Human cathepsin K cleaves native type I and II collagens at the N-terminal end of the triple helix

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Cathepsin K (EC 3.4.22.38) is a recently described enzyme that has been shown to cleave type I collagen in its triple helix. The aim of this study was to determine if it also cleaves type II collagen in the triple helix and to identify the helical cleavage site(s) in types I and II collagens. Soluble human and bovine type II collagen, and rat type I collagen, were incubated with cathepsin K before the reaction was stopped with *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64). Analysis by SDS/PAGE of the collagen digests showed that optimal activity of cathepsin K against native type II collagen was between pH 5.0 and 5.5 and against denatured collagen between pH 4.0 and 7.0. The enzyme cleaved telopeptides as well as the $\alpha 1(\text{II})$ chains, generating multiple fragments in the range 90–120 kDa. The collagenolytic activity was not due to a contaminating metallo-

enzyme or serine proteinase as it was not inhibited by 1,10-phenanthroline, EDTA or 3,4-dichloroisocoumarin. Western blotting with anti-peptide antibodies to different regions of the $\alpha 1(\text{II})$ chain suggested that cathepsin K cleaved native $\alpha 1(\text{II})$ chains in the N-terminal region of the helical domain rather than at the well-defined collagenase cleavage site. This was confirmed by N-terminal sequencing of one of the fragments, revealing cleavage at a Gly-Lys bond, 58 residues from the N-terminus of the helical domain. By using a similar approach, cathepsin K was found to cleave native type I collagen close to the N-terminus of its triple helix. These results indicate that cathepsin K could have a role in the turnover of type II collagen, as well as type I collagen.

INTRODUCTION

Cartilage is a highly specialized connective tissue that is elaborated at sites where a semisolid architecture is required to provide shape and form, yet ensure flexibility, durability and deformability. Cartilage tissue consists of a relatively small number of cells and an abundant extracellular matrix. Type II collagen and aggrecan are the two major components of the extracellular matrix of cartilage. The collagen, which is present in a fibrillar form, provides tensile strength, whereas aggrecan is responsible for compressive stiffness of cartilage [1,2]. Degradation and loss from the matrix of either of these molecules contributes significantly to the destruction of articular cartilage and a loss of its function in patients with arthritic diseases. Type I collagen is far more widely distributed. It is the major collagen of skin, bone, tendon and ligaments. There is evidence of increased degradation of bone type I collagen in patients with osteoporosis, Paget's disease and metastatic bone disease [3].

The mechanism of degradation of fibrillar collagen has been the subject of considerable interest because in the native state it is resistant to attack by most proteolytic enzymes. Fibrillar collagen can be damaged by helical cleavage, resulting in denaturation, or by telopeptide cleavage, which can lead to the removal of cross-links and depolymerization of the fibrillar network [4]. It has been shown that the extracellular breakdown of fibrillar collagen helices at neutral pH is often accomplished by the action of at least three specific collagenases: interstitial collagenase (MMP-1; EC 3.4.24.7), neutrophil collagenase (MMP-8; EC 3.4.24.34) and collagenase-3 (MMP-13; EC 3.4.24.-) (where MMP stands for matrix metalloproteinase) [5–8]. They all initially cleave at a specific Gly-Leu/Ile bond to

generate characteristic 1/4 and 3/4 fragments that can then be degraded further by the collagenase itself [9] or by gelatinolytic enzymes such as gelatinase A (EC 3.4.24.24) [10] and B (EC 3.4.24.35) [11], neutrophil elastase (EC 3.4.21.37) [12] and plasmin (EC 3.4.21.7) [13] at neutral pH, and cathepsins B (EC 3.4.22.1) [14], S (EC 3.4.22.27) [15] and L (EC 3.4.22.15) [16] at acidic pH.

The identity of the proteinases involved in extracellular matrix destruction remains to be definitively established. In synovial joints, proteolytic activity might be derived either from extracellular cells such as infiltrating polymorphonuclear leucocytes and synoviocytes, or from chondrocytes that have been activated by inflammatory cytokines such as interleukin 1 [17]. In bone, both osteoblasts and osteoclasts are capable of synthesizing MMPs and cysteine proteinases [18,19]. However, in soft connective tissues an intracellular route of type I collagen breakdown also exists, which employs both metalloproteinases and cysteine proteinases [3].

Although MMPs clearly have an important role in joint destruction, there are several lines of evidence to suggest that certain cysteine proteinases such as cathepsins B and L can contribute to the destruction of extracellular matrix [19–26].

Cathepsin K is a recently identified enzyme showing homology with members of the papain family of cysteine proteases (Family C1), which includes cathepsins S, L and B [27,28]. The mRNA for cathepsin K has been detected in a variety of tissues including heart, placenta, lung, skeletal muscle and small intestine [27]. However, it is thought to be particularly highly expressed in osteoclasts [27,29] and ovaries [27]. Deficiency of cathepsin K causes pycnodysostosis, an autosomal recessive osteochondrodysplasia characterized by osteosclerosis and short stature [30]. More recently, mouse hypertrophic chondrocytes [31] and

Abbreviations used: 3,4-DCI, 3,4-dichloroisocoumarin; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; MMP, matrix metalloproteinase; NHMec, aminomethyl coumarylamide; rhCK, recombinant human cathepsin K; Z, benzyloxycarbonyl.

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synovial tissue fibroblasts from osteoarthritis and rheumatoid arthritis patients were shown to express cathepsin K mRNA [32,33].

Cathepsin K has potent collagenolytic activity against type I collagen even at pH values as high as 6.5 [15]. So far there have been no published reports of cathepsin K cleavage of type II collagen. The aim of this study was to determine whether cathepsin K cleaves native type II collagen in its triple helix and to identify the cleavage sites in collagen types I and II.

MATERIALS AND METHODS

Materials

Recombinant human cathepsin K (rhCK) was produced as described previously [15]. Native bovine type II collagen, 3,4-dichloroisocoumarin (3,4-DCI), tosylphenylalanylchloromethane (Tos-Phe-CH₂Cl)-treated trypsin (EC 3.2.21.4), hyaluronidase (EC 3.2.1.35) from bovine testes, *Clostridium histolyticum* collagenase (EC 3.4.24.3), *trans*-epoxysuccinyl-L-leucyl-amido-(4-guanidino)butane (E-64), EDTA, dithiothreitol and disodium *p*-nitrophenyl phosphate were all purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Z-F-R-NHMec (in which Z stands for benzyloxycarbonyl and NHMec for aminomethyl coumarylamide) was purchased from Bachem Feinchemikalien AG (Bulbendorf, Switzerland). Alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin was purchased from Southern Biotechnology (Birmingham, AL, U.S.A.). Coomassie Brilliant Blue R-250, nitrocellulose membranes, and alkaline phosphatase substrate solution kit were all purchased from Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K.). All other reagents were of analytical grade.

Preparation of collagens

Purified bovine type II collagen was a gift from Dr. Philippe Dekeyser (University of Gent, Gent, Belgium). Human type II collagen was prepared by differential salt precipitation as described previously [34,35]. Native rat type I collagen was isolated from tail tendons as described previously [36].

Active-site titration

The concentration of active rhCK was determined essentially as described previously [37], with Z-F-R-NHMec as substrate.

Cleavage of soluble collagen by rhCK

Triple helical collagen was dissolved in 0.2 M acetic acid then dialysed at 4 °C against 100 mM sodium acetate buffer (pH 4.0, 5.0 and 5.5), 100 mM potassium phosphate buffer (pH 6.0 and 6.5) or 100 mM Tris/HCl buffer (pH 7.0). All buffers contained

2 mM dithiothreitol and 2 mM EDTA. Reaction mixtures contained the indicated concentrations of collagen and rhCK and the reactions proceeded at 25 °C for 6 h unless otherwise stated. E-64 was used to stop the enzyme reactions at a final concentration of 1 mM. Tos-Phe-CH₂Cl-treated trypsin was dissolved in the same buffer(s) and used in control reactions, which were stopped by the addition of 3,4-DCI (1 mM). For comparative purposes, MMP-13-cleaved type II collagen (a gift from Dr. Philippe Dekeyser) was used. Bovine type II collagen dissolved in 100 mM Tris/HCl, pH 7.6, containing 10 mM CaCl₂, was incubated with active MMP-13 for 40 h at 30 °C, after which EDTA was added to a final concentration of 20 mM. The final molar ratio of collagenase to collagen was 1:50.

Antipeptide antibodies for the detection of denatured type II collagen

Mouse monoclonal antibody COL2-3/4m and rabbit anti-peptide antibodies AH9L2 and AH12L3 were prepared and characterized as described previously [2], and details available from the authors on request. COL2-3/4m reacts with a defined epitope in the CB11 region of the $\alpha 1(\text{II})$ chain, AH9L2 reacts with a helical epitope nine residues away from the C-terminal telopeptide, and AH12L3 reacts with a helical epitope 15 residues away from the N-terminal telopeptide of the bovine $\alpha 1(\text{II})$ chain. The relative locations of these epitopes are shown in Figure 1. All three antibodies were purified on a protein G column before use (details available from the authors on request).

SDS/PAGE and immunoblotting

SDS/PAGE was performed by the method of Laemmli [38]. Collagen fragments were analysed on 6 % (w/v) polyacrylamide gels (except where indicated otherwise) under reducing conditions. The electrophoresed samples were transferred to nitrocellulose membranes and blocked for 1 h at ambient temperature with PBS/3 % (w/v) BSA before immunoblotting. Blocked membranes were incubated for 1 h at ambient temperature with antibodies or control sera diluted in PBS/3 % (w/v) BSA. The rabbit anti-peptide antibodies, AH12L3 and AH9L2, were diluted 1:10000 and 1:1000, and antibody COL2-3/4m was diluted 1:2000. After three washes with PBS/Tween, the membranes were incubated for 30 min at room temperature with alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse second antibody, as appropriate, each diluted to 1:1000 with PBS/3 % BSA. The membranes were washed three times with PBS/Tween and once with distilled water. Alkaline phosphatase substrate solution was prepared from the Bio-Rad commercial kit. It was incubated with the membranes at ambient temperature until colour had developed, then the membranes were rinsed with distilled water.

Figure 1 Relative locations of epitopes AH12L3, CB11B and AH9L2 on the $\alpha 1(\text{II})$ chain

The approximate locations of epitopes AH12L3, CB11B (recognized by antibody COL2-3/4m) and AH9L2 are shown in relation to the collagenase cleavage site. Collagenases remove the C-terminal end of the molecule, resulting in a 3/4 fragment containing epitopes AH12L3 and CB11B and a 1/4 fragment containing epitope AH9L2.

Protein sequencing

Bovine type II or rat type I collagens (20 μ M) were each incubated with 20 nM rhCK in 100 mM sodium acetate buffer, pH 5.0, containing 2 mM dithiothreitol and 2 mM EDTA, for 16 h at 25 °C. The digests were electrophoresed and electroblotted on PVDF membranes as previously described by Dunbar and Wilson [39]. N-terminal sequence analysis was performed by Bryan Dunbar in the Protein Sequencing Unit of the University of Aberdeen (Aberdeen, U.K.).

RESULTS

Degradation of soluble type II collagen by rhCK

The collagenolytic activity of rhCK against type II collagen was determined over the pH range 4.0–7.0 at 25 °C. Bovine type II collagen (20 μ M) was incubated with 100 nM purified rhCK for 6 h and the reaction was stopped by adding 1 mM E-64. rhCK extensively degraded type II collagen between pH 5.0 and 6.0, whereas the degradation at pH 6.5 and 4.0 was less prominent; at pH 7.0 it was undetectable (Figure 2A). Under optimal

Figure 2 Cleavage of type II collagen by rhCK

Samples were analysed by SDS/PAGE (6% gel) as described in the Materials and methods section. **(A)** Native soluble bovine type II collagen (20 μ M) was digested at 25 °C by rhCK (100 nM) for 6 h at a range of pH values. Lanes 1 and 2, collagen incubated without rhCK at pH 4.0 and 7.0 respectively; lanes 3–8, collagen after incubation with rhCK at pH 4.0, 5.0, 5.5, 6.0, 6.5 and 7.0 respectively. The four major fragments are indicated as F1–F4. **(B)** Soluble bovine type II collagen was denatured by heating for 10 min at 80 °C. The resulting gelatin (20 μ M) was incubated at 25 °C with rhCK (100 nM) for 2 min at a range of pH values. Lane 1, denatured collagen incubated without rhCK; lanes 2–7, the results of incubation with rhCK at pH 4.0, 5.0, 5.5, 6.0, 6.5 and 7.0 respectively. In both panels the position of molecular mass standards are indicated at the left.

Figure 3 Time course and effect of enzyme concentration for type II collagen cleavage by rhCK

(A) Native bovine type II collagen (20 μ M) was digested with 200 nM rhCK at 25 °C and pH 5.0. The reaction was stopped by the addition of 1 mM E-64 after various periods and the samples were analysed by SDS/PAGE as described in the Materials and methods section. **(B)** Native bovine type II collagen (20 μ M) was digested with various concentrations of rhCK at 25 °C and pH 5.0 for 16 h. The reactions were stopped with 1 mM E-64 and the samples were analysed by SDS/PAGE (6% gel). In both panels the positions of molecular mass standards are indicated at the left.

conditions (pH 5.0; see lane 4 of Figure 2A), four major fragments were generated (F1–F4) with molecular masses of 90–120 kDa, similar to those generated by rhCK from type I collagen (see below and [15]), indicating cleavage by rhCK at helical site(s). Further, rhCK modified the γ and β components. There was a loss of intensity of these bands and a decrease in their molecular mass (Figure 2A). An identical pattern of degradation was obtained when rhCK was incubated with human type II collagen (results not shown). When collagen was incubated with 100 nM trypsin, no degradation of type II collagen was observed (results not shown), indicating that the collagen preparation used here was not denatured.

rhCK (100 nM) had potent gelatinolytic activity against 20 μ M heat-denatured collagen. The collagen was totally degraded within 2 min over a pH range of 4.0–7.0 (Figure 2B).

Although the cysteine proteinase inactivator E-64, at a concentration of 1 mM, fully inhibited the degradation of type II collagen by rhCK (results not shown), we still considered it particularly important to rule out any effect of contaminating

MMPs or other metalloenzymes. To this end, the rhCK preparation was preincubated with 1 mM 1,10-phenanthroline or 20 mM EDTA. Neither of these inhibitors had any detectable effect on the cleavage of type II collagen by rhCK. Similarly, the serine proteinase inhibitor 3,4-DCI (1 mM) had no effect (results not shown).

When 20 μ M bovine type II collagen was incubated with rhCK (200 nM) for various periods (1–7 h), fragments F1–F4 appeared concurrently after 1 h and all of them had an increased intensity of staining with Coomassie Brilliant Blue with time (Figure 3A). These observations suggest that rhCK cleaves the triple helix at multiple sites.

Figure 3(B) shows the effect of increasing concentrations of rhCK on the degradation of 20 μ M bovine type II collagen over 16 h. Fragments F1–F4 were all detectable at enzyme concentrations of 62.5–500 nM. At 1000 nM rhCK, F4 was the only major fragment detected, indicating that it is relatively resistant to further proteolysis.

Immunological detection of rhCK-cleavage products of type II collagen

We have made use of antibodies to type II collagen epitopes to probe for fragments of type II collagen generated by rhCK. Antibodies AH12L3 and COL2-3/4m are specific to epitopes in the α 1(II) chain that are located 15 and 369 residues respectively from the first (N-terminal) residue of the triple helix (bovine sequence) (L. J. Croucher and A. P. Hollander, unpublished work). In contrast, antibody AH9L2 is specific to an epitope located 10 residues from the last (C-terminal) residue of the triple helix (L. J. Croucher and A. P. Hollander, unpublished work). Digests of bovine type II collagen (20 μ M) by rhCK (100 nM) at pH 5.0 for 6 h at 25 °C were electrophoresed and electroblotted to nitrocellulose as described in the Materials and methods section. For comparative purposes, a lane containing MMP-13-cleaved type II collagen was included. Figures 4(A) and 4(B) show immunoblots with COL2-3/4m and AH12L3 as primary antibodies. Both antibodies specifically detected denatured, uncleaved γ , β and α components of type II collagen, as well as the 3/4-length fragment of MMP-13-cleaved type II collagen. Fragments F1–F4 generated by rhCK cleavage of type II collagen (see Figure 2A) were all detected by antibody COL2-3/4m but not by AH12L3. This suggests that rhCK cleaves type II collagen at a site between the epitopes recognized by these two antibodies (see Figure 1), presumably in the N-terminal region of the triple helix.

Antibody AH9L2 detected denatured, uncleaved γ , β and α components of type II collagen and it also detected the 1/4-length fragment of type II collagen generated by MMP-13, as shown on an immunoblot from a 12% (w/v) gel (Figure 4C). The higher-molecular-mass band detected by this antibody in MMP-13-cleaved type II collagen is a cross-linked dimer of the 1/4-length fragment [40]. AH9L2 detected all four fragments, F1–F4, generated by rhCK as well as four other fragments that were not visible on the Coomassie-stained 6% gels. These findings suggest that rhCK does not cleave type II collagen at the C-terminal end of the triple helix, although we cannot rule out the possibility that the enzyme makes a cleavage within the 10-residue sequence between the AH9L3 epitope and the C-terminus of the helix (see Figure 1 for the relative positions of the epitopes).

N-terminal sequencing of rhCK-cleaved type II collagen

To identify helical cleavage site(s) generated by rhCK in type II collagen, the N-terminal sequence of fragment F1 was obtained. The 14-residue sequence obtained matched exactly a sequence in

Figure 4 Detection of different epitopes in type II collagen fragments with anti-peptide antibodies

After digestion of native soluble type II collagen with rhCK, the reaction was stopped with 1 mM E-64 and the samples were analysed by SDS/PAGE (6% or 12% gel) and immunoblotting. Lane 1, uncleaved collagen; lane 2, rhCK-cleaved collagen; lane 3, MMP-13-cleaved collagen. (A) Detection of epitope CB11B with monoclonal antibody COL2-3/4m. Samples were separated by SDS/PAGE (6% gel). The 3/4 fragment from collagenase cleavage and the four major fragments, F1–F4, from rhCK cleavage are indicated. (B) Detection of epitope AH12L3 in the N-terminal region of type II collagen: samples were separated by SDS/PAGE (6% gel). The migration position of the 3/4 fragment from collagenase cleavage is indicated. (C) Detection of epitope AH9L2 in the C-terminal region of type II collagen. Samples were separated by SDS/PAGE (12% gel). The migration position of the 1/4 fragment (and its cross-linked dimer) from collagenase cleavage and the four major fragments, F1–F4, from rhCK cleavage are indicated.

Figure 5 Comparison of the cleavage fragments produced from collagen types I and II by rhCK

Native soluble rat type I collagen and bovine type II collagen were each digested by rhCK (100 nM) at 25 °C and pH 5.0 for 6 h. The reaction was stopped by the addition of 1 mM E-64 and the samples were analysed by SDS/PAGE (6% gel). Lane 1, type I collagen incubated without enzyme; lane 2, rhCK-cleaved type I collagen; lane 3, type II collagen incubated without enzyme; lane 4, rhCK-cleaved type II collagen. The major fragments are indicated as F1'–F4' for type I collagen and F1–F4 for type II collagen.

the bovine α 1(II) chain located 58 residues from the N-terminus of the triple helix (see Figure 6). This result indicates that rhCK cleaved a Gly-Lys bond, thus removing epitope AH12L3 but retaining epitopes CB11B (detected with antibody COL2-3/4m) and AH9L2. For comparison, the uncleaved sequence of pepsin-extracted bovine type II collagen was obtained (results not

Figure 6 Comparison of rhCK cleavage sites in collagen types I and II

Sequence alignments for the rat type I collagen $\alpha 1$ chain, rat type I collagen $\alpha 2$ chain fragment and bovine type II collagen $\alpha 1$ chain are shown. The complete rat $\alpha 2$ (I) chain sequence is not currently available. The $\alpha 2$ (I) chain fragment, which we identified by N-terminal sequencing, has been aligned with the other collagen α chains by comparison with the mouse $\alpha 2$ (I) chain sequence (not shown). Arrows indicate rhCK cleavage sites identified by N-terminal sequencing of fragments. The box indicates the location of epitope AH12L3 in the $\alpha 1$ (II) chain, which is removed by rhCK cleavage.

shown), and this confirms that the collagen preparation used was not fragmented before digestion with rhCK.

rhCK cleavage sites in type I collagen

Although it has previously been shown that cleavage of type I collagen by rhCK is probably in the triple helix, this finding was not confirmed by identification of the actual cleavage site(s). In the light of our finding that this enzyme cleaves type II collagen in the N-terminal region of its helix, we considered it important also to identify the cleavage site(s) in type I collagen. For this purpose we used acid-extracted rat tail collagen, which is essentially free of contamination from collagen types III and IV.

rhCK cleaved type I collagen to generate a number of fragments (Figure 5). Two of the fragments migrating closest to the intact α -chains, F2' and F3', were excised after transfer to a PVDF membrane and sequenced. The 20-residue sequence obtained for fragment F2' exactly matched a sequence in the rat $\alpha 1$ (I) chain, starting 189 residues from the N-terminus of the triple helix (see Figure 6). The 20-residue sequence of fragment F3' exactly matched a sequence in the mouse $\alpha 2$ (I) chain, starting 144 residues from the N-terminus of the triple helix [see Figure 6; the complete rat $\alpha 2$ (I) chain amino acid sequence is not currently available for comparison]. We were unable to obtain a sequence from the band labelled F1' in Figure 5. Figure 6 illustrates the relative locations of the rhCK cleavage sites identified for types I and II collagen.

DISCUSSION

Our results demonstrate clearly for the first time that rhCK is capable of cleaving within the helical portion of native type II collagen under conditions that preclude any helix denaturation [41]. The collagenolytic action of the rhCK preparation was not

due to a contaminating serine proteinase or metalloproteinase; this was confirmed by the lack of any inhibition by 3,4-DCI, EDTA or 1,10-phenanthroline. The fragments were generated from type II collagen and not a contaminating protein, as shown by immunoblotting with $\alpha 1$ (II)-specific antibodies and N-terminal sequencing. In addition we have shown that the characterized rhCK cleavage sites in types I and II collagen are located close to the N-terminus of the triple helix.

Type II collagen is still efficiently hydrolysed by rhCK above pH 5.5. Under similar conditions, only low or no activity is observed for cathepsin L [42] or cathepsin B [42,43], both of which act on native collagen under more acidic conditions. In most soft connective tissues, the intracellular route for the degradation of fibrillar type I collagen mediated by lysosomal cysteine proteinases prevails, but in periods of accelerated breakdown, such as in inflammation, morphogenesis and metastasis, the extracellular route becomes important [3]. An intracellular route for type II collagen degradation in cartilage has not been described. However, the ability of cathepsin K to degrade type II collagen over the pH range 4.0–6.5 raises the possibility of an extracellular as well as intracellular role for the enzyme.

Cathepsin K is notable for its very effective gelatinolytic activity in the pH range 4.0–7.0. This is in agreement with previous findings with denatured type I collagen as substrate [15]. This activity adds to the potential ability of cathepsin K to remodel the extracellular matrix, either on its own or in concert with other collagenolytic and gelatinolytic enzymes.

Cleavage of types I and II collagen near the N-terminal region within the helical domain of the $\alpha 1$ (II), $\alpha 1$ (I) and $\alpha 2$ (I) chains seems to be a unique characteristic of cathepsin K, although the possibility of the discovery of other enzymes with the same characteristic cannot be dismissed. The cleavage by the mammalian collagenases towards the C-terminal end of fibrillar collagens is suggested to be due to laxity of the helix around the cleaved Gly-Leu/Ile bond caused in part by the presence of imino acid residues, allowing access of the enzyme to its target site [44]. It is currently not clear how cathepsin K is able to interact with its target site in the intact triple helix, but presumably there is enough laxity of the helix close to the N-terminus to facilitate this enzyme–substrate interaction. In type I collagen the sequences obtained for cleavage of the $\alpha 1$ and $\alpha 2$ chains did not align with each other (see Figure 6). Mammalian collagenases initially cleave type I collagen at a single, homologous site in the $\alpha 1$ and $\alpha 2$ chains. This is presumably due to the specific interaction between native collagen and the haemopexin domain of these proteinases [45]. The cleavage of type I collagen α chains at non-homologous sites might be due to the absence of a haemopexin domain in cathepsin K. However, we cannot rule out the possibility that the fragments we sequenced are not the earliest ones: the primary cleavage sites in type I collagen might be even closer to the N-terminus.

The cleavage sites identified in type I and II collagens are in agreement with published specificity data of cathepsin K. The $\alpha 1$ chain cleavages in both type I and type II collagen have a proline or hydroxyproline residue at the P2 position. P2 proline-containing synthetic substrates have been identified as specific for cathepsin K [46].

This is the first study demonstrating the possible involvement of human cathepsin K in cartilage breakdown. Using reverse transcriptase-mediated PCR (RT-PCR) we have found that freshly isolated human chondrocytes express cathepsin K constitutively (results not shown). This is in agreement with a previous report describing the presence of mouse cathepsin K in some hypertrophic chondrocytes [31]. There is also evidence that synoviocytes from rheumatoid arthritis patients express cathepsin

K mRNA [33]. In the light of the data reported here, future studies will need to focus on the potential role of cathepsin K in the turnover of type II collagen in normal and pathological states.

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