

Matrix metalloproteinases 19 and 20 cleave aggrecan and cartilage oligomeric matrix protein (COMP)

Jan O. Stracke^{a,*}, Amanda J. Fosang^b, Karena Last^b, Francesca A. Mercuri^b,
Alberto M. Pendás^c, Elena Llano^c, Roberto Perris^{d,e}, Paul E. Di Cesare^f, Gillian Murphy^a,
Vera Knäuper^a

^a*School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK*

^b*Orthopaedic Molecular Biology Research Unit, University of Melbourne, Department of Paediatrics and Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville, Vic. 3052, Australia*

^c*Departamento de Bioquímica y Biología Molecular, Universidad de Oviedo, 33006 Oviedo, Spain*

^d*Department of Evolutionary and Functional Biology, University of Parma, 43100 Parma, Italy*

^e*Division for Experimental Oncology 2, The National Cancer Institute Aviano, IRCSS, Aviano, Italy*

^f*Musculoskeletal Research Centre, Department of Orthopaedic Surgery, New York University-Hospital for Joint Diseases, New York, NY 10003, USA*

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Abstract Matrix metalloproteinase (MMP)-19 and MMP-20 (enamelysin) are two recently discovered members of the MMP family. These enzymes are involved in the degradation of the various components of the extracellular matrix (ECM) during development, haemostasis and pathological conditions. Whereas MMP-19 mRNA is found widely expressed in body tissues, including the synovium of normal and rheumatoid arthritic patients, MMP-20 expression is restricted to the enamel organ. In this study we investigated the ability of MMP-19 and MMP-20 to cleave two of the macromolecules characterising the cartilage ECM, namely aggrecan and the cartilage oligomeric matrix protein (COMP). Both MMPs hydrolysed aggrecan efficiently at the well-described MMP cleavage site between residues Asn³⁴¹ and Phe³⁴², as shown by Western blotting using neo-epitope antibodies. Furthermore, the two enzymes cleaved COMP in a distinctive manner, generating a major proteolytic product of 60 kDa. Our results suggest that MMP-19 may participate in the degradation of aggrecan and COMP in arthritic disease, whereas MMP-20, due to its unique expression pattern, may primarily be involved in the turnover of these molecules during tooth development. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Matrix metalloproteinases (MMPs) are a group of homologous zinc-dependent endopeptidases that can degrade all major components of the extracellular matrix (ECM). This class of enzymes has been implicated in connective tissue remodelling during normal and pathological conditions [1,2]. Elevated levels of some MMPs have been detected in the cartilage and

synovial fluid of arthritis patients, indicating a role for these enzymes during cartilage destruction in this type of degenerative disease and in vitro models support this role by showing increased MMP-mediated hydrolysis of cartilage macromolecules upon treatment with cytokines or retinoic acid [3–6].

MMP-19 and MMP-20 are newly identified members of the MMP family [7–9]. Despite their classical MMP domain structure, including a signal sequence, a latency domain, a catalytic domain and a COOH-terminal domain with sequence similarity to haemopexin, these enzymes lack a series of structural features distinctive of the diverse MMP subfamilies. Therefore, it was proposed that MMP-19 and MMP-20 belong to new MMP subfamilies. MMP-19 might play a role in arthritis since serum anti-MMP-19 autoantibody titres seem to be frequent among rheumatoid arthritis (RA) patients [10]. Furthermore, MMP-19 mRNA has been found constitutively expressed in RA and traumatic synovial membranes [11]. MMP-20 was cloned from odontoblastic cells, which are involved in matrix remodelling during enamel maturation, and the enzyme was shown to cleave the major component of teeth ECM, amelogenin [9].

Degradation of aggrecan, the major proteoglycan of the cartilage ECM responsible for the load-bearing and elastic properties of this tissue, is one of the earliest detectable events in arthritic cartilage degeneration. MMPs have been implicated in proteolysis and the subsequent loss of aggrecan from cartilage during arthritis [12–15]. The loss of this hyaluronan-bound macromolecule from cartilage tissue is due to proteolytic cleavage within the inter-globular domain (IGD) of its core protein, thereby releasing a major fragment containing most of the glycosaminoglycan chains into the joint fluid (Fig. 1). The major MMP cleavage site within the IGD is between residues N³⁴¹ and F³⁴². Stromelysins, collagenases, gelatinases, MT1-MMP and matrilysin cleave at this site, generating new NH₂- or COOH-terminal sequences that are recognised by specific neo-epitope antibodies [12,16–19]. Neo-epitope antibodies have been used to detect aggrecan fragments in cartilage and synovial fluid from RA patients, providing evidence for cleavage at this site in vivo and implicating MMPs in aggrecan turnover during arthritic disease [12,14,15,20,21]. Analysis of the aggrecan degradation products found in the joint space during arthritis indicated, however, that another distinct class of enzymes, hydrolysing the

*Corresponding author. Fax: (44)-1603-592250.
E-mail: j.stracke@uea.ac.uk

Abbreviations: MMP, matrix metalloproteinase; IGD, inter-globular domain; COMP, cartilage oligomeric matrix protein; ECM, extracellular matrix; RA, rheumatoid arthritis; OA, osteoarthritis

Glu³⁷³–Ala bond within the IGD, contributes towards the loss of cartilage aggrecan (Fig. 1). The enzymes responsible for this proteolysis were recently identified as members of the ADAMTS group of metalloproteinases [22,23]. Large hyaluronan-binding proteoglycans, like aggrecan, are also major components of the early tooth ECM and proteolytic processing by MMPs may play a part in the turnover of this specific ECM during development [24,25].

Another important component of the ECM of joint tissues, is cartilage oligomeric matrix protein (COMP), a pentameric protein predominantly expressed in cartilage and tendon [26,27]. This molecule plays an important role in the accumulation and integrity of the cartilage ECM. Fragments of COMP were found in serum and synovial fluid from RA and osteoarthritis (OA) patients [28–32], and MMPs have been implicated in the cleavage of COMP during the arthritides, since hydroxamic acid-based MMP inhibitor BB-94 inhibited degradation in a cartilage explant model [33,34].

To investigate the possible roles of MMP-19 and -20 in the progression of RA and the turnover of tooth ECM, respectively, we examined the catalytic domains of these two enzymes in terms of their ability to cleave aggrecan and COMP in vitro using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis.

2. Materials and methods

A chemiluminescence blotting substrate kit was purchased from Pierce, USA. Nitrocellulose membrane was from Amersham, USA. Agarose type HSC was from PS Park Scientific (Northampton, UK). Native pig aggrecan G1–G2 and recombinant human G1–G2 fragments have been obtained as described previously [35,36]. Bovine aggrecan (A1D1) was a generous gift from Professor Michael Bayliss and Dr. Jay Dudhia, Royal Veterinary College, London, UK. COMP was purified from human articular cartilage as previously described [27]. All other reagents were of analytical grade.

2.1. Expression, refolding and purification of the catalytic domains of MMP-19 and MMP-20

COOH-terminal deletion mutants of MMP-19 (pro $\Delta_{260-508}$ MMP-19) (GenBank/EBI accession number x92521) and MMP-20 (EMBL accession number y12779) were expressed in *Escherichia coli*, refolded and purified as described previously [7,9].

2.2. MMP cleavage of G1–G2 aggrecan, aggrecan and COMP

Digestion of native pig aggrecan G1–G2, recombinant human G1–G2 aggrecan and bovine aggrecan was performed at 37°C in buffer containing 50 mM Tris–HCl pH 7.6, 150 mM NaCl, 10 mM CaCl₂, 0.05% (v/v) Brij 35. Apart from recombinant human G1–G2, which was digested for 3.5 h, incubation was performed overnight. Bovine aggrecan (A1D1) was used at 100 μ g/ml, human and pig G1–G2 aggrecan were used at 250 μ g/ml in the cleavage assay. MMP-19 catalytic domain (4 μ M) and MMP-20 catalytic domain (30 μ M) were used at various concentrations (4 nM, 12 nM, 24 nM, 40 nM and 120 nM) in the assay. Cleavage was stopped by addition of EDTA and 1,10-phenanthroline to final concentrations of 10 mM and 2 mM, respectively. COMP was incubated in a time-course experiment at 37°C with MMP-19 and MMP-20 catalytic domain, respectively, in the above buffer using an enzyme/substrate ratio (w/w) of 1/20 prior to analysis by SDS–PAGE and Western blotting.

2.3. Western blot analysis of aggrecan cleavage

Samples were electrophoresed on 5% SDS gels or agarose–acrylamide composite gels [37] prior to electroblotting onto nitrocellulose membrane and analysed for AF-28 epitope (1:1000), ...DIPEN epitope (1:1000) and ...ITEGE epitope (1:1000). Characterisation of monoclonal antibody AF-28 [38], and polyclonal anti-ITEGE and anti-DIPEN antisera [36] have been described. Rabbit anti-mouse and goat anti-rabbit horseradish peroxidase conjugated immunoglob-

ulin were purchased from Dako (Denmark). The monoclonal antibody AF-28 detects the NH₂-terminal sequence FFGVG... of G2 fragments derived from MMP cleavage. Polyclonal anti-ITEGE antibody recognises the COOH-terminal epitope ...ITEGE of G1 fragments produced by aggrecanase cleavage. Polyclonal anti-DIPEN antibody recognises the COOH-terminal epitope ...DIPEN of G1 fragments produced by MMP cleavage.

3. Results

3.1. Digestion of native and recombinant G1–G2 aggrecan and whole aggrecan with the catalytic domains of MMP-19 and MMP-20

To determine the ability of MMP-19 and MMP-20 to cleave aggrecan at the major ...DIPEN³⁴¹–FFGVG... site identified for cleavage by other MMPs, two aggrecan G1–G2 fragments, one isolated from pig laryngeal cartilage [35] and the other expressed in a baculovirus expression system [36], were incubated with various amounts of these enzymes. The cleavage products were analysed by Western blotting to identify the generated fragments. Both MMP-19 and MMP-20 were able to digest native (data not shown) and recombinant G1–G2 efficiently (Figs. 2 and 3). Cleavage of both G1–G2 substrates by MMP-19 resulted in the appearance of a product of 55 kDa on silver-stained SDS gels at enzyme/substrate ratios of approximately 1/500 (data not shown). The size of the fragment coincides with the predicted mass of the MMP-generated G1 fragment. This was confirmed by Western blot analysis using the anti-DIPEN antibody (Figs. 2A and 3A, not shown for pig G1–G2). Digest of the recombinant G1–G2 substrate resulted in a product containing G2 of about 60 kDa, which was detected by the AF-28 antibody (Figs. 2B and 3B). In addition, cleavage of native pig G1–G2 by MMP-19 generates a product of about 120 kDa, corresponding with the size of the G2 fragment, as shown by SDS–PAGE and Western blot using the same antibody (data not shown). Western blot analysis using the anti-ITEGE antibody, recognising the aggrecanase cleavage site, revealed that neither MMP-19 nor MMP-20 were able to cleave at this site (data not shown). Here we showed that MMP-19 generates the same cleavage products from both substrates as MMP-20, as shown by Western blot analysis.

After employing the truncated aggrecan substrates, we investigated whether MMP-19 and MMP-20 also cleave native aggrecan within the IGD. Therefore, whole bovine aggrecan (A1D1) was incubated with the catalytic domains of MMP-19

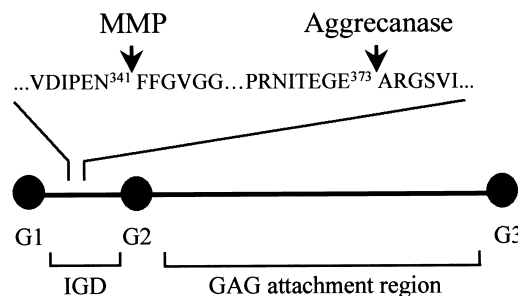


Fig. 1. Schematic representation of the cleavage sites in the aggrecan IGD. G1, G2 and G3 represent the globular domains of the aggrecan protein core and IGD is the inter-globular domain between G1 and G2. The main MMP and aggrecanase cleavage sites in the IGD are indicated with arrows.

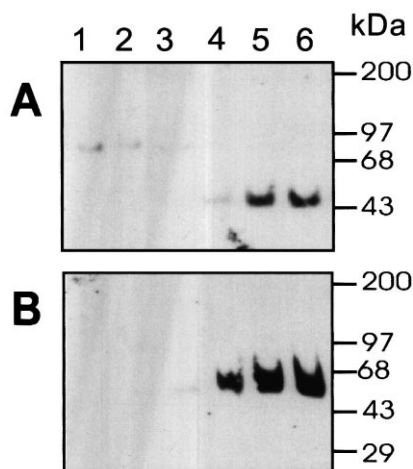


Fig. 2. Western blot analysis of the digestion of recombinant G1-G2 aggrecan (250 µg/ml) with the catalytic domain of MMP-19. Cleavage was performed for 3.5 h at 37°C with various concentrations of enzyme. A: Western blot analysis using Anti-DIPEN neo-epitope antibody. B: Western blot analysis using anti-FFGVG... neo-epitope antibody AF-28. The enzyme concentrations used, were: lanes 1: no enzyme, lanes 2: 4 nM, lanes 3: 12 nM, lanes 4: 24 nM, lanes 5: 40 nM, lanes 6: 120 nM.

and MMP-20 in a time-dependent fashion and analysed for the COOH-terminal G1 neo-epitope ...DIPEN (Fig. 4). The digestions generated G1 fragments that were immunodetected with the anti-DIPEN antibody. The fragment displayed a mass of approximately 60 kDa which corresponds to the G1 fragment of MMP-cleaved aggrecan.

These results show that MMP-19 and MMP-20 display the same specificity for the aggrecan IGD as other members of the MMP family [16,39]. Neither MMP-19 nor MMP-20 catalytic domain were able to cleave aggrecan at the aggrecanase site ($E^{373}-A^{374}$) since no signal was obtained by Western blotting

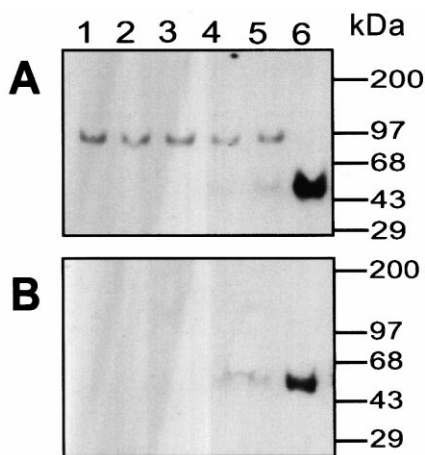


Fig. 3. Western blot analysis of the digestion of recombinant G1-G2 aggrecan (250 µg/ml) with the catalytic domain of MMP-20. Cleavage was performed for 3.5 h at 37°C with various concentrations of enzyme. A: Western blot analysis using Anti-DIPEN neo-epitope antibody. B: Western blot analysis using anti-FFGVG... neo-epitope antibody AF-28. The enzyme concentrations used, were: lanes 1: no enzyme, lanes 2: 4 nM, lanes 3: 12 nM, lanes 4: 24 nM, lanes 5: 40 nM, lanes 6: 120 nM.

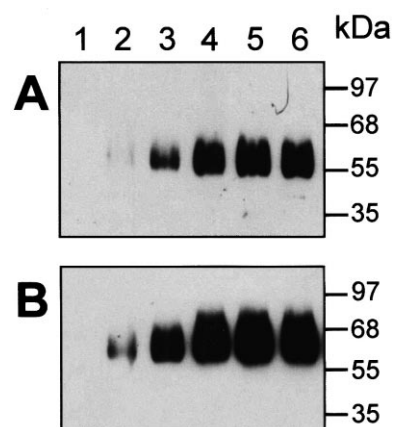


Fig. 4. Western blot analysis of the digestion of aggrecan (A1D1, 100 µg/ml) purified from bovine nasal cartilage with the catalytic domains of MMP-19 and MMP-20. Cleavage was performed overnight at 37°C with various concentrations of enzyme. Anti-DIPEN neo-epitope antibody was used for the detection of cleavage products. A: Digestion with MMP-19 catalytic domain. B: Digestion with MMP-20 catalytic domain. The enzyme concentrations used, were: lanes 1: no enzyme, lanes 2: 4 nM, lanes 3: 12 nM, lanes 4: 24 nM, lanes 5: 40 nM, lanes 6: 120 nM.

with anti-ITEGE antibody, which recognises aggrecanase-generated G1 neo-epitopes (data not shown).

3.2. Digestion of COMP with the catalytic domains of MMP-19 and MMP-20

The COMP, a pentameric protein involved in the crosslinking of cartilage components, was digested in a time-dependent manner with the catalytic domains of MMP-19 and MMP-20, using an enzyme/substrate ratio (w/w) of 1/20 (Fig. 5). Similar cleavage patterns were obtained with both enzymes. The 100 kDa monomer was degraded by both enzymes almost completely after 20 h of incubation to one major fragment of 60 kDa.

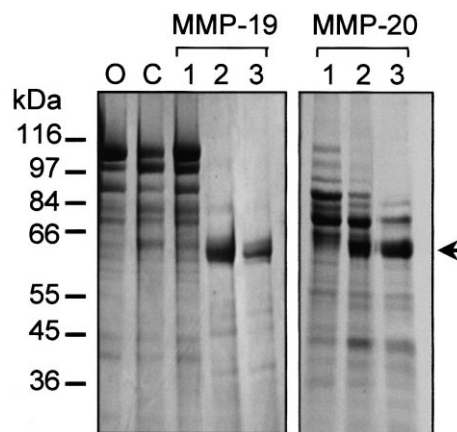


Fig. 5. SDS-PAGE (6%) analysis: COMP cleavage by the catalytic domains of MMP-19 and MMP-20 at 37°C. The enzyme/substrate ratio used in this experiment was 1/20 (w/w). Lane O: original protein, lane C: control, lanes 1: 1 h incubation, lanes 2: 5 h incubation, lanes 3: overnight incubation.

4. Discussion

In recent years MMPs have been implicated in the degradation of aggrecan since elevated levels of several MMPs are found in cartilage, synovial fluids and serum from arthritis patients. Furthermore, synthetic MMP inhibitors were shown to prevent aggrecan loss from cartilage. Neo-epitope antibodies recognising aggrecan fragments generated by the action of MMPs, were used to confirm the presence of specific MMP degradation products *in vivo* [14,15,20,21]. Furthermore, all MMPs tested exhibit the same specificity for the Asn³⁴¹–Phe bond located within the IGD of aggrecan (Fig. 1). Although the recently discovered aggrecanases 1 and 2, both members of the ADAMTS family [22,23], are thought to be the major players in aggrecan loss from cartilage, it is evident that MMPs have a distinct role in cartilage proteoglycan turnover [14,15,21,39]. The quantification of aggrecanase and MMP involvement in aggrecan loss has not been done and further studies are needed to determine which of these enzyme families plays the major role during pathological conditions and normal aggrecan metabolism. MMP-19 is a recently described member of the MMP family [8] and the mRNA was found associated with proliferating chondrocytes in the chondroepiphysis of mouse embryos during musculoskeletal development (Suneel Apte, personal communication). Since proliferating chondrocytes are a hallmark of cartilage destruction during arthritis, MMP-19 has been suggested to play a distinctive role in this process. Interestingly, MMP-19 mRNA is constitutively expressed in arthritic and traumatic synovial membranes [11], a tissue which expresses high levels of stromelysin-1 (MMP-3). In contrast to stromelysin-1, which is found in high concentrations as a latent zymogen in synovial fluid from arthritis patients [40], MMP-19 is able to autoactivate and could therefore exhibit a considerable impact on cartilage aggrecan [7].

MMP-20 is also a newly discovered MMP, cloned from odontoblastic cells and implicated in the matrix turnover during teeth development [9,41,42]. Since MMP-20 mRNA expression was only found in the enamel organ and not in RA cartilage or synovium, the enzyme may be involved during aggrecan turnover in the developing teeth [11,41,42].

In this study, the ability of both enzymes to cleave the large ECM proteoglycan aggrecan was investigated. MMP-19 and MMP-20 were both able to cleave native bovine aggrecan, pig G1-G2 and human recombinant G1-G2 within the IGD. Both enzymes demonstrate specificity for the MMP cleavage site Asn³⁴¹–Phe whereas they were not able to hydrolyse aggrecan at the aggrecanase site Glu³⁷³–Ala as shown by immunodetection of the generated neo-epitopes. The two enzymes therefore conform with the specificity profile of all other MMPs tested so far, for cleavage at the Asn³⁴¹–Phe bond. The possibility that these two enzymes hydrolyse aggrecan at other sites, as shown for other MMPs [16,39], remains to be investigated and could lead to new clues about the function of MMP-19 and MMP-20 during aggrecan metabolism in tissues where they are prominently expressed.

We could also show in this study that both, MMP-19 and MMP-20 were able to cleave the cartilage macromolecule COMP in a distinct manner. Although both MMPs generate one major cleavage product of about 60 kDa, MMP-19 seems to be slightly more efficient in cleaving COMP than MMP-20. COMP fragments in serum and synovial fluid from OA and

RA patients have earlier been identified as markers for these diseases [28–32]. It has been suggested that MMPs play a role in the hydrolysis of COMP and, therefore, compromise the integrity of the cartilage ECM structure leading to the ultimate loss of joint function [33,34].

However, the role of these two enzymes *in vivo* against aggrecan and COMP substrates is not known and further work is required to establish the function of the here-described MMPs in human disease.

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