Cathepsin B in osteoarthritis: zonal variation of enzyme activity in human femoral head cartilage

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Abstract

Objectives—To determine the quantitative topographical distribution of cathepsin B in human femoral head cartilage by measuring the zonal variation of enzyme activity in specimens taken from various anatomical regions of normal and osteoarthritic (OA) tissues, and to correlate this parameter with the severity of the OA lesions.

Methods—OA articular cartilage was obtained at surgery for total hip replacement and control cartilage obtained at post-mortem. Cylinders of full thickness cartilage with underlying bone were retrieved with a biopsy trephine. Sections of cartilage were produced by cryocutting the tissue as slices parallel to the articular surface and assayed for cathepsin B with a specific, highly sensitive fluorogenic substrate. The severity of the OA lesions was graded according to the histopathological-histochemical method of Mankin.

Results—Zonal cathepsin B activity of normal cartilage was uniform and low in all regions of the femoral head. In apparently intact OA cartilage and in severely degraded tissue the zonal distribution and the amounts of enzyme were similar to control values. At sites with active disease, cathepsin B activity was much greater than in controls and its irregular zonal distribution correlated with tissue degeneration, hypercellularity, or cloning of chondrocytes as determined histochemically. Particularly high enzyme levels were observed at sites with regenerating cartilage, where some zonal peaks attained 20-fold activity with respect to controls.

Conclusion—Cathepsin B may play a role in sustaining the chronicity of OA, not as an initiator, but rather as a perpetuator of the disease and as an antagonist of regeneration.

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Chondrocyte mediated breakdown of cartilage has classically been studied in the context of cell stimulation by cytokines.^{1,2} Matrix degradation has been attributed to metalloproteinases stimulated by cytokines from synovium,³ monocyte-macrophage lineage cells,⁴ or polymorphonuclear leucocytes,⁵ or of autocrine origin.⁶ A rich literature and modern trends in the pathophysiology of articular cartilage have been comprehensively

reviewed.^{7,8} Enzymes other than metalloproteinases have received less attention, but the discovery that endogenous hydrolases are responsible for cartilage autodegradation^{9,10} stimulated research on the possible pathological significance of this phenomenon.^{11,12} Cathepsin B and cathepsin D are able to degrade both collagen and aggrecan in vitro,^{13–15} but cathepsin D, although present at extracellular sites in rheumatoid synovium,¹⁶ is not responsible for the resorption of cartilage in organ culture.¹⁷ Despite higher levels of cathepsin D in osteoarthritis (OA) compared with normal cartilage,^{18,19} its action is restricted to intracellular catabolism of matrix macromolecules within chondrocytes.²⁰

Increased concentrations of cathepsin B have been demonstrated in rheumatoid synovium^{16,21,22} and synovial fluid,^{21,23–25} while much smaller amounts were found in OA synovial fluid.^{24,25} Increased concentrations of cathepsin B in human OA cartilage^{18,26,27} were accompanied by a decrease in cysteine endopeptidase inhibiting activity in severely damaged tissue.²⁷ Chondrocytes can be stimulated to increase dramatically the synthesis, storage, and secretion of cathepsin B through modulation of their differentiated phenotype to a more primitive one.²⁸ This property is reversible: cathepsin B levels revert to normal after the phenotype is modulated back to its original state.^{28,29} After stimulation with interleukin-1 β , cathepsin B biosynthesis and storage are slightly increased, while secretion is not.³⁰

We describe below zonal variations of cathepsin B activity in normal and OA human articular cartilage and suggest that this enzyme may play a pathological role by sustaining and perpetuating the OA process.

Materials and methods

CARTILAGE

Femoral heads were obtained from patients who underwent total hip replacement for both primary and secondary OA. After resection, the specimens were immediately immersed in sterile Hank's balanced salt solution supplemented with penicillin 50 IU/ml and streptomycin 50 μ g/ml, and processed within two hours. Control femoral heads were obtained at postmortem, usually four to five hours, but never in excess of eight hours, after death, immediately placed in Hank's balanced salt solution with penicillin-streptomycin (as above) and amphotericin B 2.5 μ g/ml, and processed within 30 minutes. Only femoral heads of individuals with no history of joint

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Figure 1 Superior extremity of a right human femur showing the four regions from which articular cartilage was sampled. A: Anterior view of an osteoarthritic head showing bone eburnation of the superior region (dashed area), 'shelving' cartilage (dotted area beneath the dashed area) and marginal osteophytes. P: Posterior view of a normal head.

disease were considered as controls. Occasionally, femoral heads obtained at post-mortem presented clear signs of OA. The diagnosis was confirmed by an experienced pathologist and, accordingly, the specimens were attributed to the OA group. (To avoid confusion, normal cartilage specimens were identified by the prefix 'control' before their number, while OA specimens were assigned a three digit number.) The anatomical position (fig 1) from which cartilage was obtained was documented by photographing the femoral heads from all sides before and after sampling, and designated anterior, posterior, superior, or inferior.³¹ Usually the superior region of OA femoral heads was levigated, with exposure of an eburnated bone surface, and the inferior region, mainly towards the margin, was covered by osteophytes.

The cartilage type¹⁸ was determined for each specimen: normal articular cartilage from relatively young subjects without history of joint diseases (type I); slightly soft or fibrillated cartilage from normal femoral heads obtained from elderly subjects and different from OA cartilage (type III); residual (degenerating) cartilage obtained from OA femoral heads after total hip replacement (type IV, typically having a yellow colour); and nascent (regenerating) cartilage from OA femoral heads (type V, with a white colour). Often, type V cartilage was clearly found growing above and in close contact with residual (type IV) cartilage; these specimens were designated type IV + V. Type II cartilage (from fractures of the neck of the femur) was not available to us and osteophytic cartilage (type VI) could not be investigated with the technique used in this study because the cartilaginous layer was very thin. Cylinders of full thickness cartilage and underlying bone with a diameter of 4.0 mm were obtained using a hollow biopsy trephine (Straumann Ltd, Waldenburg, Switzerland). With this method the cartilage remained intact, and local tissue damaging compression forces could be avoided. The samples were immediately immersed in physiological saline, thoroughly rinsed, trimmed to leave 5–7 mm of bone underneath the cartilage, and blotted dry. The cylindrical samples were then rapidly attached

at the bone side to a cork disk, using a small drop of Tissue-Tek® OCT compound (Miles, IN, USA), frozen in a quick freeze carbon dioxide chamber and stored at -70°C . The specimens were allowed to reach the cabinet temperature of -25°C before being cryosectioned as 20 μm thick slices parallel to the articular surface. This is a modification of a previously published method.²⁶ Five consecutive sections were collected in conical, 1 ml Eppendorfer vials until the subchondral bone was reached, and left at -25°C . Five sections obtained as described have a volume of 1.26 mm^3 and an area of 126.9 mm^2 , whereas a single, 100 μm thick section would have the same volume, but an area of only 26.4 mm^2 . The presence of the subchondral bone was easily perceived by the resistance offered to the knife and the colour of the sections, so that one or two sections, at the most, with mixed bone and calcified cartilage tissue were eventually present in the deepest sample analysed in each cylinder. Cartilage slices included superficial, mid and deep layers—more precisely, zones I–V.³²

HISTOLOGY

Measurement of cathepsin B in a cartilage plug did not allow simultaneous histological analysis within the same specimen. Nevertheless, the severity of the OA lesions was estimated at sites immediately adjacent to the sampled cylinders according to the histological-histochemical grading method of Mankin *et al.*³³ To avoid artificial loss of proteoglycans during preparation for paraffin embedding, cartilage samples (either cylinders obtained as described above, or slices with underlying bone resected from the area between two adjacent holes produced for cylinder extraction) were snap frozen and cryosectioned at a thickness of 14–18 μm . After 15 minutes fixation with 4% formaldehyde in 0.1 mol/l sodium phosphate buffer, pH 7.4, sections were rinsed with this buffer, brought to 50% and then to 80% ethanol (five minutes each), and sequentially stained with Weigert's iron chloride haematoxylin, fast green FCF, and safranin O before being permanently mounted.³⁴ Under these conditions glycosaminoglycans were stained by safranin O in the orthochromatic form³⁵ and their loss was made visible by the presence of the green counterstain. The only disadvantage of this method is that the subchondral bone may be damaged in some sections and the tidemark integrity could not be evaluated.

CATHEPSIN B ASSAY

The frozen cartilage sections were thawed rapidly to disrupt cells by suspending them in 0.50 ml of 0.10 mmol/l Z-Arg-Arg-NMec (abbreviations of amino acids and substituents according to the Joint Commission on Biochemical Nomenclature;³⁶ -NMec = 7-(4-methyl)coumarylamide) (Bachem Ltd, Bubendorf, Switzerland) in 0.1 mol/l Na^+/K^+ phosphate buffer containing 2 mmol/l EDTA and 2 mmol/l dithiothreitol (DTT), pH 6.0.

After incubation at 37°C under gentle shaking for exactly four hours the reaction was stopped with 10 µl of 0.1 mol/l iodoacetamide in water. The fluorescence of liberated 7-amino-4-methylcoumarin was read in an Aminco SPF-500 fluorimeter operating in the ratio mode with excitation and emission wavelengths set at 383 and 455 nm, respectively. The recorder scale was calibrated with 7-amino-4-methylcoumarin solutions of known concentration determined using an absorption coefficient of 16 000 mol⁻¹ l cm⁻¹ at 342 nm for this substance. The described assay was optimised in preliminary experiments using cartilage specimens with high and low cathepsin B activities, and using purified human spleen cathepsin B.³⁷ In order for the reaction to be linear with time and enzyme concentration, an excess of substrate was ensured.

Z-Arg-Arg-NMec is a substrate with high specificity for cathepsin B; it does not react with cathepsin H³⁸ and has only trace activity with cathepsin L.³⁹ Possible interference by these cysteine endopeptidases or other enzymes was excluded by control studies. For this purpose, four adjacent plugs of cartilage were taken from a region of an OA femoral head with uniform thickness. Two plugs were used to determine the uniformity of the zonal variation of cathepsin B activity and two plugs were used to determine the effect of inhibitors included in the assay solution in alternate samples. The inhibitors used were 3,4-dichloroisocoumarin, L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E-64) (Sigma, St Louis, MO, USA), Z-Phe-Ala-CHN₂, Z-Phe-Phe-CHN₂, Pro-Phe-Har-CH₂Cl (a gift of Dr E Shaw, Basle, Switzerland), and leupeptin (Bachem Ltd).

One unit of cathepsin B activity was defined as the amount of enzyme that hydrolysed 1 nmole of substrate in four hours at pH 6.0 and 37°C. The activities shown in the figures are expressed as units released by a 'sample'—that is, by five consecutive sections of cartilage obtained as described above. The activities could not be normalised to the cellularity of the samples because the fluorimetric assay did not allow simultaneous determination of the DNA content.

STATISTICAL METHODS

Statistical comparisons were performed with a two tailed Student's *t* test, taking into account equality or inequality of variances as determined with Fisher's *F* test. Probability values (*p*) were explicitly calculated; *p* < 0.05 was considered significant.

Results

The method for measuring the zonal distribution of cathepsin B activity in articular cartilage was highly sensitive and could be performed by using just one section of tissue 4 mm in diameter and 20 µm thick. However, to save time and reduce costs (100 sections are obtained from a 2 mm deep plug of cartilage),

five consecutive sections were pooled for analysis. Actual concentrations of 7-amino-4-methylcoumarin released in the assay were in the range 0.2–80 µmol/l, thus allowing accurate readings with any commercial fluorimeter. This method allowed the topographical mapping of cathepsin B in the cartilage of a whole joint to show its quantitative distribution in the various zones.

Both thiol activation (by DTT) and the presence of EDTA were necessary to ensure measurement of cathepsin B activity. In the control studies for assay specificity, the activity was completely abolished by Pro-Phe-Har-CH₂Cl, a potent inhibitor of cathepsin B,⁴⁰ and by leupeptin. Good inhibition was obtained with Z-Phe-Ala-CHN₂, and E-64, whereas no inhibition was observed with Z-Phe-Phe-CHN₂ or with the general serine endopeptidase inhibitor 3,4-dichloroisocoumarin.⁴¹ These data (fig 2) confirmed the cysteine endopeptidase character of the measured enzyme and are highly characteristic of cathepsin B.³⁸

Cathepsin B activity in zones of normal cartilage (type I) never exceeded seven units/sample (fig 3). Activity as a function of the distance from the subchondral bone was quite regular, generally greater towards the articular surface (zone I) and lower in the mid zones (II and III). In the deeper zones (IV and V), catheptic activity was either the same as observed in the central zones, or slightly greater. Cell density in zones of articular cartilage is greater in the superficial layer than in the mid and deep zones, where it is quite uniform.⁴² Zonal variations of cathepsin B activity in normal cartilage can thus be attributed to cell heterogeneity, different metabolism, and different density. As already suggested,²⁶ greater activity in the superficial layer is a property of the fibrocyte type chondrocytes of this zone. Type III cartilage, obtained from elderly subjects (controls 6 and 8 in figure 3) was not fibrillated and was still considered to be 'normal' on the basis of macroscopic morphology, the only difference

Figure 2 Zonal variation of cysteine endopeptidase activity in two plugs (continuous and dotted lines) of OA femoral head cartilage with uniform thickness showing the effect of selected substances, added to alternate samples as shown, in inhibiting enzyme activity. Two other plugs were used to show the continuity of cathepsin B activity in the same area (not shown). A sharply decreased activity in a given sample indicates inhibition. DTT = Dithiothreitol.

Figure 3 Zonal variation of cathepsin B activity in normal (control) femoral head cartilage. A, P, S, and I indicate the anterior, posterior, superior, and inferior regions, respectively. R = right, L = left femoral head.

from young cartilage being a slightly softer consistency.

The zonal variation of cathepsin B activity in OA cartilages was less regular (figs 4–6). While some OA specimens had cathepsin B profiles similar to those of the controls, others had considerably greater activities both towards the surface (examples: fig 4, 011; fig 5, 019 and 023; fig 6, 015) and in the deep zones (examples: fig 4, 032 and 028; fig 5, 026; fig 6, 018). Large variations in the activity profiles were sometimes observed in samples from the same anatomical site, as shown in figure 6, where the axes of the two cartilage plugs from the posterior region of patient 029,

and those of the anterior region of patient 018, were about 9 mm apart from each other. In this study we examined cartilages from 20 OA femoral heads (54 samples) and from both the left and right femoral heads of nine controls (42 samples). (For reasons of space not all of the specimens analysed are shown in figures 3–6, but all the available data have been used in figure 7 and 8 for purposes of statistical analysis.) Although the primary indication for surgery of patient 011 was hip necrosis, the cartilage had typical OA lesions.

Statistical evaluation of zonal cathepsin B activity in cartilage (figs 3–6) poses a practical problem. While comparison of activity between

Figure 4 Zonal variation of cathepsin B activity in osteoarthritic femoral head cartilage from regions A (anterior) and P (posterior). A short diagnosis is given: Pr = primary idiopathic, Se = secondary, OA = osteoarthritis. R = right, L = left femoral head.

Figure 5 Zonal variation of cathepsin B activity in osteoarthritic femoral head cartilage from regions A (anterior), P (posterior), and I (inferior). Other abbreviations as in figure 4.

zones of diseased and control tissue sounds mathematically correct, this comparison would result in an anatomical nonsense. The 'zones' in articular cartilage are a descriptive concept, but there are no boundaries between them and their thickness varies within a joint and within individuals. Moreover, the original thickness of partly degraded OA cartilage, where some layers have been worn away, is unknown. Therefore, statistical comparison of cathepsin B activities in normal and OA cartilages was based on a normalised (fractional) activity of each specimen, namely the ratio of the area under the curves shown in figures 3–6 to the cartilage depth in mm.

The dependence of the normalised activities upon the anatomical site within the femoral head is shown in figure 7A, together with the cartilage type. In normal subjects cathepsin B activity was similar in the four regions of the femoral head: the mean values for the posterior, anterior and inferior regions were statistically indistinguishable from one another, while the superior region had significantly less activity than the others ($p = 0.0041$ compared with posterior). The posterior, anterior and inferior regions of OA cartilage had statistically indistinguishable mean values of cathepsin B activity when compared with one another. Comparison of the activities from femoral head

Figure 6 Zonal variation of cathepsin B activity in osteoarthritic femoral head cartilage from regions A (anterior), P (posterior), and I (inferior). Other abbreviations as in figure 4.

regions in OA with those in normal cartilage revealed significant differences between posterior ($p = 0.0040$) and anterior ($p = 0.0014$) sites. A p value of 0.0618 for the two-tailed comparison at the inferior regions became significant when subjected to a one tailed t test. The superior region of all OA femoral heads was eburnated, with too little residual 'shelving' cartilage to allow analysis for zonal variations of enzyme activity. The mean of all 42 normal (control) samples (2.8 (SD 0.8, SEM 0.13)) and the mean of all 54 OA samples (5.5 (SD 3.1, SEM 0.4)) were significantly different ($p = 9 \times 10^{-8}$).

The data of figure 7A are plotted as a function of age in figure 7B, in which the lack of correlation between cathepsin B activity and donor's age in the cartilage types I and III is evident. OA cartilages from relatively young subjects had the greatest activities and a large proportion of typical OA samples from elderly subjects had activities comparable to those of the controls. The slope of the curve, significantly different from zero, suggests an

Figure 7 Normalised cathepsin B activity in femoral head cartilage. A: All data available for normal and osteoarthritic specimens showing regional variation (P = posterior, A = anterior, S = superior, I = inferior). B: Data from A plotted as a function of the donor's age. The linear regression lines (solid) are surrounded by their respective 95% confidence interval envelopes, shown as dashed lines for the cartilage types IV, V and IV + V (OA group) and as dotted lines for cartilage types I and III (controls). Control group: correlation = 0.267, $t = 1.751$, $p = 0.0876$; OA group: correlation = -0.388, $t = -3.039$, $p = 0.0037$.

Figure 8 Data of figure 7 plotted according to cartilage type. The number of specimens in each group is shown within the boxes, bars indicate SD, and p values show the statistical significance of differences of the means with respect to type I cartilage.

inverse correlation between enzyme activity and age in the OA group.

Figure 8 shows the dependence of cathepsin B activity on cartilage type using all pooled data. With cartilage type I as the reference, the other types showed significantly higher activities, although the difference between the means of types I (2.7 (SD 0.8)) and III (3.5 (0.8)) was small.

The histological-histochemical grade of the OA lesions³³ was determined for all of the patients and controls represented in figures 3–6, in at least one cartilage specimen immediately adjacent to a plug analysed for cathepsin B activity (fig 9). Many more samples were analysed than are shown here; for all of them, structure, cellularity, and safranin O staining could be determined and these data were used for evaluation. For reasons explained in the Methods section, it was not always possible to judge tidemark integrity of the samples; figure 9 shows the samples

Figure 9 Dependence of cathepsin B activity upon the severity of the lesions in femoral head cartilage. The point for type I cartilage was averaged from six specimens while the others represent single observations. The Mankin histopathological score was independently determined by two observers and the mean value is shown (accounting for the occasional fractional values). The slope of the regression line (correlation = 0.283, $t = 1.319$, $p = 0.217$) surrounded by the 95% confidence interval envelope, is not significantly different from zero.

for which all four parameters could be unequivocally determined. The slope of the curve was statistically indistinguishable from zero and no correlation was found between cathepsin B activity and the Mankin score.

Discussion

Our results agree with and extend previous studies on cathepsin B in human articular cartilage that were performed with tissue pooled from femoral heads of different subjects^{18,26} or with cartilage pooled from various regions and zones of tibial plateaus and femoral condyles.²⁷ Pathological specimens obtained at surgery represent just one particular moment of a disease the origins of which are remote and which has a past history that can probably not be traced back in its particular pathogenetic traits. The very different cathepsin B activities observed in OA cartilages indicated great disease heterogeneity, not only between different patients, but also between regions and zones from the same femoral head, thus confirming the focal character of the OA process, which may show a wide spectrum of activity in closely juxtaposed anatomical positions.⁴³ We exploited this property for interpreting the possible pathogenetic role of cathepsin B in various phases of the disease. Each specimen of OA cartilage represented a case that could be understood and interpreted after a close examination of the medical history, and the macroscopic, enzymatic, and histological-histochemical properties. Thus the lack of correlation between cathepsin B activity and the Mankin score³³ is not surprising and makes sense when consideration is given to the individual contributions of the four parameters that make up Mankin's grading system. Although the present data do not demonstrate absolute facts, we observed some significant trends, as described below.

The mean cathepsin B activity in all pooled data of residual cartilage (type IV) was only slightly greater than that of normal (type I) cartilage because the activity of a large proportion of the type IV specimens was in the range of the controls, despite clear signs of OA. While this is an effect of data pooling, we stress the importance of individual evaluation of zonal cathepsin B distribution for identifying focal sites of high activity. Type IV cartilage represents the old, residual tissue that may show all possible stages of degradation, from an almost intact structure to complete disorganisation. Type IV samples with low grade destruction, almost normal cellularity, slightly impaired safranin O staining in the superficial or deeper zones, and undamaged tidemark, had low Mankin scores and low cathepsin B levels. This cartilage can be considered to represent a tissue in an initial phase of OA. Other type IV specimens showed clear signs of degeneration, hypercellularity or cloning, impaired safranin staining, tidemark destruction without duplication, and high cathepsin B activity. We consider this cartilage as representative of active OA in a destructive

phase, but not yet with a reparative reaction. The type IV cartilage specimens with clear signs of degeneration and low levels of cathepsin B were either hypocellular or hypercellular, but with many remnants of chondrons and cell clusters containing highly degenerated, atrophied, and possibly dead cells. The overall structure of these specimens was from irregular to completely disorganised, with severely reduced safranin O staining and the tidemark duplicated, triplicated, or damaged. This cartilage, representing a highly degenerated tissue with a past history of active disease, thus had high histological scores and low cathepsin B levels.

The greatest enzyme activities were found in type V cartilage and in those specimens having cartilage type V overgrowing type IV. These samples had zonal cathepsin B activities that were up to 20-fold greater than those found in corresponding zones of control cartilage. Histological analysis suggested that particularly high activity was present in zones where hyperactive chondrocytes were involved in the reparative processes by synthesising cartilage of a 'juvenile' type.²⁶ At these sites the chondrocytes were in clusters within a completely disorganised interterritorial matrix showing strong pericellular safranin O staining. These cartilage specimens covered a broad spectrum of cathepsin B activities that were always greater than controls, and a broad range of histological scores.

The distribution of enzyme activity in OA cartilages with various degrees of degeneration suggests a pathological role for cathepsin B. The relatively low activities in apparently intact and severely degraded OA cartilages, in contrast with high levels in tissues with active disease and at regenerating sites, suggest that cathepsin B may act as a perpetuator rather than an initiator of cartilage destruction in OA and may be at least partly responsible for unsuccessful tissue repair. This concept is supported by cytochemical and histochemical results discussed in the accompanying paper.⁴⁴

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