggests that cathepsin K may have a role in the pathogenesis of osteoarthritis. In mice, it is the most abundant cysteine proteinase in the knee joint during growth and aging. During the early stages of articular cartilage degeneration, the expression of cathepsin K mRNA was up regulated both in the transgenic Del1 and in aging control mice. Its localisation in and around uncalcified cartilage chondrocytes as well as in the matrix of calcified cartilage also supports this theory. However, at present we cannot rule out the possibility that physical damage to articular cartilage triggers up regulation of cathepsin K in adjacent areas and in synovial tissue in order to remove the resultant

debris. Whichever the case, pharmaceutical inhibition of excessive cathepsin K activity might help to prevent or slow down the progression of osteoarthritic cartilage degeneration.

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ECHO

Genetic susceptibility to systemic lupus erythematosus in Finnish families

Please visit the *Annals of the Rheumatic Diseases* website (www.annrheumdis.com) for a link to the full text of this article.

The first nationwide study in Finland to map genetic factors conferring susceptibility to systemic lupus erythematosus (SLE) has been completed. Suggestive linkage was observed in three genetic regions: 5p, 6q25-q27 and 14q21-q23. A further region, chromosome 6p (locus for human leucocyte antigen (*HLA*)) was also suggestive of linkage after a marker gap was filled.

A total of 17 genes have already been linked significantly to SLE using model based and non-parametric approaches. This study adds to this evidence; two of the regions, *HLA* region and 14q21-q23 have previously been reported, while the remaining two, 5p and 6q25-q27, were novel regions.

As the model of inheritance of SLE is not known, linkage analysis with non-parametric analysis was used. In all four regions identified, non-parametric linkage scores increased as information provided by additional markers increased, but none reached the threshold for significant linkage.

The extensive hospital registration system in Finland meant that approximately 85% of all patients with SLE who needed hospital based treatment were identified for this study. Seventy three patients and 96 healthy relatives from 35 families were investigated. A further study is now planned to identify common ancestral chromosomes among a larger cohort of patients with SLE and controls by high resolution mapping.

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