Characterization of helical cleavages in type II collagen generated by matrixins

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Several vertebrate collagenases have been reported to cleave type II collagen, leading to irreversible tissue destruction in osteoarthritis. We have investigated the action of MMP-1 and MMP-13 on type II collagen by use of neoepitope antibodies and N-terminal sequencing. Previous studies have suggested that the initial cleavage of type II collagen by MMP-13 is followed by a second cleavage, three amino acids carboxy-terminal to the primary cleavage site. We show here that this cleavage is also produced by APMA-activated MMP-1 in combination with MMP-3 (i.e. fully activated MMP-1). The use of a selective inhibitor of MMP-3 has shown that it is this enzyme, rather than interstitial collagenase which had been exposed to MMP-3, which makes the second cleavage. In addition we have identified, through N-terminal sequencing, a third cleavage site, three residues carboxy-terminal to the secondary site. Since MMP-2 is thought to be responsible for gelatinolytic action on type II collagen we have investigated the effect of MMP-2 after the initial helical cleavage made by either MMP-1 or MMP-13. A combination of MMPs-1, -2 and -3 results in both the second and third cleavage sites; adding MMP-2 to MMP-13 did not alter the cleavage pattern produced by MMP-13 on its own. We conclude that none of the three cleavage sites will provide information about the specific identity of the collagenolytic enzymes involved in collagen cleavage *in situ*. Staining of cartilage sections of osteoarthritis patients with the neoepitope antibodies revealed type II collagen degradation starting at or near the articular surface and extending into the mid and deep zones with increasing degeneration of the cartilage.

INTRODUCTION

Degradation of articular cartilage is a major feature of both osteoarthritis and rheumatoid arthritis. The two main extracellular components of articular cartilage are type II collagen, which provides tensile strength and aggrecan-hyaluronate aggregates which endow cartilage with its compressive stiffness [1]. These proteins are degraded in arthritic cartilage [2] but it is the breakdown of type II collagen which leads to an irreversible loss of structural integrity of the tissue [3].

Turnover of the cartilage matrix is regulated by chondrocytes. These cells can synthesize a variety of proteolytic enzymes which are capable of degrading the extracellular matrix. Of particular importance are members of the matrixin subfamily, the matrix metalloproteinases. The soluble matrixins include the collagenases, gelatinases and stromelysins. It is now known that chondrocytes have the capacity to synthesize three different collagenases, namely interstitial collagenase (MMP-1; EC 3.4.24.7) [4], neutrophil collagenase (MMP-8; EC 3.4.24.34) [5] and collagenase-3 (MMP-13; EC 3.4.24.–) [6]. The one-quarter and three-quarter fragments generated by cleavage of fibrillar collagens by collagenases [7–9] are susceptible to further cleavage by other matrixins such as gelatinases A (MMP-2; EC 3.4.24.24) and B (MMP-9; EC 3.4.24.35) [10,11], as well as by enzymes of different classes such as neutrophil elastase (EC 3.4.21.37) [12]. There are three stromelysins: MMP-3 (EC 3.4.24.17) MMP-10 (EC 3.4.24.22) and matrilysin (MMP-7; EC 3.4.24.23) [13,14]. Stromelysin-3 (MMP-11) in fact represents a new, distinct subgroup of the MMPs [15].

It is clear from studies of cartilage and synovial fluid that both aggrecan and type II collagen are degraded, in arthritis, by proteolytic attack [16,17]. However the precise mechanisms by which these connective tissue components are degraded are not fully understood; a proteolytic cascade was suggested with an important role for the collagenases since these were thought to be primarily responsible of cleaving the intact triple helix of type II collagen at neutral pH [18].

However, a more recent report suggests an important role for Mt1-MMP (MMP-14) as well since it has collagenolytic and gelatinolytic activity, sharing the proteolytic characteristics with MMP-8 and it is highly co-expressed with MMP-2 in human osteoarthritic chondrocytes [19].

Cleavage of the triple helix of type II collagen by MMP-1, MMP-8 or MMP-13 occurs at a position approximately one-quarter of the distance from the C-terminal end, namely at the Gly^{975}-Leu^{976} bond. Mitchell et al. reported the expression of MMP-13 by chondrocytes in human osteoarthritic cartilage [20]. Their experiments demonstrated that MMP-13 cleaves type II collagen more efficiently than MMP-1 (5-fold increase in k_{cat}/K_m) and it also makes an additional cleavage, three amino acids carboxy-terminal to the first cleavage site. The same secondary cleavage was recently demonstrated for MMP-1 and MMP-8 by Billinghurst et al. [21].

We have investigated the effect of MMP-1 and MMP-13 alone or in combination with other matrixins on type II collagen to verify if any of the reported cleavage sites are specific for a particular collagenase. To this end we have obtained N-terminal sequences on the one-quarter length fragments generated under

Abbreviations used: APMA, 4-aminophenylmercuric acetate; Dpa, *N*-3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl; Fmoc, 9-fluorenylmethoxycarbonyl; IL-1, interleukin-1; KLH, keyhole limpet haemocyanin; Mca, (7-methoxycoumarin-4-yl)acetyl; MMP, matrix metalloproteinase; OA, osteoarthritis; TBS, Tris-buffered saline.

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different proteolytic conditions. In addition we have made neoepitope antibodies to the primary and secondary cleavage sites produced in type II collagen and we have used them here in combination with N-terminal sequencing. This approach has been used successfully for several other proteolytic cleavage sites, including those found in cartilage aggrecan or link protein [22–25]. We have used these antisera to screen cartilage sections from OA patients for the presence of the cleavage sites.

EXPERIMENTAL

Materials

The materials used in this study were purchased from the following sources: bromoacetic acid *N*-hydroxysuccinimide ester, Freund's complete and incomplete adjuvant, alkaline phosphatase-labelled goat anti-rabbit IgG and *p*-nitrophenyl phosphate, type I and II collagen from Sigma, Bornem, Belgium; chondroitinase ABC was from ICN; KLH from Calbiochem, Belgium; Microtitre plates from Dynatech Laboratories, Inc., Chantilly, VA, U.S.A. and ICN/Flow, Asse, Belgium; recombinant human interstitial collagenase, stromelysin-1 and gelatinase A were from Biogenesis, Poole, U.K.; recombinant human collagenase-3 was a kind gift from Dr. P. Mitchell, Pfizer Central Research, Groton CT, U.S.A. The stromelysin inhibitor BB3437 was from British Biotech Pharmaceuticals Ltd, Oxford, U.K. The quenched fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 was provided by Dr. C. Graham Knight, Strangeways Research Laboratory, Cambridge, U.K.

Collagen preparation

Bovine and human type II collagen were prepared by differential salt precipitation, as previously described [26]. HDC was prepared by heating a 1 mg/ml solution of collagen in TBS for 20 min at 80 °C. In some experiments, bovine type II collagen from Sigma was used, with identical results.

Electrophoresis and Western blotting

SDS/PAGE of purified collagens was performed using 7.5% (w/v) polyacrylamide gels, which were stained with either Coomassie Brilliant Blue or Silver Stain, as previously described [27]. The electrophoresed samples were transferred to a nitrocellulose membrane which was then blocked for 1 h at ambient temperature with TBS containing 3% (w/v) BSA (TBS-3% BSA). The membrane was incubated for 1 h at ambient temperature with the antiserum (1:2500) preincubated in 1% (w/v) gelatin in TBS at 37 °C for 20 min.

After three washes with TBS-Tween, the membrane was incubated at ambient temperature for 1 h with alkaline-phosphatase labelled donkey anti-rabbit Ig diluted 1:3000 with TBS-3% BSA. The membrane was washed twice with TBS-Tween and once with TBS. Alkaline phosphatase substrate solution was prepared from a commercial kit (Bio-Rad) employing 5-bromo-4-chloro-3-indoyl phosphate and Nitroblue Tetrazolium. It was added and incubated with the membrane at ambient temperature until optimal colour had developed (10–30 min). Further reaction was stopped by rinsing in distilled water.

Peptide synthesis, and preparation of antibodies

Two peptides: Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val-Gly-Cys and Gln-Arg-Gly-Ile-Val-Gly-Leu-Pro-Gly-Cys were synthesized using the Fmoc technique by Dr. A. Moir, Kreb's Institute, Dept. Molecular Biology and Biotechnology, University of Sheffield, U.K. The sequence of these peptides corresponds to the eight N-terminal amino acids of the one-quarter fragment of type II collagen after interstitial collagenase and collagenase-3 cleavage respectively, with an extra Gly as spacer and a Cys for coupling the peptides to the KLH carrier, with the aid of bromoacetic acid *N*-hydroxysuccinimide ester [28]. The synthesized peptides were purified chromatographically by reversed-phase HPLC on a C18 column and were verified by sequence analysis.

Two female New Zealand white rabbits (2.5 kg) per peptide were injected subcutaneously every 2 weeks with 300 μg of conjugated peptide emulsified in 0.5 ml complete (first immunization) or incomplete (second immunization) Freund's adjuvant. The injections were given 2 weeks apart and each one was followed after 10 days by a test bleed and antibody titre determination by ELISA. After three injections the antisera were of sufficient titer and 60 ml of serum was obtained from each rabbit and stored in aliquots at −20 °C.

Screening of the rabbit antisera for antibodies to the immunizing peptides

Detection of antibodies was by direct ELISA assay, as previously described [16]. Plates were coated overnight at 4 °C with 40 μg/ml of the peptide, 50 μl/well. The uncoated binding sites were blocked by incubation with 1% (w/v) BSA in TBS after which they were incubated with the diluted rabbit serum for 90 min at 37 °C. Bound antibody was quantified by a 2 h incubation at 37 °C with a 1/1000 dilution of goat anti-rabbit alkaline phosphatase, followed by the alkaline phosphatase substrate *p*-nitrophenyl phosphate. The product of the reaction was quantified after 10–20 min at ambient temperature at 405 nm in a microplate reader. All washes were done with TBS containing 0.1% v/v Tween-20.

Inhibition of Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val and Gln-Arg-Gly-Ile-Val-Gly-Leu-Pro binding in ELISA

An inhibition ELISA was developed essentially as previously described [16]. A panel of peptides was serially diluted in TBS (concentration range 0.05–300 μg/ml), and incubated overnight at 37 °C with the antiserum (final dilution 1:20000, 100 μl), after which all samples were transferred to the appropriate well of the antigen-coated (2 μg/ml, 50 μl) and blocked ELISA plates. After 30 min the plates were washed and incubated with secondary antibody as described above in the direct ELISA. Substrate was added and the plates were incubated at 37 °C until the well that contained no competing antigen gave an absorbance reading of approximately 1.0 at 405 nm. This reading was taken as 100%. The same inhibition ELISA protocol as described above was used to investigate the interference of native and heat denatured types I, II and III collagen (not shown).

Characterization of the neoepitopes of type II collagen produced by cleavage by MMP-1, MMP-13 and MMP-2

Human type I, bovine and human type II, and human type III collagens were initially dissolved in 0.5 M acetic acid and then diluted to a final concentration of 2.5 mg/ml in 0.1 M Tris/HCl, pH 7.6, containing 10 mM $CaCl_2$ and recombinant human interestitial collagenase (0.77 μM) which had been activated with 2 mM APMA for 1 h at 37 °C according to the supplier's guidelines. Collagenase-3 (0.83 μM) was activated by addition of 1 mM APMA and incubation for 1 h at 37 °C [39]. The final molar ratio of collagenase to collagen was 1:33 and 1:30 respectively. The control contained collagen in buffer without collagenase. The samples were incubated for approximately 48 h

at 30 °C after which hydrolysis was stopped by the addition of EDTA to 20 mM.

In some experiments, MMP-2 (0.5 μM final concentration) was added to the reaction mixture after degradation of the α-chains of type II collagen by fully activated MMP-1 and MMP-13, and the reaction was prolonged for another 24 h, then stopped by addition of EDTA to 20 mM. The reaction products were analysed by immunoblotting and N-terminal sequence analysis after transfer to a PVDF membrane.

Investigation of the role of MMP-3 in the collagenolytic activity of MMP-1

In some experiments MMP-1 (0.77 μM) was fully activated by incubation with 2 mM APMA and MMP-3 (0.67 μM) for 5 h at 37 °C [29,30] before adding in the type II collagen. Their respective molar ratio to type II collagen was 1:33 and 1:29.

We used a selective inhibitor of MMP-3: BB3437 (IC_{50} = 60 nM for MMP-3, IC_{50} for MMP-1 = 30 μM) in order to distinguish between the action of these two enzymes on the substrate [31]. The inhibitor was used at a concentration of 3 μM and was added to the fully active MMP-1 incubation mixture before addition of the type II collagen. At the concentration of the inhibitor used MMP-3 activity was fully inhibited whereas MMP-1 activity was essentially not affected as verified by use of a quenched fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (not shown).

An additional control was included where the inhibitor was added to MMP-3 prior to incubation with MMP-1. The reaction products were analysed by immunoblotting.

Immunohistochemistry

Human articular cartilages were obtained from the femoral condyles of adult knee joints of 10 patients undergoing total joint arthroplasty. Cartilage explants were mounted in OCT-embedding media, frozen and stored at −70 °C.

Sections (6 μm) were cut perpendicular to the articular surface at −21 °C using a Leitz cryostat. Sections were picked up on adhesive microscope slides and dried overnight at ambient temperature before they were frozen and stored at −20 °C. Before incubation with the primary antibody the sections were processed in essentially the same way as previously described [32]. Briefly, they were dried for 30 minutes at ambient temperature after which they were fixed in 4% formaldehyde. This was followed by blocking of the unreactive aldehyde groups with 1% (w/v) normal goat serum in PBS and blocking of the endogenous peroxidase activity by incubation of the sections with freshly prepared 0.5% (v/v) H_2O_2 in absolute methanol. To enhance permeability of the extracellular matrix the sections were treated with chondroitinase ABC prior to treatment with EDTA to ensure removal of any calcific deposits in the specimens. The last step before incubation with the primary antibody was blocking of the background with 10% (v/v) goat serum, 1% w/v BSA in PBS. The sections were incubated with the primary antibody or preimmune serum diluted 1:25 in 2% (v/v) goat serum, 1% (w/v) gelatin in PBS for 2 h at ambient temperature followed by three washes for 10 min with TBS. A biotin-streptavidin detection system was used according to the manufacturer's recommendations (ABC-Vectastain® kit, Vector Laboratories, Peterborough, U.K.) after which the peroxidase reaction was developed and the sections mounted permanently. Additional negative controls were performed consisting of immunoadsorption of the antisera with their respective epitope (not shown).

RESULTS

Characterization of neoepitope antibodies to collagenase cleavage sites

Antibodies were produced to the two cleavage sites previously reported to occur following incubation of type II collagen with MMP-13 [20]. Antibody MV-1 was raised against Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val and antibody MV-2 was raised against Gln-Arg-Gly-Ile-Val-Gly-Leu-Pro. In preliminary ELISA assays it was found that the inclusion of gelatin in the reaction mixture inhibited non-specific binding of antibody to denatured α-chains without inhibiting specific recognition of the collagenase cleavage site. Under these conditions, neither antibody detected native or denatured collagen types I, II or III (not shown). Inhibition ELISA assays were performed to determine the specificity of peptide sequences which could inhibit binding of each antibody to its immunizing peptide. MV-1 binding to Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val was inhibited by this peptide, but not by Gly-Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val, demonstrating the need for the α-NH_2 group on the Leu N-terminal amino acid (Figure 1a).

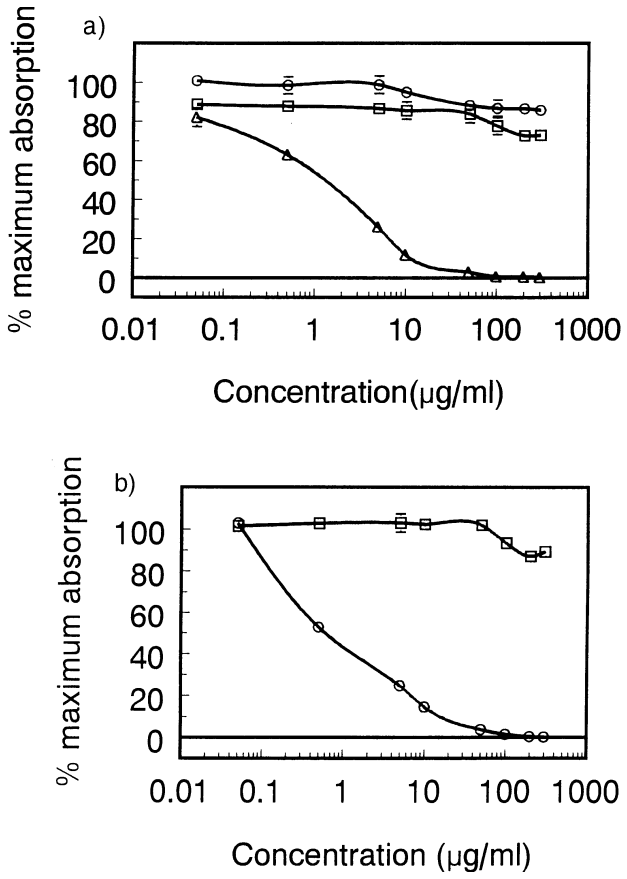

Figure 1 Characterization of antisera MV-1 and MV-2 by inhibition ELISA

(**a**) Antibody MV-1 was preincubated with the test peptides and then transferred to ELISA plates coated with the MV-1 immunizing peptide. The inhibitory peptides were the MV-1 immunizing epitope, Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val (△), the same epitope with an additional N-terminal glycine residue (○) or the MV-2 immunizing epitope, Gln-Arg-Gly-Ile-Val-Gly-Leu-Pro (□). (**b**) Antibody MV-2 was preincubated with the test peptides and then transferred to ELISA plates coated with the MV-2 immunizing peptide. The inhibitory peptides were the MV-2 immunizing epitope, Gln-Arg-Gly-Ile-Val-Gly-Leu-Pro (○), or the MV-1 immunizing epitope, Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val (□).

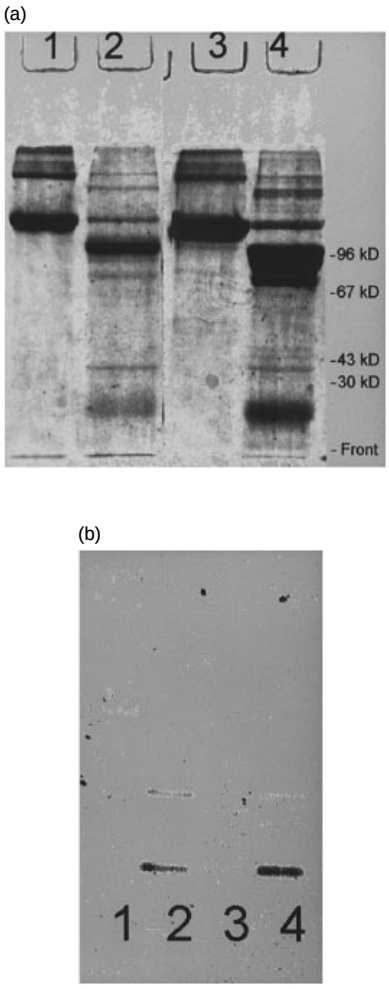

Figure 2 Cleavage by APMA-activated MMP-1 of type II collagen, detected in Western immunoblots using antibody MV-1

(**a**) Silver-stained SDS/PAGE of human and bovine collagens. (**b**) Western immunoblot using antiserum MV-1. In both figures: lane 1 is undigested human type II collagen; lane 2 is MMP-1-digested human type II collagen; lane 3 is undigested bovine type II collagen; lane 4 is MMP-1-digested bovine type II collagen.

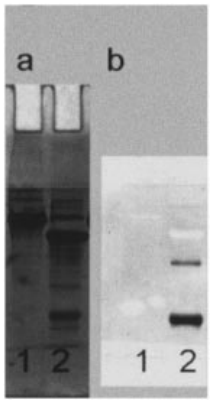

Figure 3 Cleavage by APMA-activated MMP-13 of type II collagen, detected in Western immunoblots using antibody MV-2

(**a**) Silver-stained SDS/PAGE of bovine collagen. (**b**) Western immunoblot using antiserum MV-2. In both figures, lane 1 is undigested bovine type II collagen; lane 2 is MMP-13-digested bovine type II collagen.

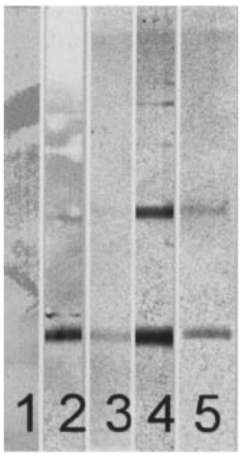

Figure 4 Cleavage of bovine type II collagen by combinations of APMA activated matrixins, detected in Western immunoblots using antibody MV-2

Lane 1, cleavage by MMP-1 only; lane 2, cleavage by MMP-1 + MMP-3; lane 3, cleavage by MMP-1 + MMP-2 + MMP-3; lane 4, cleavage by MMP-13; lane 5 is cleavage by MMP-13 + MMP-2.

In addition, MV-1 was not inhibited by Gln-Arg-Gly-Ile-Val-Gly-Leu-Pro (Figure 1a), indicating that it does not cross-react with the sequence generated by the second cleavage site. Similarly, binding of antibody MV-2 to Gln-Arg-Gly-Ile-Val-Gly-Leu-Pro was inhibited by this peptide but not by Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val (Figure 1b). Thus each antibody specifically recognized the one-quarter fragment primary or secondary neoepitope to which it was raised.

Identification of type II collagen cleavage sites by Western blotting

We tested MMP-1 and MMP-13 with type II collagen at a collagenase:collagen molar ratio of 1:33 and 1:30 respectively. Under these conditions, APMA-activated MMP-1 cleaved bovine and human type II collagen to generate one-quarter fragments that reacted with antibody MV-1 (Figure 2), demonstrating presence of the primary cleavage site. At the concentration of MMP-1 used, we were not able to detect with MV-2 a band corresponding to the secondary cleavage site (see lane 1 in Figure 4).

APMA-activated MMP-13 cleaved bovine type II collagen to generate one-quarter fragments that reacted with antibody MV-2 (Figure 3), demonstrating presence of the second cleavage site, but not with MV-1 (not shown). Presumably the lack of any primary cleavage site was due to efficient cleavage at the secondary site by MMP-13 following the prolonged incubation time in these experiments. The one-quarter fragments detected by MV-1 and MV-2 migrated at a distance equivalent to a M_r of 25000. These two antibodies also detected a band at about 50000 in both the MMP-1 and MMP-13 digests (see Figures 2 and 3), which are presumably cross-linked dimers of the one-quarter fragments. To test the effects of stromelysin-1-activation of

Figure 5 Role of MMP-3 in producing the secondary cleavage site

Lane 1, cleavage of type II collagen by APMA-activated MMP-1 only; lane 2, cleavage of type II collagen by APMA-activated MMP-1 and MMP-3 after addition of BB3437 (3 μM); lane 3, cleavage of type II collagen by APMA-activated MMP-1 and MMP-3; lane 4, cleavage of type II collagen by APMA-activated MMP-1 and MMP-3 inhibited with BB3437 (3 μM) before adding it to MMP-1. Bands were detected on a western blot with MV-2.

MMP-1 on type II collagen cleavage, APMA-activated MMP-1 and MMP-3 were incubated together prior to the addition of type II collagen. Under these conditions the secondary cleavage site was detected in the one-quarter fragments, using antibody MV-2 (Figure 4, lane 2), confirming that this cleavage site is not specific to the action of MMP-13. In order to discriminate between an activator-role for MMP-3 resulting in a fully active form of MMP-1 [29,30] and the direct effect of MMP-3 on MMP-1-cleaved type II collagen we added a selective MMP-3 inhibitor, BB3437, prior to addition of type II collagen but after activation of MMP-1 with MMP-3 [31]. Under these conditions we were unable to detect the second cleavage site (Figure 5, lane 2), indicating that MMP-3 was itself responsible for the generation of this fragment, presumably following initial cleavage of the helical collagen by MMP-1.

Cleavage of the triple helix of type II collagen results in unwinding of the helix, making the three-quarter and one-quarter fragments susceptible to attack by gelatinolytic proteinases. Therefore we investigated the role of MMP-2 in degradation of one-quarter fragments. A combination of APMA-activated MMPs-1, -2 and -3 or MMPs-13 and -2 each generated one-quarter fragments that were detected using antibody MV-2 (Figure 4). However the intensity of staining was much lower than with MMP-13 alone or MMPs-1 and -3 in combination. This suggests that MMP-2 cleaves the one-quarter fragment further, resulting in loss of the MV-2 epitope. The results of all the Western blotting experiments are summarized in Table 1.

Identification of type II collagen cleavage sites by N-terminal sequencing

In order to verify the Western blotting results using a more definitive technique, we determined the N-terminal sequence of one-quarter fragments generated under the same conditions as described above. When MMP-13 was used to cleave human type II collagen we were unable to detect the one-quarter fragment produced by cleavage at the primary type II collagen cleavage site (Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val). However we did detect fragments generated from the secondary cleavage, with an N-terminal sequence of Gln-Arg-Gly-Ile-Val-Gly-Leu-Pro (Table 1). In addition we detected one-quarter fragments with the amino-terminus Ile-Val-Gly-Leu-Pro-Gly-Gln-Arg (Table 1), indicating an additional cleavage three residues carboxy-terminal to the secondary site.

Quantitative recovery of the residues produced by the first round of Edman degradation also allowed us to derive the sensitivity of the antibodies in Western blotting on type II collagen samples that had been exposed to identical conditions: antibody MV-1 detected 0.52 μg of cleaved type II collagen; antibody MV-2 detected 0.48 μg of cleaved type II collagen.

We next analysed collagen samples that had been treated with a combination of APMA-activated MMPs-1 and 3. This mixture generated one-quarter fragments with amino-termini from both the primary and secondary cleavage sites in equimolar proportions (Table 1). This confirms the finding by Western blotting that the second cleavage site is not specific to MMP-13 but can be generated by the action of MMP-3 on MMP-1-cleaved type II collagen as well. The third cleavage site was not detected. Finally, we tested the effects of MMP-2 on collagenolytic cleavage sites. When type II collagen was cleaved by a combination of MMPs-1, -2 and -3, the primary cleavage site was not detected but the second and third cleavage sites were (Table 1). Thus this mixture reproduced exactly the effect of MMP-13. The result suggests that the primary cleavage site produced by cleavage with MMP-1 and -3 is completely removed by the addition of MMP-2. The same secondary and tertiary cleavages were pro-

Table 1 N-terminal sequences of type II collagen one-quarter fragments following treatment with matrixins

	Amino-terminal sequences								
	LAGQRGIV			QRGIVGLP			IVGLPGQR		
Treatment of type II collagen	By Western blot	By sequence	Yield (pmol)	By Western blot	By sequence	Yield (pmol)	By Western blot	By sequence	Yield (pmol)
MMP-1 + APMA	Yes	—	—	N.D.	—	—	—	—	—
MMP-13 + APMA	N.D.	N.D.		Yes	Yes	3.04	—	Yes	3.53
MMP-1 + MMP-3 + APMA	Yes	Yes	6.71	Yes	Yes	6.52	—	N.D.	
MMP-1 + MMP-3 + MMP-2 + APMA	N.D.	N.D.		Yes	Yes	3.13	—	Yes	4.76
MMP-13 + MMP-2 + APMA	N.D.	N.D.		Yes	Yes	5.2	—	Yes	5.67

—, no sequence-analysis or Western blotting was performed.
N.D., no fragments were detected.

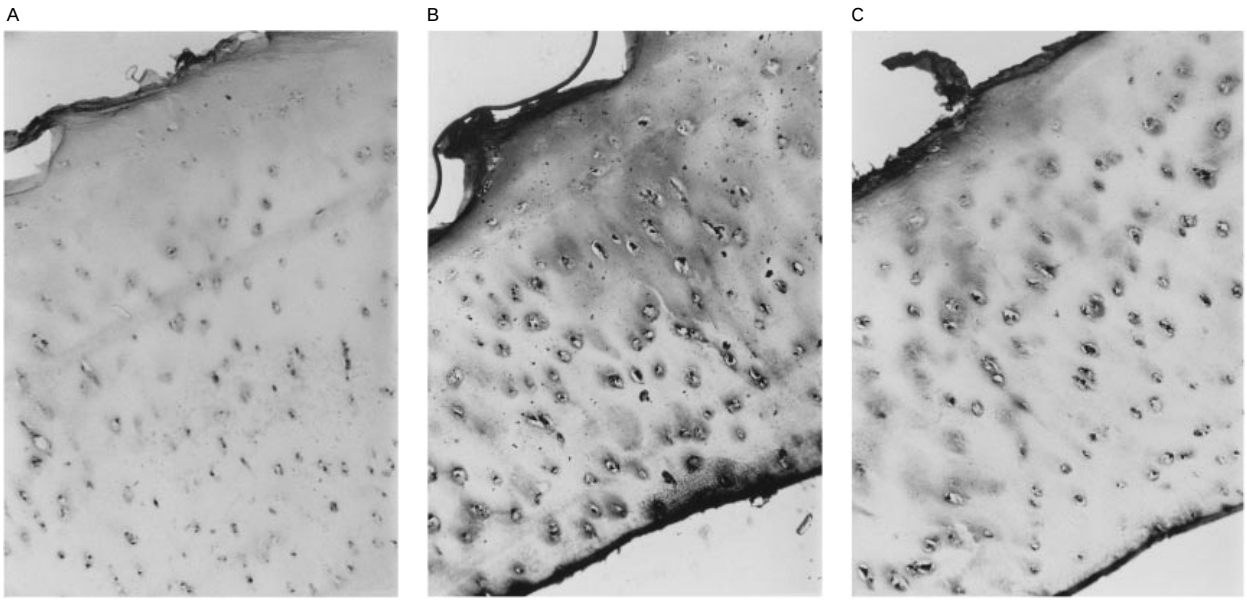

Figure 6 Immunohistochemical analysis of cartilage sections of OA patients

(The top of the figures corresponds with the articular surface.) (**A**) Cryostat section of femoral condylar articular cartilage from a 74-year-old female stained with preimmune serum (× 10). (**B**) Cartilage section from the same patient stained with MV-1 (× 10). (**C**) Cartilage section from the same patient stained with MV-2 (× 10). (The intense staining seen at the cutting edge is non-specific staining.)

duced by a combination of APMA-activated MMPs-13 and -2 as by APMA-activated MMP-13 alone (Table 1).

Immunohistochemical analysis of OA cartilage

We examined 10 OA cartilages; all patients were diagnosed as having classical non-inflammatory arthritis. When cartilage sections of OA patients were stained with the antibody MV-1, the staining appeared to be most intense in the superficial zone extending to the mid and deep zone where the staining was more restricted to the pericellular region of the chondrocytes. Typical results for one patient are shown in Figure 6B. In some specimens staining was mainly observed in the superficial and upper mid zones whereas the mid and deep zones were essentially unstained. Control sections stained with preimmune serum showed very little background staining (Figure 6A). Cartilage sections stained with antibody MV-2 gave essentially the same staining pattern, but staining was less pronounced and more restricted to the pericellular region of the chondrocytes (Figure 6C).

DISCUSSION

It is known from previous studies that the concentration of denatured type II collagen in OA cartilage is higher than in normal cartilage and this is presumably the result of collagenolytic activity [16,33,34]. It is not known which collagenase(s) cleave type II collagen in OA. This will depend on their relative expression levels, their degree and type of activation, the presence of inhibitors and their relative efficiency at cleaving type II collagen. These collagenases show distinct substrate specificities: MMP-1 cleaves type III collagen in preference to type II collagen, whereas MMP-13 has the most efficient action against type II collagen [8,20]. Very little is known as to what extent other matrixins contribute to the further degradation of collagenase-cleaved type II collagen.

Both MMP-1 [34] and MMP-13 [6,30] are expressed by chondrocytes in human OA cartilage; chondrocytes from normal healthy cartilage express MMP-1 and MMP-8 [5] but not MMP-13 and their expression in isolated chondrocytes is significantly upregulated by cytokines such as IL-1 and TNF-α [35–38].

MMP-3 activates pro-MMP-13 by a 2-step mechanism *in vitro*, although relatively high amounts of MMP-3 are required [39]. MMP-3 alone cannot efficiently activate pro-MMP-1 [40,41]. However it is known that catalytic amounts of MMP-3 can generate the fully active form of MMP-1 in combination with APMA, trypsin or plasmin [29,30,41–43]. Recently it was shown by Knäuper et al. [44] that Mt1-MMP (MMP-14) as well as gelatinase A (MMP-2) may be able to activate pro-MMP-13 alone or in concert. This novel activation mechanism is potentially important *in vivo*. In contrast, pro-MMP-1 is resistant to proteolysis by Mt1-MMP [44].

The finding by Mitchell et al. [20] that MMP-13 cleaves at a second site that was apparently not cleaved by MMP-1 raised the possibility of distinguishing between these proteolytic activities using neoepitope antibodies. During the preparation of this manuscript Billinghurst et al. [21] reported that both MMP-1 and MMP-13 were able to produce the primary and secondary cleavage.

In addition it was shown by use of a preferential inhibitor for MMP-13 that this matrixin is likely to play a significant role in type II collagen degradation in OA. We were not able, under the conditions used, to detect the second cleavage site when MMP-1 alone was incubated with type II collagen. This could be due to the 6-fold lower molar ratio of enzyme to substrate that was used. In this study we have confirmed the observation of a second cleavage site three residues carboxy-terminal to the primary site and in addition we have identified, through N-terminal sequencing, a novel third cleavage site, three residues carboxy-terminal to the secondary site. A fragment with an N-terminus corresponding to this tertiary site was identified in the

study of Mitchell et al. [20] by mass spectroscopy, when MMP-13 was used to cleave a synthetic peptide substrate.

We have shown here by N-terminal sequencing of type II collagen cleavage products and by using neoepitope antibodies that the secondary cleavage produced by MMP-13 on type II collagen is also produced by APMA-activated MMP-1 in combination with MMP-3. It is known that MMP-3 can generate the fully active form of MMP-1 [29,30,41–43] and it seemed likely that this fully active collagenase produced the secondary cleavage. However when we investigated this possibility by use of a selective inhibitor for MMP-3, we found that the second cleavage site was produced by the gelatinolytic action of MMP-3.

Since MMP-2 is thought to be responsible for additional processing of the unwound α-chains after cleavage of the triple helix, we have investigated the action of this enzyme in combination with fully active MMP-1. A combination of MMPs-1, -2 and -3 produced both the second and third cleavage sites and in this case the most likely mechanism was generation of the third cleavage directly by MMP-2.

MMP-13 is an efficient gelatinase [39] and cleavage of the Gly^{978}-Gln^{979} bond occurs after initial cleavage of the Gly^{975}-Leu^{976} bond in peptide substrates and in type II collagen [20,21]. We have shown here that MMP-13 also cleaves at a third site, Gly^{981}-Ile^{982} in type II collagen. Taken together these results suggest that initial cleavage of the triple helix at the primary site is followed by unwinding of the α-chains and subsequent cleavage at the second and third sites by tripeptidyl-peptidase activity of the matrixins. Thus none of the known sites of cleavage are specific for any one matrixin, and therefore they cannot be used as evidence of a role for a specific member of this sub-family of metalloproteinases in type II collagen degradation.

The results of immunohistochemical analysis demonstrated that damage to type II collagen in OA cartilage originates around the chondrocytes, starting in the superficial layers, where staining was most intense, and extending into the mid zone with progressive degeneration, as previously described [27,32]. Moreover antibodies against MMP-1 stained the immediate pericellular region of the chondrocytes (not shown) demonstrating that the chondrocytes are the main mediators of type II collagen breakdown by producing the enzymes responsible for it.

In some OA cartilage samples analysed, the deep zones were mostly unstained, in contradiction to the results obtained with an antibody to denatured type II collagen [32]. This may indicate that a different mechanism of collagen breakdown is occurring in the deep zones which leads either to the destruction of the epitopes we have been analysing or a lack of their generation. We have reason to believe that the one-quarter fragments diffuse very rapidly out of the cartilage: although several combinations of matrixins are able to produce the third cleavage site, when we used a recently developed antibody directed against this cleavage site (data not shown), very little staining appeared presumably due to loss of the epitope from the cartilage.

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REFERENCES

1 Kempson, G. (1980) in The Joints and Synovial Fluid, (Sokoloff, L., ed.), pp. 238–239, Academic Press Inc., New York
2 Poole, A. R. (1993) in Arthritis and Allied Conditions (McCarty, D. J. and Koopman, W. G., eds.), pp. 279–315
3 Saxne, O. and Heinegård, D. (1995) J. Rheumatology **22**, suppl. 43, 71–74
4 Murphy, G., Hembry, R. M. and Reynolds, J. J. (1986) Collagen Relat. Res. **6**, 351–364
5 Cole, A. A., Chubinskaya, S., Schumacher, B., Huch, K., Csszabo, G., Yao, J., Mikecz, K., Hasty, K. A. and Kuettner, K. E. (1996) J. Biol. Chem. **271**, 11023–11026
6 Reboul, P., Pelletier, J. P., Tardif, G., Cloutier, J. M. and Martelpelletier, J. (1996) J. Clin. Invest. **97**, 2011–2019
7 Fields, G. B., Netzel-Arnett, S. J., Windsor, L. J., Engler, J. A., Birkedal-Hansen, H. and Van Wart, H. E. (1990) Biochemistry **29**, 6670–6677
8 Welgus, H. G., Jeffrey, J. J. and Eisen, A. Z. (1981) J. Biol. Chem. **256**, 9511–9515
9 Hasty, K. A., Jeffrey, J. J., Hibbs, M. S. and Welgus, H. G. (1987) J. Biol. Chem. **262**, 10048–10052
10 Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A. and Goldberg, G. I. (1989) J. Biol. Chem. **264**, 17213–17221
11 Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C., Bauer, E. A. and Goldberg, G. I. (1988) J. Biol. Chem. **263**, 6579–6587
12 Gross, J., Highberger, J. H., Johnson-Wint, B. and Biswas, C. (1980) in Collagenase in Normal and Pathological Connective Tissues, (Wooley, D. R. and Evanson, J. M., eds.), pp. 11–36, Wiley and Sons, New York
13 Muller, D., Quantin, J., Gesnel, M. C., Millon-Collard, R., Abecassis, J. and Breathnach, R. (1988) Biochem. J. **253**, 187–192
14 Quantin, J., Murphy, G. and Breathnach, R. (1989) Biochemistry **28**, 5327–5334
15 Basset, P., Bellocq, J. P., Wolf, C., Stoll, I., Hutin, P., Limacher, J. M., Podhajcer, O. L., Chenard, M. P., Rio, M. C. and Chambon, P. (1990) Nature **348**, 699–704
16 Hollander, A. P., Heathfield, T. F., Webber, C., Iwata, Y., Bourne, R., Rorabeck, C. and Poole, A. R. (1994) J. Clin. Invest. **93**, 1722–1732
17 Lohmander, L. S., Hoerrner, L. A. and Lark, M. W. (1993) Arthritis Rheum. **36**, 181–189
18 Buttle, D. J., Bramwell, H. and Hollander, A. P. (1995) Clin. Mol. Pathol. **48**, 167–177
19 Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. (1997) J. Biol. Chem. **272**, 2446–2451
20 Mitchell, P. G., Magna, H. A., Reeves, L. M., Lopresti, M. L. L., Yocum, S. A., Rosner, P. J., Geoghegan, K. F. and Hambor, J. E. (1996) J. Clin. Invest. **97**, 761–768
21 Billinghurst, R. C., Dahlberg, L., Reiner, A., Bourne, R., Rorabeck, C., Mitchell, P. G., Hambor, J. E., Diekmann, O., Tschesche, H., Chen, J.-M., Van Wart, H. E. and Poole, A. R. (1997) J. Clin. Invest. **99**, 1534–1545
22 Hughes, C. E., Caterson, B., White, R. J., Roughley, P. J. and Mort, J. S. (1992) J. Biol. Chem. **267**, 16011–16014
23 Hughes, C. E., Caterson, B., Fosang, A. J., Roughley, P. J. and Mort, J. S. (1995) Biochem. J. **305**, 799–804
24 Lark, M. W., Williams, C. H., Hoerrner, L. A., Weidner, J. R., Ayala, J. M., Harper, C., Christen, A., Olszewski, J., Konteatis, Z., Webber, R. and Mumford, R. A. (1995) Biochem. J. **307**, 245–252
25 Fosang, A. J., Last, K., Gardiner, P., Jackson, D. C. and Brown, L. (1995) Biochem. J. **310**, 337–343
26 Miller, E. J. (1971) Biochemistry **10**, 1652–1659
27 Dodge, G. R. and Poole, A. R. (1989) J. Clin. Invest. **83**, 647–661
28 Bernatowicz, M. S. and Matsueda, G. R. (1986) Anal. Biochem. **155**, 95–102
29 Murphy, G., Cockett, M. I., Stephens, P. E., Smith, B. J. and Docherty, A. J. P. (1987) Biochem. J. **248**, 265–268
30 Suzuki, K., Enghild, J. J., Morodomi, T., Salvesen, G. and Nagase, H. (1990) Biochemistry **29**, 10261–10270
31 Patent No. WO 96/33165 (1996) Derivatives of succinamide: use as metalloproteinase inhibitors
32 Hollander, A. P., Pidoux, I., Reiner, A., Rorabeck, C. and Poole, A. R. (1995) J. Clin. Invest. **96**, 2859–2869
33 Kozaci, L. D., Buttle, D. J. and Hollander, A. P. (1996) Arthritis Rheum. **40**, 164–174
34 Pelletier, J.-P., Martel-Pelletier, J., Howell, D. S., Ghandur-Mnaymneh, L. G., Enis, J. E. and Woessner, Jr., J. F. (1983) Arthritis Rheum. **26**, 63–68
35 Borden, P., Solymar, D., Sucharczuk, A., Lindman, B., Cannon, P. and Heller, R. A. (1996) J. Biol. Chem. **271**, 23577–23581
36 Mitchell, P. G. and Cheung, H. S. (1991) J. Cell. Physiol. **149**, 132–140
37 Gowen, M., Wood, D. D., Ihrie, E. J., Meats, J. E. and Russell, R. G. G. (1984) Biochim. Biophys. Acta **797**, 186–193
38 Mort, J. S., Dodge, G. R., Roughley, P. J., Liu, J., Finch, S. J., DiPasquale, G. and Poole, A. R. (1993) Matrix **13**, 95–102
39 Knäuper, V., Lopezotin, C., Smith, B., Knight, G. and Murphy, G. (1996) J. Biol. Chem. **271**, 1544–1550
40 Wilhelm, S. M., Collier, I. E., Kronberger, A., Eisen, A. Z., Marmer, B. L., Grant, G. A., Bauer, E. A. and Goldberg, G. I. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6725–6729

41 He, C., Wilhelm, S. M., Pentland, A. P., Marmer, B. L., Grant, G. A., Eisen, A. Z. and Goldberg, G. I. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2632–2636
42 Nagase, H., Enghild, J. J., Suzuki, K. and Salvesen, G. (1990) Biochemistry **29**, 5783–5789
43 Brinckerhoff, C. E., Suzuki, K., Mitchell, T. I., Oram, F., Coon, C. I., Palmiter, R. D. and Nagase, H. (1990) J. Biol. Chem. **265**, 22262–22269
44 Knäuper, V., Will, H., Lopezotin, C., Smith, B., Atkinson, S. J., Stanton, H., Hembry, R. M. and Murphy, G. (1996) J. Biol. Chem. **271**, 17124–17131

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