

Degradation of cartilage aggrecan by collagenase-3 (MMP-13)

Amanda J. Fosang^{*a}, Karena Last^a, Vera Knäuper^b, Gillian Murphy^b, Peter J. Neame^c

^aOrthopaedic Molecular Biology Research Unit, Melbourne University Department of Paediatrics, Royal Children's Hospital, Parkville 3052, Australia

^bStrangeways Research Laboratory, Cambridge CB1 4RN, UK

^cShriners Hospital for Crippled Children, Tampa Unit, FL 33612, USA

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Abstract Degradation of the large cartilage proteoglycan aggrecan in arthritis involves an unidentified enzyme aggrecanase, and at least one of the matrix metalloproteinases. Proteinase-sensitive cleavage sites in the aggrecan interglobular domain (IGD) have been identified for many of the human MMPs, as well as for aggrecanase and other proteinases. The major MMP expressed by chondrocytes stimulated with retinoic acid to degrade their matrix is collagenase-3 or MMP-13. Because of its potential role in aggrecan degradation we examined the specificity of MMP-13 for an aggrecan substrate. The results show that MMP-13 cleaves aggrecan in the IGD at the same site (..PEN₃₄₁–FFG..) identified for other members of the MMP family, and also at a novel site ..VKP₃₈₄–VFE.. not previously observed for other proteinases.

Key words: Aggrecan; Matrix metalloproteinase; Collagenase; Arthritis; Cartilage degradation

1. Introduction

Aggrecan is the major proteoglycan found in cartilage and it is the structural molecule that contributes most significantly to the weight-bearing properties of the tissue. A major feature of cartilage degeneration associated with arthritis is loss of aggrecan due to proteolytic cleavage within the interglobular region between the G1 and G2 globular domains. The proteinase(s) responsible for aggrecan degradation in the tissue have not been identified, but much interest has focused on the role of the matrix metalloproteinases in arthritic disease [1–10]. A predominant MMP cleavage site in the aggrecan interglobular domain has been identified between N₃₄₁ and F₃₄₂. The stromelysins, collagenases, gelatinases, and matrilysin are all active at this site [11–14]. MMP cleavage at N₃₄₁–F₃₄₂ produces aggrecan fragments with N-terminal FFGVG... sequence and there is evidence for cleavage at this site in vivo [12,15]. Products of cleavage at this site have also been identified in culture medium from unstimulated porcine cartilage explants [16] and in synovial fluids from arthritis patients [17].

The major aggrecan fragments found in human synovial fluids from OA and joint injury patients however, do not correspond with MMP cleavage products, but rather result from cleavage at the E₃₇₃–A₃₇₄ bond in the IGD [18,19]. Similarly,

under conditions of normal and IL-1 stimulated turnover cartilage explants release aggrecan fragments with N-terminal sequences corresponding to cleavage between E₃₇₃ and A₃₇₄ [20–22]. The putative enzyme responsible for this cleavage has been named aggrecanase but its identity remains unknown. Sites in the IGD that are cleaved by cathepsin B [13], leukocyte elastase [23], plasmin and urokinase [24] have also been described, but do not correlate with aggrecanase cleavage. Interestingly, one member of the MMP family, MMP-8, has been shown to have aggrecanase activity in vitro [25].

Collagenase-3, or MMP-13, was recently cloned from a cDNA library derived from a human breast tumour [26] and shown to contain structural motifs that identify it as a new member of the collagenase subfamily of MMPs. MMP-13 exhibits 86% homology in amino acid sequence to rat and mouse MMP-1 [27,28] suggesting that rodent collagenase and human MMP-13 represent a gene product that is distinct from MMP-1 [28]. MMP-13 is also the major MMP expressed by retinoate-stimulated chondrocyte monolayers [29]. Because of its potential role in aggrecan degradation it was of interest to examine the specificity of this new MMP for an aggrecan substrate. Our results show that MMP-13 cleaves the aggrecan IGD at the same major (..PEN₃₄₁–FFG..) and minor (...SED₄₄₁–LVV...) MMP cleavage sites identified for other members of the MMP family, and also at a novel site, ..VKP₃₈₄–VFE..., not previously observed for other proteinases.

2. Materials and methods

An enhanced chemiluminescence (ECL) Western blotting kit, cysteine proteinase inhibitor E-64 and pepstatin were from Boehringer Mannheim, Germany. Keratanase (*Pseudomonas* sp.) (EC 3.2.1.103), keratanase II (*Bacillus* sp.) and chondroitin ABC lyase (*Proteus vulgaris*) (EC 4.2.2.4) were from Seikagaku Kogyo, Japan. Agarose type HSC was from PS Park Scientific (Northampton, UK). Trypsin (diphenylcarbamoyl chloride treated) (EC 3.4.21.4), soybean trypsin inhibitor, 1,10-phenanthroline, 3-dimethylaminopropionitrile, 6-aminohexanoic acid, phenylmethanesulphonyl fluoride, benzamidinium hydrochloride and 4-aminophenylmercuric acetate (APMA) were from Sigma Chemical Co. (St. Louis, MO). [4-(2-Aminoethyl)benzene]-sulfonylfluoride (AEBSF) was from Calbiochem-Novabiochem, Australia. Human umbilical chord hyaluronan was from BDH (Poole, Dorset, UK). A Biosep-SEC S4000 hplc column (300 × 7.8 mm) was from Phenomenex, USA. Rabbit anti-mouse horseradish peroxidase (HRP) conjugated immunoglobulin was from Dako (Denmark) and a goat anti-mouse-HRP conjugate was from Promega Corporation (Australia). Polyvinylidene difluoride membrane (Immobilon) was from Millipore-Waters (Sydney, Australia). All other reagents were of analytical grade.

2.1. Expression of recombinant MMP-13

Human recombinant procollagenase-3 was expressed using stably transfected NSO mouse myeloma cells and purified from serum-free cell culture medium using S-Sepharose fast flow (Knäuper et al., 1995, submitted).

*Corresponding author. Fax: (61) (3) 9345 6668

Abbreviations: IGD, interglobular domain of aggrecan; MMPs, matrix metalloproteinases; HRP, horseradish peroxidase; APMA, 4-aminophenylmercuric acetate; AEBSF, [4-(2-aminoethyl)benzene]-sulfonylfluoride; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence.

2.2. Enzyme digestions

MMP-13 digestions were in buffer containing 10 mM calcium chloride, 100 mM sodium chloride, 50 mM Tris-HCl, pH 7.5, at 37°C, for the times specified. The digests also contained 1.25 mM AEBSF, 5 μ M pepstatin and 20 μ g/ml E64 as proteinase inhibitors. G1–G2 samples were keratanase-treated prior to SDS-PAGE. Keratanase digestions were in 50 mM Tris/acetate buffer, pH 7.2, at 37°C overnight with 0.025 units of keratanase/30 μ l, in the presence of 10 mM EDTA, 20 μ g/ml E64, 5 μ M pepstatin and 1.25 mM AEBSF. For sequencing experiments, a high concentration (160 μ g/ml) of MMP-13 was used and 184 μ g purified G1–G2 was digested for 16 h at 37°C. The digestion products were then mixed overnight with hyaluronan and purified on a Biosep-SEC S4000 hplc column [13] and sequenced directly from immobilon membranes following SDS-PAGE and electrotransfer.

2.3. Activation of pro-MMPs

ProMMP-13 (244 μ g/ml) was activated with either 1 mM APMA for 2 h at room temperature in a total volume of 62.5 μ l, or 1 μ g trypsin for 5 min at 37°C in a total volume of 46 μ l. Trypsin was inactivated with 12.5 μ g of soybean trypsin inhibitor.

2.4. Immunodetection with monoclonal antibodies AF-28 and BC-3

Samples electrophoresed on 5% SDS gels [30] or agarose/acrylamide composite gels [31] were transferred onto Immobilon membrane and analysed for AF-28 epitope [17] or BC-3 epitope [32]. After blocking with 5% skim milk powder in PBS, the membranes were incubated with AF-28 antibody (1:1,000 dilution) or BC-3 antibody (1:1,000) in 0.5% skim milk powder in PBS for 1 h at room temperature then washed six times in buffer containing 0.1% tween-20 in PBS. Membranes for AF-28 detection were incubated for a further one hour with the Dako anti-mouse-HRP conjugate (1:10,000 dilution) at room temperature in 0.5% skim milk powder in PBS and membranes for BC-3 detection were incubated with the Promega anti-mouse-HRP conjugate (1:7,500 dilution). After six washes in PBS with 0.1% tween-20 the membranes were treated with ECL reagent according to the manufacturers instructions.

Prior to immunodetection with BC-3 antibody, glycosaminoglycan chains present on whole aggrecan (but not G1–G2) were removed by digesting the membranes with 0.01 U/ml chondroitin ABC lyase, 0.01 U/ml keratanase and 0.001 U/ml keratanase II in 50 mM Tris/Acetate pH 7.4 for 2 h at room temperature [32].

3. Results

3.1. MP-13 activation by APMA or trypsin

ProMMP-13 was activated with either APMA or trypsin to determine which activation procedure gave the maximum amount of proteinase activity (Fig. 1). The results show that trypsin-activated enzyme was more active than APMA-activated enzyme against an aggrecan G1–G2 substrate, since 1.4 μ g/ml APMA-activated MMP-13 failed to degrade G1–G2, whereas 1.4 μ g/ml trypsin-activated MMP-13 gave abundant 50 kDa G1 product and 85 kDa G2 product. When a twenty-fold higher concentration of enzyme was used, G1–G2 was almost completely degraded by APMA and trypsin-activated enzyme although a small amount of undigested substrate still remained. At 28 μ g/ml, trypsin-activated enzyme, but not APMA-activated enzyme, produced a small amount of a 68 kDa band (Figs. 1 and 3, arrow). These results indicate that trypsin treatment yields a more active MMP-13 than APMA, and trypsin activation was used for all subsequent experiments. There was no difference in the pattern or ratio of bands produced by MMP-13 digestion when the digests were done at pH 5.5 (results not shown).

3.2. Digestion of aggrecan with MMP-13

To determine whether MMP-13 would cleave aggrecan at the major ..PEN₃₄₁–FFG.. site identified for other MMPs, whole aggrecan purified from human, pig, calf, rat and shark cartilage

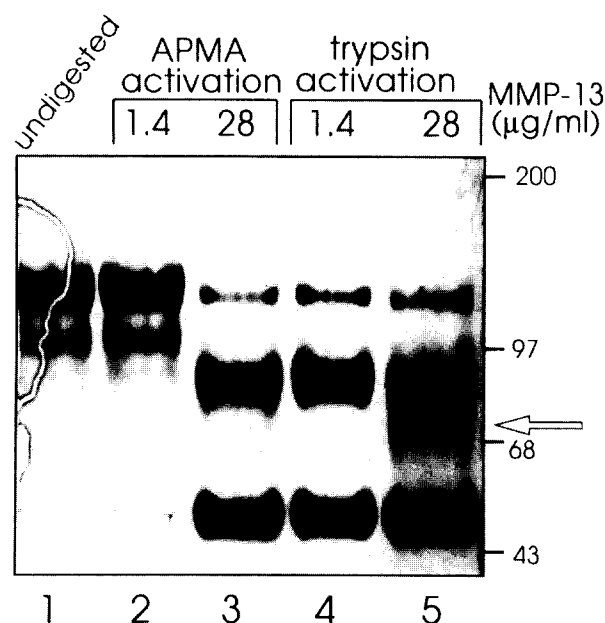


Fig. 1. Purified G1–G2 (2.5 μ g) was digested with 1.4 μ g/ml (lanes 2 and 4) or 28 μ g/ml (lanes 3 and 5) of MMP-13 which had been activated with either APMA (lanes 2 and 3) or trypsin (lanes 4 and 5). Aliquots of digested (lanes 2–5) and undigested (lane 1) G1–G2 were electrophoresed on 5% SDS gels and visualised with silver stain.

was digested and analysed on dissociative agarose/acrylamide composite gels. MMP-13 digestion of all the mammalian aggrecans (Fig. 2a) and the shark aggrecan (not shown) generated products of faster electrophoretic mobility as seen by toluidine blue staining. Immunodetection with monoclonal antibody AF-28, specific for cleavage at the major MMP site which creates the neo-epitope FFGVG... N-terminal sequence [17], revealed specific staining in MMP-13-digested aggrecan from human, pig, bovine and rat aggrecan (Fig. 2b), but no AF-28 immunoreactivity was detected with the shark substrate, suggesting that the ..DIPENFFGVG.. sequence is absent or modified in this primitive species. The ..DIPENFFGVG.. sequence is not found in avian aggrecan [33]. No AF-28 immunoreactiv-

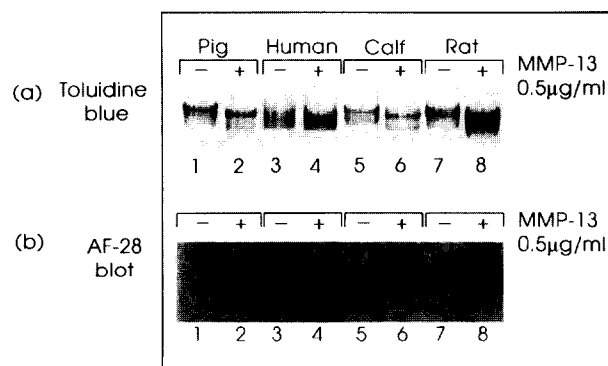


Fig. 2. Aggrecan (640 μ g/ml) purified from human, pig, calf, and rat chondrosarcoma cartilage was digested overnight with 0.5 μ g/ml trypsin-activated MMP-13. Aliquots of digested (lanes 2, 4, 6 and 8) and undigested (lanes 1, 3, 5 and 7) material were electrophoresed in duplicate on agarose/acrylamide composite gels. Based on their dye-binding content, 5 μ g of sample was loaded for toluidine blue staining (a) and 1 μ g was loaded for AF-28 immunoblotting (b).

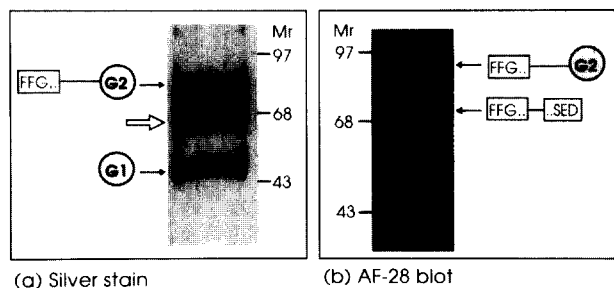


Fig. 3. Purified G1–G2 (106 µg) was digested with 148 µg/ml trypsin-activated MMP-13, followed by keratanase digestion and the pattern of digestion analysed on 5% SDS gels by silver staining (a) and AF-28 immunoblotting (b). The G1 and G2 globular domains are represented schematically. The FFG.. N-terminal sequences (determined experimentally) and the ..SED C-terminal sequences (predicted [17,25,45]) are also shown schematically. The unlabelled arrow indicates the band analysed by sequencing.

ity was detected in undegraded aggrecan as reported previously [17]. These results show that MMP-13 shares the same specificity for the ..PEN₃₄₁–FFG.. cleavage site in aggrecan as other members of the MMP family. Immunoblotting with monoclonal antibody BC-3, which recognises the neo-epitope created by cleavage at the aggrecanase site, did not reveal any immunoreactivity indicating that low concentrations of MMP-13 (0.5 µg/ml) did not cleave at the E₃₇₃–A₃₇₄ aggrecanase site in any of these species.

3.3. Digestion of G1–G2 with MMP-13

An aggrecan G1–G2 substrate [34] was digested with a high concentration of MMP-13 (160 µg/ml) to determine whether this enzyme was able to cleave at the aggrecanase site in the IGD. The pattern of digestion products was analysed by SDS-PAGE with silver staining, or Western blotting with monoclonal AF-28 or monoclonal BC-3. Three MMP-13 digestion products were detected by silver stain (Fig. 3a). The large G2-containing product (*M_r* approximately 85 kDa) and the fastest migrating G1 product (*M_r* approximately 50 kDa) have been identified previously [17] by sequencing [11,25] and immunoblotting [11]. The middle band was of a similar size to the 75 kDa aggrecanase fragment produced by MMP-8 digestion [25]. However, immunodetection with BC-3 monoclonal antibody, specific for the N-terminal ARGSVL.. sequence generated by cleavage at the aggrecanase site, was negative, indicating that even at high concentrations MMP-13 failed to cleave at the aggrecanase site. The identity of this band was therefore determined by N-terminal sequence analysis (see below).

AF-28 immunoblotting of the MMP-13 digested G1–G2 showed two products (Fig. 3b). The largest band represents the same 85 kDa G2 band that was detected by silver stain (Fig. 3a). A significantly smaller proportion of AF-28 epitope was present on a band of approximately 68 kDa. Based on its size and N-terminal sequence, this band is most likely to correspond with a fragment derived from the interglobular domain that is the product of cleavage at both the major and minor MMP sites ([17], fragment 6). As shown in Fig. 4, a minor activity of interstitial and neutrophil collagenases [14] and matrilysin [13] is cleavage at the minor MMP site. This action produces a 68 kDa IGD product similar to that seen in Fig. 3b, as well as a smaller 40 kDa G2 product ([17], fragment 4) with an LVVQV.. N-terminal sequence [14,25]. The MMP-13 di-

gests, however, did not show any 40 kDa G2 band on the silver stain (Fig. 3a), even though the enzyme appeared to have cleaved at the minor MMP site. One explanation for this is that the proportion of substrate cleaved at the minor MMP site was very low (based on the relative intensity of the two AF-28 bands; Fig. 3b). The relative abundance of the two AF-28 bands suggests that cleavage at this site is also a minor activity of MMP-13. A second explanation is that silver stain is significantly less sensitive than immunoblotting (based on the relative intensity of the 85 kDa G2 bands in Fig. 3a,b), and the amount of the 40 kDa G2 band was too low to detect by this method.

3.4. N-terminal sequence analysis of an MMP-13 digestion product

N-terminal sequencing of the unknown band identified on the silver-stained gel (Fig. 3a) revealed a VAL-PHE-GLU-xxx-xxx N-terminal sequence corresponding to MMP-13 cleavage at ..VKP₃₈₄ ↓ VFE.. (Fig. 4). This novel cleavage site in the aggrecan IGD has not been reported previously for any proteinase.

4. Discussion

The report by Flannery and Sandy [29] that MMP-13 is the predominant MMP expressed by chondrocytes under conditions of accelerated matrix resorption, raises important questions about the role of MMP-13 in normal and pathological aggrecan catabolism. Recent data has shown that in addition to the predominant involvement of aggrecanase [18,19,35], there is also a significant involvement of one or more MMPs in aggrecan degradation. MMP inhibitors can specifically block release of aggrecan from resorbing cartilage [16,36–38] but more compelling evidence for the involvement of MMPs in cartilage degradation is that N- and C-terminal aggrecan neo-epitope sequences, derived specifically from MMP activity, have been found in cartilage [12,15,39] and synovial fluids [17]. Since MMP-13 shares the same sequence specificity for an aggrecan substrate as other members of the MMP family, it must be considered as one of the candidate mediators of aggrecan degradation.

This study has identified a new cleavage site in the IGD. Cleavage between P₃₈₄ and V₃₈₅ in aggrecan has not been reported previously. Peptide bond cleavage at proline residues occurs during MMP-8 [40] and MMP-1 [41] autoproteolysis at P₂₅₀–I₂₅₁ and P₂₄₇–I₂₄₈, respectively. Interestingly MMP-13 does not cleave after proline in the hinge region, but at S₂₄₅–L₂₄₆

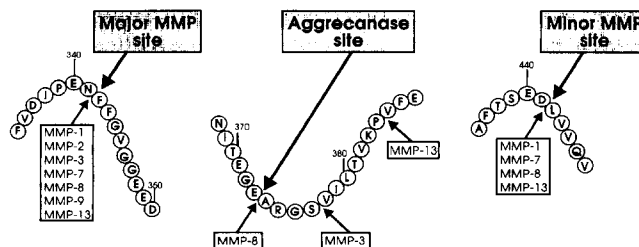


Fig. 4. Schematic representation of a non-continuous amino acid sequence in the aggrecan IGD showing the major MMP site that is cleaved by all MMPs, the aggrecanase site [20–22], the minor MMP site that is cleaved by matrilysin [13] and the collagenases [14,25] and additional minor sites that have been identified for MMP-3 [25] and MMP-13.

(Knäuper and Murphy, unpublished results). MMP-7 [42] and MMP-8 [43] have also been shown to cleave between proline and methionine residues. The cleavage at P₃₈₄–V₃₈₅ in aggrecan is clearly a minor activity of MMP-13 since high enzyme concentrations are required for cleavage at this site to occur. This is similar to the finding that, in vitro MMP-3 and MMP-8, at high concentration or in the presence of polyethyleneglycol, exhibit additional minor activities, cleaving at S₃₇₇–V₃₇₈ and E₃₇₃–A₃₇₄, respectively [25]. The in vivo significance of these minor MMP actions is not clear; there is no direct evidence in vivo for cleavage at sites in the IGD other than at the major MMP site and the aggrecanase site. However some studies have shown that at least seven different G1 fragments can be found in human articular cartilage extracts [44], suggesting that there are probably multiple and complex catalytic processes contributing to the net loss of aggrecan from cartilage.

There are currently no clues as to which of the MMPs in cartilage may be involved in aggrecan degradation. There is no way of discriminating between the actions of individual MMPs on the basis of their degradation products. Discrimination of this kind awaits the development of inhibitors highly specific for a single MMP. Similarly the presence of high levels of MMP mRNA in joint tissues may not be reflected in high levels of translated enzyme, and high levels of pro-enzyme may not result in high catalytic activity if the enzyme remains in its inactive pro-form. Thus the identity of the MMP(s) involved in aggrecan degradation and its mechanism of action relative to the putative aggrecanase enzyme is still to be resolved.

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