RESEARCH COMMUNICATION

Neutrophil collagenase (MMP-8) cleaves at the aggrecanase site E^{373}–A^{374} in the interglobular domain of cartilage aggrecan

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Native and recombinant neutrophil collagenase (MMP-8) was shown to cleave at the E^{373}–A^{374} 'aggrecanase' site in the interglobular domain of aggrecan. The time course of digestion *in vitro* showed that MMP-8 cleaved initially at N^{341}–F^{342}, the predominant metalloproteinase site, before cleaving at the E^{373}–A^{374} site. A synthetic peptide, IPENFFG, inhibited cleavage at E^{373}–A^{374} but not N^{341}–F^{342} *in vitro*, indicating that the E^{373}–A^{374} sequence was a less preferred site for MMP-8 cleavage than N^{341}–F^{342}. IPENFFG also inhibited release of A^{374} RGSVI fragments from cartilage in explant culture, suggesting that a metalloproteinase cleaved at the aggrecanase site *in situ*. The possibility remains that 'aggrecanase' may be a metalloproteinase in cartilage.

INTRODUCTION

Aggrecan is the major proteoglycan in cartilage and is responsible for its resilience and load-bearing properties. The loss of aggrecan is a major feature of cartilage degeneration associated with arthritis. Normal turnover and pathological loss of aggrecan from cartilage involves proteolytic cleavage of the core protein and the most susceptible region is located at the N-terminus where two globular domains, G1 and G2, are separated by a short interglobular domain (IGD) (Figure 1). The IGF has been identified as a key site of proteolytic attack, however the proteinase(s) responsible for cleavage in the tissue have not been identified.

The matrix metalloproteinases (MMPs) are thought to be involved in aggrecan degradation, however there is controversy as to which of the MMPs (1,2,3,8 or 9) are primarily responsible, or whether several MMPs work in combination. In studies *in vitro* using a purified G1–G2 substrate prepared from cartilage aggrecan [1] we have identified cleavage sites specific for MMP-1 (interstitial collagenase; EC 3.4.24.7), MMP-2 (gelatinase A; EC 3.4.24.24), MMP-3 (stromelysin 1 EC 3.4.24.17), MMP-7 (matrilysin; EC 3.4.24.23), MMP-9 (gelatinase B; EC 3.4.24.35) and MMP-8 (neutrophil collagenase; EC 3.4.24.34) [2–4]. The results show that these MMPs cleave between N^{341} and F^{342} (based on the human aggrecan sequence [5]), and that this is the preferred and predominant site of cleavage for this class of enzyme. A minor MMP cleavage-site between D^{441} and L^{442} is also present, however the incidence of cleavage at this site is low, and not all the MMPs show this activity [3,4].

Other laboratories have identified a major cleavage-site within aggrecan IGD that is different to the size identified for the MMPs [6–10]. These studies have shown that under conditions of normal and interleukin-1 (IL-1)-stimulated turnover, bovine cartilage explants release aggrecan fragments with N-terminal sequences corresponding to cleavage between E^{373} and A^{374} [6–8]. In addition, the major aggrecan fragments found in synovial fluids from osteoarthritis and joint-injury patients result from cleavage at the same E^{373}–A^{374} bond [9,10]. The enzyme responsible for this cleavage has been named aggrecanase but its identity remains unknown. Sites in the IGD that are cleaved by cathepsin B [3], leukocyte elastase [11], plasmin and urokinase [12] have also been described, but do not correlate with aggrecanase cleavage.

In this paper we report that native and recombinant human MMP-8 cleaves a G1–G2 substrate at the aggrecanase site. This important finding establishes that an MMP is able to cleave at the aggrecanase site whereas previous results suggested that this class of enzyme was not responsible. Our results further show that cleavage *in vitro* at the aggrecanase site occurs after cleavage at the adjacent MMP site.

EXPERIMENTAL

Cystatin, E-64 and pepstatin were from Boehringer Mannheim, Germany. Keratanase (*Pseudomonas sp.*) (EC 3.2.1.103) was from Seikagaku Kogyo, Japan. Chondroitin ABC lyase (*Proteus vulgaris*) (EC 4.2.2.4) and keratanase II (*Bacillus sp.*) were from Seikagaku, U.S.A. Agarose type HSC was from PS Park Scientific (Northampton, U.K.). Methods for the preparation of the G1–G2 substrate and polyclonal rabbit anti-G2 antiserum have been described [1]. The purification of native MMP-8 from human neutrophils [13], recombinant MMP-8 catalytic domain fragment [14], rabbit-bone prostomelysin [15], recombinant human prostromelysin [16,17], recombinant human fibroblast procollagenase [18], human progelatinase A [19] and recombinant tissue inhibitor of metalloproteinase-1 (TIMP-1) [20] was as described.

Abbreviations used: IGD, interglobular domain; MMPs, matrix metalloproteinases; PMSF, polymethanesulphonyl fluoride; SBTI, soybean trypsin inhibitor; PEG, polyethylene glycol; IL-1, interleukin-1; TIMP-1, tissue inhibitor of metalloproteinase-1.

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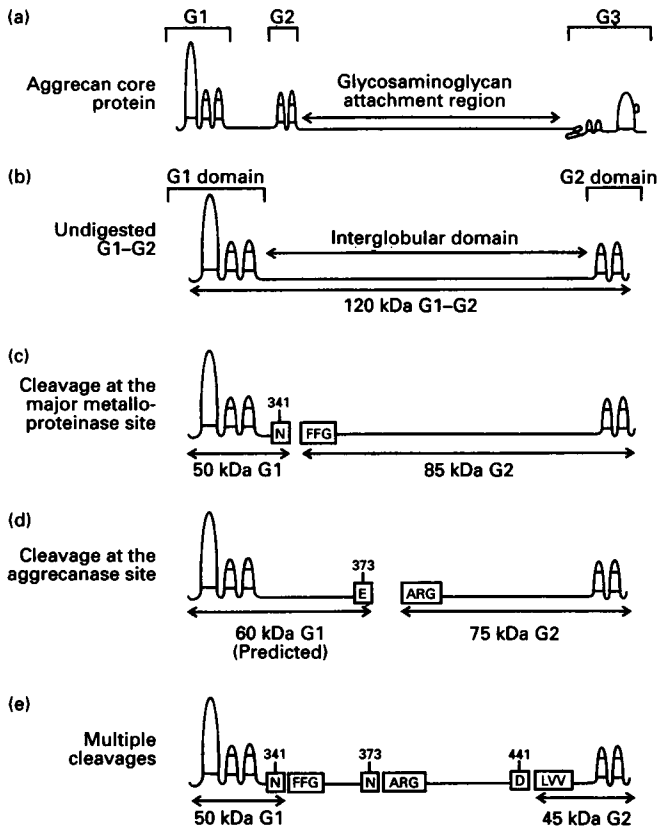

Figure 1 Schematic representation of cleavage sites in the aggrecan interglobular domain

The G1, G2 and interglobular domains (**b**) are shown in relation to the complete aggrecan core-protein (**a**). The cleaved fragments generated by the action of MMPs (**c,e**) and aggrecanase (**d,e**) are shown. The only G1 product found was 50 kDa. The 60 kDa fragment predicted for a primary cleavage at the aggrecanase site (**d**) was not detected. Cleavage at D^{441}–L^{442} (**e**) occurred at low incidence (20% or less). Small IGD fragments lacking globular domains (**e**) F^{342}–E^{373} (predicted relative molecular mass 5000–10000 Da) and A^{374}–D^{441} (8000–15000) were not detected. The size of the fragments is shown as their apparent relative molecular mass on 5% SDS gels after keratanase digestion.

Enzyme digestions

MMP digestions were carried out in buffer containing 10 mM calcium chloride, 100 mM sodium chloride and 50 mM Tris/HCl, pH 7.5, at 37 °C, for the times specified. The digests contained 1 mM phenylmethanesulphonyl fluoride (PMSF), 2 μM pepstatin and either 40 μg/ml cystatin or 20 μg/ml E-64 as proteinase inhibitors. Keratanase digestions were done in 50 mM-Tris/acetate buffer, pH 7.5, at 37 °C overnight with 0.025 units of keratanase per 30 μl, in the presence of 10 mM EDTA/100 μg/ml cystatin/1 mM PMSF. All MMP digested G1–G2 samples were keratanase-treated prior to SDS/PAGE unless otherwise stated. Keratanase-treated G2 digestion products were sequenced directly from Immobilon membranes following SDS/PAGE and electrotransfer. Immunodetection with the BC-3 monoclonal antibody (1:1000) was as described [21].

Activation of pro-MMPs

The procollagenases (10 μg) were activated with 1 mM $HgCl_2$ for 1 h at 37 °C, or alternatively, by incubation with 0.1 μg of recombinant human prostromelysin and 0.5 μg of trypsin for 30 min at 37 °C in a total volume of 50 μl. Trypsin was inactivated with 5 μg of soybean trypsin inhibitor (SBTI). Control-tubes containing prostromelysin, trypsin and SBTI did not degrade the G1–G2 substrate. Prostromelysin (0.25 μg) was activated by incubation with 70 mg of plasmin for 1 h at 37 °C, or 0.25 μg of trypsin for 20 min at room temperature, followed by inhibition with a 10-fold molar excess of aprotinin or SBTI respectively.

Explant cultures

Cartilage taken from the metacarpophalangeal joints of young pigs was maintained in explant culture in Dulbecco's modified Eagle's medium with supplements as described [22]. The explants were equilibrated for 3 d in serum-free medium, then incubated in medium containing human recombinant IL-1α (10 mg/ml) for 24 h in the presence or absence of peptide (4 mg/ml). Control cultures were maintained in serum-free medium with or without peptide. After 24 h the medium was removed, dialysed against distilled water and the concentration of sulphated glycosaminoglycan determined using the 1,9-Dimethylmethylene Blue assay [23]. Aliquots containing 5 μg of glycosaminoglycan were freeze-dried and reconstituted in 8 M urea prior to electrophoresis on composite gels [24]. Duplicate gels were either stained with Toluidine Blue (0.27%, w/v in acetic acid) or electrophoretically transferred onto Hybond N+ membrane (Amersham). Before immunodetection with BC-3 antibody, glycosaminoglycan chains were removed from aggrecan fragments by digesting the membranes with 0.01 units/ml chondroitin ABC lyase in 0.1 M Tris/acetate, pH 8.0/1% w/v BSA for 1 h at room temperature, followed by digestion with keratanase (0.01 units/ml) and keratanase II (0.002 units/ml) in 50 mM sodium acetate, pH-6.5/1% w/v BSA.

RESULTS AND DISCUSSION

Previous experiments digesting G1–G2 with low concentrations (< 40 μg/ml) of MMPs had identified a major cleavage site EN^{341}–FFG where all MMPs showed activity. When MMP-8 was incubated with G1-G2 at high concentration (160 μg/ml) a different pattern of fragments emerged (Figures 1 and 2). A major product containing the G2 domain formed a broad band on SDS/PAGE at 100–120 kDa (Figure 2A, a, lane 1) which resolved into two components after keratanase treatment (Figure 2A, a, lane 2). The first band at 85–90 kDa was immunoreactive with an anti G2 antiserum (Figure 2A, c) and contained the N-terminal sequence FFGVG corresponding to the major MMP cleavage site. The second band at 75–80 kDa was also G2 positive on immunoblotting and contained the N-terminal sequence ARGSV1 corresponding to the aggrecanase site. This fragment also reacted strongly with a monoclonal antibody BC-3 (Figure 2A, b, lane 2) which specifically recognises a peptide with the ARGSVI N-terminal sequence [25]. Non-keratanase treated digests were not immunolocalised with BC-3 antibody (Fig. 2A, b, lane 1) indicating that the epitope was masked by keratan sulphate chains. A third band (41–46 kDa) was identified as a small G2-containing fragment with N-terminal sequence LVVQV corresponding to the second, minor MMP site. There was also a small amount of undigested G1–G2 and a single G1-containing fragment with 50–56 kDa.

The ARGSVI-G2 fragment was produced exclusively by MMP-8 and was not found in G1–G2 samples digested with human recombinant fibroblast collagenase ($\leqslant$ 390 μg/ml) (Figure 2C, lanes 4 and 5), stromelysin (rabbit bone, $\leqslant$ 40 μg/ml; human recombinant, $\leqslant$ 100 μg/ml) (Figure 2D) or human gelatinase A ($\leqslant$ 25 μg/ml) (results not shown) and these are all concentrations of MMPs that produce extensive digestion of the

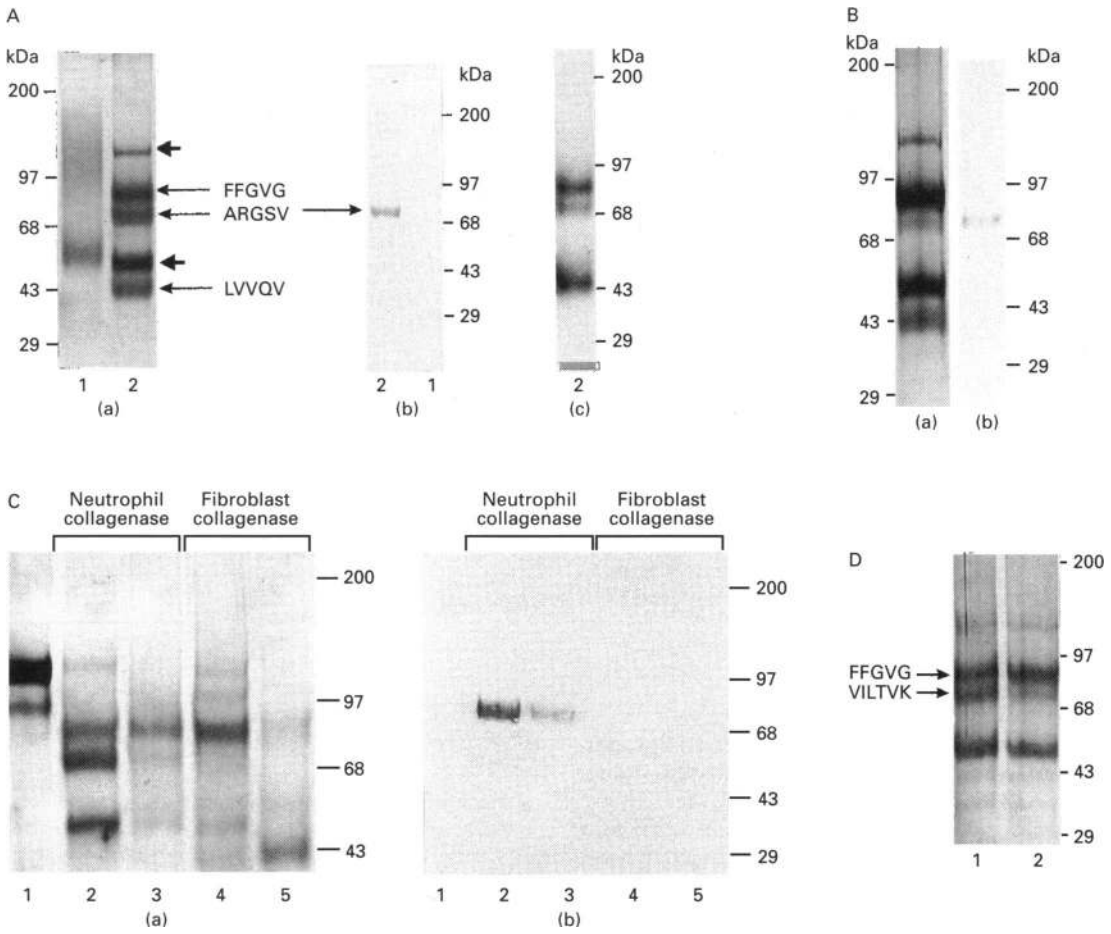

Figure 2 Degradation of aggrecan G1–G2 by MMPs and identification of an aggrecanase-type product following digestion with MMP-8

A, Digestion of G1–G2 (4 mg/ml) with $HgCl_2$-activated human MMP-8 (160 μg/ml), SDS/PAGE and analysis by silver stain (**a**) or immunodetection with BC-3 (**b**) before (lane 1) and after (lane 2) keratanase treatment, (**c**) immunodetection with polyclonal anti-G2 antiserum. The N-terminal sequences of the G2 bands are shown. The short arrows mark undigested G1–G2 and the G1 domain bands. B, Digestion of G1–G2 (4.2 mg/ml) with a purified, recombinant catalytic-domain fragment of human MMP-8 (192 μg/ml) and analysis by silver stain (**a**) or BC-3 immunoblot (**b**). C, Digestion of G1–G2 (1 mg/ml) with MMP-8 (140 μg/ml) (lanes 2 and 3) or MMP-1 (140 μg/ml) (lanes 4 and 5), following activation of the enzymes by $HgCl_2$ (lanes 2 and 4) or stromelysin and trypsin (lanes 3 and 5); lane 1 is undigested. Analysis by silver stain (**a**) or BC-3 immunoblot (**b**). D, Digestion of G1–G2 (0.25 mg/ml) with purified rabbit-bone stromelysin (25 μg/ml), following activation of the proenzyme with either plasmin (lane 1) or trypsin (lane 2), with analysis by silver stain. The N-terminal sequence of the G2 product derived from a trypsin-activated stromelysin digest is shown.

G1–G2 substrate. The MMP-2 digests following keratanase treatment produced a single 85 kDa G2 product. The MMP-3 digests (25–40 μg/ml) (Figure 2D) produced two bands (75 kDa and 85 kDa) that appeared similar to those produced by MMP-8. However, N-terminal sequence analysis of the 75 kDa MMP-3-derived band revealed an N-terminal VILTVKP sequence (Figure 2D), and the band was not immunodetected with BC-3 antibody irrespective of whether the prostromelysin was activated with *p*-aminophenylmercuric acetate, plasmin or trypsin. These results show that stromelysin, gelatinase and fibroblast collagenase failed to cleave at the aggrecanase site.

MMP-8 was able to generate the ARGSVI-fragment following activation with either $HgCl_2$, or trypsin and stomelysin (Figure 2C, lanes 2 and 3). A recombinant catalytic domain or human MMP-8 [14] was also able to produce the 75 kDa ARGSVI-fragment (Figure 2B). Proteinase inhibitor experiments provided further evidence that the cleavage was not due to a contaminating proteinase of another class. The specific MMP inhibitor TIMP-1, and the metal chelators EDTA and 1,10-phenanthroline each blocked MMP-8 activity and no G1 or G2 products were formed. When the ratio of TIMP-1:MMP-8 was less than 1:1, the activity of the enzyme was partially blocked, resulting in the production of the 85 kDa band, but not the 75 kDa band (results not shown). In contrast, 2 mM PMSF, 1 μM pepstatin, 10 mM *n*-ethylmaleimide μg/ml cystatin and 20 μg/ml E-64 were unable to inhibit its action and in each case the 75 kDa ARGSVI-band was detected by silver stain and immunoreactivity with BC-3. Together with evidence from the recombinant enzyme these results provide strong proof that MMP-8 was responsible for this activity.

Although the aggrecanase activity of MMP-8 was detected only at high enzyme concentration (160 μg/ml), this activity could also be detected at concentrations as low as 10 μg/ml if polyethylene glycol (PEG) 4000 was included in the digests. Aggrecan is an expanded macromolecule with a large hydrodynamic domain which excludes other macromolecules (such as enzymes) and concentrates them in a smaller excluded volume. The addition of PEG 4000 *in vivo* thus mimics the effect of aggrecan in cartilage *in vivo*. The results show that at 10 μg/ml, in the absence of PEG 4000, MMP-8 did not produce any

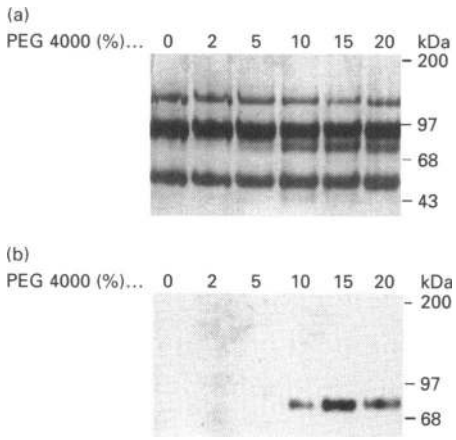

Figure 3 Volume exclusion effect of polyethylene glycol 4000

G1–G2 (0.5 mg/ml) was digested overnight at 37 °C with $HgCl_2$-activated MMP-8 (10 μg/ml) either alone (lane 1) or in the presence of PEG 4000 at a final concentration of 2%, 5%, 10%, 15% or 20% (lanes 2–6 respectively). Aliquots were electrophoresed in duplicate on 5% SDS gels and analysed by silver stain (**a**) or BC-3 blot (**b**).

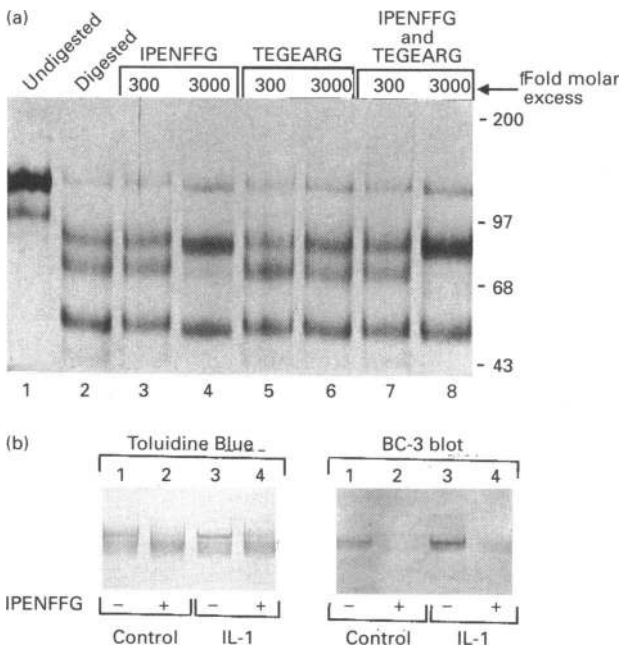

Figure 4 Effect of synthetic peptides IPENFFG and TEGEARG on MMP-8 action *in vitro* and on release of aggrecan fragments from cartilage explant cultures

(**a**) G1–G2 (0.25 mg/ml) was digested with $HgCl_2$-activated MMP-8 (100 μg/ml) for 16 h at 37 °C in the presence (lanes 3–8) or absence (lane 2) of either the IPENFFG peptide (lanes 3 and 4), the TEGEARG peptide (lanes 5 and 6) or both peptides together (lanes 7 and 8). The peptides were present in 300-fold or 3000-fold molar excess over G1–G2. (**b**) Aliquots of dialysed medium from cartilage explant cultures incubated with (lanes 3 and 4) or without (lanes 1 and 2) interleukin 1α, and with (lanes 2 and 4) or without (lanes 1 and 3) the IPENFFG peptides were electrophoresed on composite gels and analysed by Toluidine Blue staining or BC-3 immunodetection.

75 kDa ARGSVI-fragments (Figure 3a and b, lane 1), however when PEG 4000 was included in the digests at final concentrations of 10% or greater, ARGSVI-fragments were readily detected by silver stain (Figure 3a) and immunodetection with BC-3 (Figure 3b). These results show that polymers such as PEG can increase the local concentration of enzyme by excluded-volume effects and thereby affect their activity. Thus, the effective concentration of proteinase in cartilage matrix may be high because of the volume-exclusion effects of aggrecan, even though the total issue concentration of enzyme might be quite low.

The 75 kDa ARGSVI-fragment was not present in digests that did not also contain the 85 kDa FFGVG-fragment. Furthermore the G1 fragment released was always of similar size (~ 50 kDa) to that released by stromelysin and therefore did not contain the 32 amino acid F^{342}–E^{373} sequence (Figure 1e). This suggested that under the conditions tested, cleavage at the major MMP site preceded cleavage at the E^{373}–ARGSVI site. This was investigated further in a time-course experiment, digesting G1–G2 with MMP-8 (results not shown). At early times (4 h) the major fragment was the 85 kDa G2 product, however with longer digestion times (10 h and 21 h) the 75 kDa fragment was gradually produced at the expense of the 85 kDa fragment, suggesting that the enzyme cleaves initially at the MMP site and subsequently processes the 85 kDa FFGVG fragment to the 75 kDa ARGSVI fragment. Other experiments showed that the G2 fragment with N-terminal FFGVG (released by MMP-3 digestion of G1–G2) was a substrate for further cleavage by MMP-8 at the aggrecanase site.

Further evidence that MMP-8 cleaves preferentially at the major MMP site was provided by experiments in which two synthetic 7-near peptides, IPENFFG and TEGEARG, substrate analogues for each catabolic site, were tested as competitive substrates. At a high molar excess (3000-fold) the IPENFFG peptide reduced the amount of ARGSVI-fragment produced and increased the amount of FFGVG-fragment present in digests of G1–G2 (Figure 4a). This result suggests that the IPENFFG peptide is a poor competitor for MMP-8 cleavage at the MMP site, but a better competitor of enzyme action at the aggrecanase site. This indicates that MMP-8 favours cleavage at the MMP site because the peptide is able to compete for cleavage at E^{373}–ARG, but it is not able to compete for cleavage at N^{341}–FFG. This is similar to the results obtained with low levels of TIMP which also preferentially blocked cleavage at the aggrecanase site. The TEGEARG peptide had no effect on MMP-8 action on G1–G2, suggesting it is a weaker competitor than IPENFFG.

The results show that *in vitro* MMP-8 cleaves primarily at N^{341}–FFGVG and that cleavage at the aggrecanase site occurs as a secondary event. To investigate whether a similar pattern of enzyme action occurred in tissue, cartilage explants were cultured in the presence and absence of the IPENFFG peptide and IL-1α. The released aggrecan fragments were analysed for BC-3 epitope (Figure 4b). Over a 24 h culture-period the IPENFFG peptide was found to inhibit appearance of the ARGSVI epitope in aggrecan products released from cartilage cultured with and without IL-1. This is the same effect that was observed *in vitro* with MMP-8, and suggests that in cartilage the IPENFFG peptide is able to competitively inhibit an enzyme, presumably an MMP, for cleavage at the aggrecanase site.

The identification of aggrecanase-derived fragments in human synovial fluids initially suggested that aggrecanase was the enzyme responsible *in vivo* for loss of aggrecan from cartilage. However other data show that MMPs are also directly involved in aggrecan degradation *in vivo* since G1 fragments with C-terminal sequences corresponding to MMP cleavage have been extracted from [26] and immunolocalized in [27] human articular cartilage. These findings suggest that both MMP and aggrecanase

activities are involved in aggrecan degradation *in vivo*, and readily explain the occurrence of C-terminal DIPEN fragments in cartilage matrix and N-terminal ARGSV fragments in joint fluids. Other studies have also shown that MMPs have an important role in aggrecan degradation as hydroxamic acid-containing MMP inhibitors effectively reduce aggrecan release in IL-1 and retinoate-stimulated matrix degradation [28–30].

Whether MMP-8 is the enzyme in cartilage that is responsible for aggrecanase activity is unresolved, however there are no reports of MMP-8 protein or antigen in cartilage, and we have been unable to demonstrate MMP-8 mRNA in IL-1-stimulated and control chondrocytes using reverse transcription-PCR (J. Dudhia and T. E. Hardingham, personal communication). Some results suggest that aggrecanase is not an MMP, for although most aggrecan fragments isolated from synovial fluids or explant medium have N-terminal ARGSVI sequences, the purified MMPs (with the exception of MMP-8) appear unable to cleave at this site *in vitro*. If, however, aggrecanase is one of the members of the MMP family found in cartilage why has its action not been identified *in vitro*? Possible reasons are (1) that pure MMPs isolated from cartilage have not been tested; it is possible that their substrate specificities differ from bone, fibroblast or other cell sources, (2) that conditions in cartilage matrix such as enzyme:substrate ratio and concentration, pH and ionic environment, and routes of activation may influence the rates of attack at different substrate sites. Our data with PEG 4000 confirm that physical environment is an important aspect in determining enzyme action. Until these questions are resolved it would be premature to conclude that aggrecanase is not an MMP.

We are grateful to Paula Hazell for carrying out the cartilage culture experiments, and to T. Kleine for providing the recombinant catalytic domain of MMP-8. We thank Professor John Hamilton for his support and acknowledge financial assistance from the National Health and Medical Research Council (Australia), the Victorian Health Promotion Foundation (Australia), the Arthritis Foundation of Australia, the Arthritis and Rheumatism Council (U.K.), the Arthritis Foundation (U.S.A.), N.I.H. grant AR 32666, Shriners of North America and the Deutsche Forschungsgemeinschaft (Federal Republic of Germany).

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Received 11 August 1994/5 September 1994; accepted 7 September 1994