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Membrane-type 1 MMP (MMP-14) cleaves at three sites in the aggrecan interglobular domain

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Abstract An aggrecan G1-G2 substrate was used to determine sites within the interglobular domain that were susceptible to cleavage by MT1-MMP. Degradation products were identified by Western blotting with neo-epitope antibodies specific for MMP-derived N- and C-terminal sequences. The results showed that MT1-MMP cleaved at the N_{341}-F_{342} and D_{441}-L_{442} bonds, as shown for other MMPs, and also at a site 13 amino acids C-terminal to the N_{341}-F_{342} site. The G2 product of this additional cleavage was identified by sequence analysis and revealed an N-terminus commencing T_{355}VxxPDVELPLP. The data are consistent with MT1-MMP cleavage at three sites in the aggrecan interglobular domain; one at N_{342}-F_{342}, a second at D_{441}-L_{442} and a third at Q_{354}-T_{355}.

Key words: Matrix metalloproteinase; Membrane-type matrix metalloproteinase; Aggrecan; Neo-epitope; Arthritis

1. Introduction

Matrix metalloproteinases (MMPs) are important enzymes involved in remodelling of the extracellular matrix under both normal and pathological conditions. A number of MMPs is expressed in cartilage and can be detected at elevated levels in synovial fluids from arthritis patients, suggesting a role for these enzymes in the cartilage destruction associated with joint disease. Other studies have demonstrated increased levels of MMPs in vitro under conditions where cartilage degradation is stimulated by treatment with cytokines and retinoids.

Aggrecan is the major proteoglycan in cartilage and is essential for tissue turgor and weight-bearing. Recent studies utilising neo-epitope antibodies, specific for the newly created N- or C-termini of degraded fragments, have shown that MMPs have a direct role in the proteolysis and subsequent loss of aggrecan from cartilage in arthritis [1–5]. The predominant site at which MMPs cleave in the aggrecan interglobular domain (IGD) is between N_{341} and F_{342}. The stromelysins, collagenases, gelatinases, and matrilysin are all active at this site [1,6–8]. MMP cleavage at N_{341}-F_{342} produces aggrecan fragments with N-terminal FFGVG... sequence and there is evidence for cleavage at this site in vivo [1–5]. The major high-buoyant-density aggrecan fragments found in human synovial fluids from osteoarthritis (OA) and joint injury patients, however, do not correspond with MMP cleavage products, but rather result from cleavage at the E_{373}-A_{374} bond in the IGD [9,10]. The putative enzyme responsible for this cleavage is 'aggrecanase' but its identity is unknown.

Sato and co-workers identified a novel membrane-type MMP (MT-MMP) by library screening and RT-PCR [11]. This first membrane-type MMP (MT1-MMP) was shown to induce specific activation of progelatinase A (MMP-2), thus identifying a potentially critical mechanistic step in the regulation of MMP activity in vivo. Subsequent studies have revealed that MT1-MMP can also activate procollagenase-3 (MMP-13) and that progelatinase A considerably potentiates this rate of activation [12]. Three additional membrane type MMPs, MT2-MMP [13], MT3-MMP [14] and MT4-MMP [15] have now been cloned. MT2-MMP and MT3-MMP are also able to activate progelatinase A [16,17].

MT1-MMP is of particular interest since MT1-MMP mRNA [18,19] and enzyme [18] are expressed in human OA cartilage, and immunohistochemical studies have shown that MT1-MMP localises to chondrocytes in the superficial and transitional zones, corresponding with regions of cartilage degradation [18]. MT1-MMP gene expression is also induced by IL-1α and TNFα [18] both known to promote cartilage degradation in vitro, but not by IL-1β [19]. In addition to its progelatinase A activating activity, MT1-MMP exhibits classic collagenase activity by digesting fibrillar collagens into characteristic 3/4 and 1/4 fragments [20]. Fibronectin, vitronectin, laminin, α_1-proteinase inhibitor, α_2-macroglobulin, and aggrecan are also substrates for MT1-MMP [20,21]. In this study we have determined the specificity of MT1-MMP for aggrecan cleavage in the IGD. We report that MT1-MMP cleaves at the N_{341}-F_{342} bond, typical of all MMPs investigated so far, at the D_{441}-L_{442} bond cleaved by MMP-2, MMP-7 and the collagenases and also at a novel site located approximately half way between the major MMP site and the $E_{373}\downarrow A_{374}$ aggrecanase site.

2. Materials and methods

Chemiluminescence blotting substrate kit and cysteine proteinase inhibitor E-64 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. Rabbit anti-mouse horseradish peroxidase

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Abbreviations: IGD, interglobular domain of aggrecan; MMPs, matrix metalloproteinases; MT-MMPs, membrane-type matrix metalloproteinases; OA, osteoarthritis; IL-1, interleukin-1; TNF, tumour necrosis factor; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; AEBSF, [4-(2-aminoethyl)benzene]sulfonylfluoride; PBS, phosphate-buffered saline; ΔMT1, deletion mutant of MT1-MMP lacks the COOH-terminal transmembrane and cytoplasmic domain (Δ Ala^{536}–Val^{582}) (see [20])

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(HRP) conjugated immunoglobulin was from Dako (Denmark). Polyvinylidene difluoride (PVDF) membrane was from Micron Separations Inc. (Westborough, MA, USA). ΔMT1-MMP [22] was purified as described previously [20]. ProMMP-2 was prepared from culture media conditioned by human gingival fibroblasts [23] and was a generous gift from Prof. G. Murphy, University of East Anglia, Norwich, UK. Characterisation of monoclonal antibody AF-28 [24] and monoclonal antibody BC-3 [25] has been described. Characterisation of polyclonal anti-VDIPEN antisera will be described elsewhere. All other reagents were of analytical grade.

2.1. Enzyme digestions

MMP digestions were in buffer containing 10 mM calcium chloride, 100 mM sodium chloride, 50 mM Tris-HCl, pH 7.5, for 16 h at 37°C, and were stopped by the addition of EDTA and 1,10-phenanthroline to final concentrations of 10 mM and 2 mM, respectively. Digested samples were treated with keratanase prior to analysis by SDS-PAGE. Keratanase digestions were in 50 mM Tris-acetate buffer, pH 7.2, at 37°C overnight with 0.02 units of keratanase/30 μl, in the presence of 10 mM EDTA, 20 μg/ml E64, 5 μM pepstatin and 1.25 mM AEBSF.

2.2. N-terminal sequence analysis of MT1-MMP digested G1-G2

For sequencing experiments, 150 μg G1-G2 was digested with 35.5 μg/ml ΔMT1-MMP for 24 h at 37°C in 10 mM calcium chloride, 100 mM sodium chloride, 50 mM Tris-HCl, pH 7.5. The sample was not treated with EDTA, 1,10-phenanthroline or keratanase but immediately freeze-dried, electrophoresed on 5% SDS gels [26] and transferred to PVDF membrane. G1-G2 fragments were visualised by staining with methylene blue and excised bands analysed on a Procise 491 Protein Sequencer (Perkin-Elmer, USA).

2.3. Western blot analysis

Samples electrophoresed on 5% SDS gels were transferred to PVDF membrane and analysed for AF-28 epitope (1:1000), ...DIPEN epitope (1:1000) and BC-3 epitope (1:1000). Monoclonal AF-28 detects the N-terminal FFGVG... sequence present on IGD fragments derived from MMP cleavage [24]. Polyclonal anti-DIPEN detects the C-terminal ...DIPEN sequence present on G1 fragments derived from MMP cleavage. Monoclonal BC-3 detects the N-terminal ARGSV... sequence present on IGD fragments derived from aggrecanase cleavage [25]. Prior to immunodetection with BC-3, glycosaminoglycan chains were removed by digesting the membrane 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 [25].

3. Results

3.1. Digestion of G1-G2 with ΔMT1-MMP and MMP-2

An aggrecan G1-G2 substrate [27] was digested with ΔMT1-MMP and MMP-2, separately and in combination, to determine the ability of ΔMT1-MMP to cleave aggrecan in the IGD. The pattern of digestion products was analysed by SDS-PAGE with silver staining, and fragments identified by Western blotting (Fig. 1). Each band in Fig. 1 has been numbered and the fragments represented schematically in Fig. 2. MMP-2 digestion of G1-G2 without ΔMT1-MMP (Fig. 1a, lane 4), yielded a 50-kDa G1 product (fragment 1) and an 85-kDa G2 product (fragment 2), as seen by silver stain. These products have been characterised previously for MMP-2 [7] and several other MMPs [6,7,24,28,29]. When ΔMT1-MMP was included with MMP-2 in the digests (Fig. 1a, lanes 5 and 6), a third band, fragment 3, was visible by silver stain. The size of fragment 3 was comparable with the size of small G2 products from MMP-13 [29] and MMP-8 [30] digests.

Digestion of G1-G2 with ΔMT1-MMP in the absence of MMP-2 (Fig. 1a, lanes 2 and 3) produced G1 domain (fragment 1), a small amount of the large G2 domain (fragment 2), and significant amounts of fragment 3. This pattern of silver-stained fragments suggests that ΔMT1-MMP cleaves at

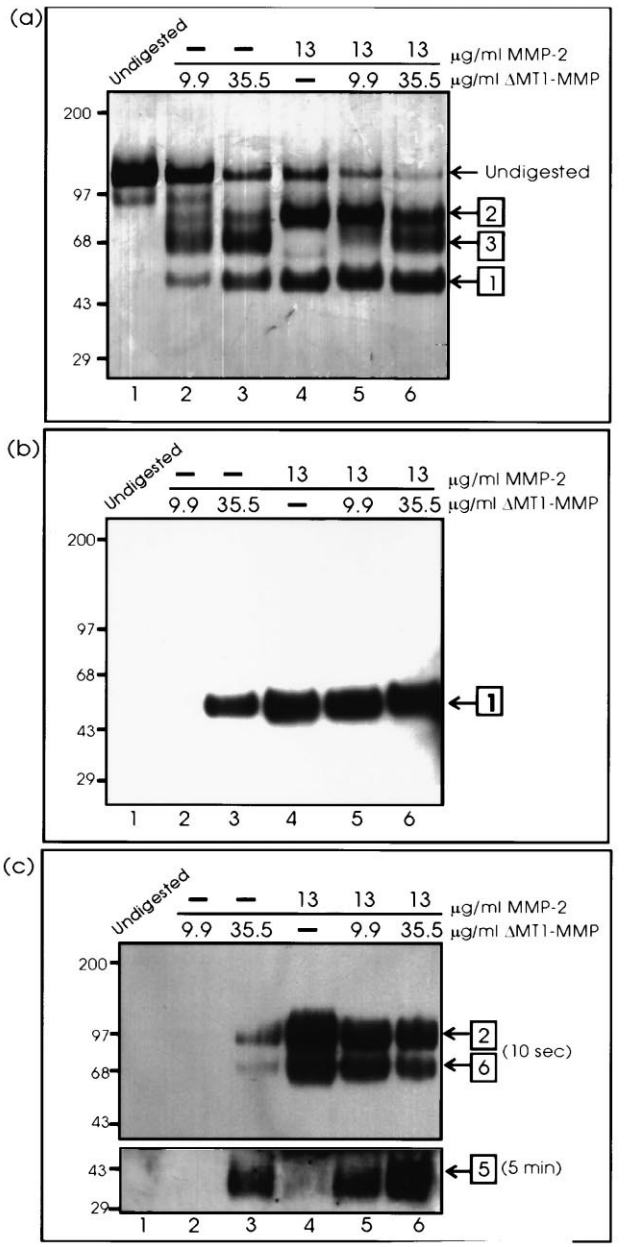

Fig. 1. Digestion of G1-G2 with MT1-MMP and MMP-2. Purified pig G1-G2 was digested for 16.5 h at 37°C with 9.9 μg/ml (lanes 2 and 5) or 35.5 μg/ml (lanes 3 and 6) MT1-MMP. MMP-2 was included in lanes 5 and 6, at a final concentration of 13 μg/ml. Lane 4: MMP-2 alone at 13 μg/ml. Samples were electrophoresed on 5% SDS gels and analysed by silver stain (a), or Western blotting with anti-...DIPEN antisera (b), or monoclonal antibody AF-28 (c). c: AF-28 immunoreactive bands developed with chemiluminescence reagent were exposed to X-ray film for either 10 s (upper panel) or 5 min (lower panel).

$PEN_{341}\downarrow FFG$ to generate fragments 1 and 2, and also at another site in the IGD to generate fragment 3. The results show that fragment 3 is a product of ΔMT1-MMP digestion, but not MMP-2 digestion.

Immunoblotting with polyclonal antisera specific for the ...DIPEN C-terminus revealed a single band of approximately 57 kDa in both the ΔMT1-MMP and MMP-2 digested samples (Fig. 1b). These results show that ΔMT1-MMP cleaves

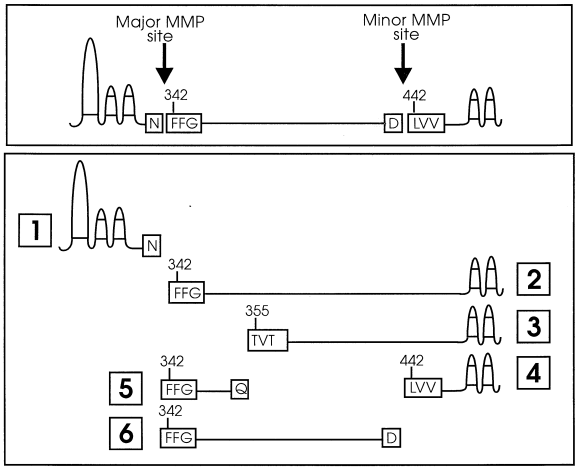

Fig. 2. Products of MT1-MMP cleavage of aggrecan G1-G2. Schematic representation of aggrecan G1-G2 substrate showing the products of ΔMT1-MMP digestion, identified by Western blotting (fragments 1, 2, 5 and 6), sequence analysis (fragment 3), or predicted (fragment 4). Fragment 4 is predicted based on identification of fragment 6, and in comparison with the products of MMP-1, -7 and -8 digestion [7,8,30].

the N_{341}-F_{342} bond in the IGD to generate a G1 fragment with a ...DIPEN C-terminus.

AF-28 immunoblotting of the MMP-2 digested G1-G2 showed two major products (Fig. 1c, upper panel, lane 4). The largest band represents the same 85-kDa G2 band that was detected by silver stain, and is therefore fragment 2. A proportion of AF-28 epitope was present as a band of approximately 68 kDa, and is labelled fragment 6. Based on its size and N-terminal sequence, this band most likely corresponds with a fragment derived from the interglobular domain that is the product of cleavage at both the major ($PEN_{341}\downarrow FFG$) and minor ($SED_{441}\downarrow LVV$) MMP cleavage sites, and has been described previously [8,24,30]. Note that fragment 3, which is approximately the same size but lacks the AF-28 epitope is readily detected by silver stain because of its G2 globular domain. Fragment 6 on the other hand is readily detected by AF-28 but poorly detected by silver stain because it lacks a globular domain.

When the AF-28 immunoblot was allowed to develop with the ECL reagent for a longer period of time (Fig. 1c, lower panel), a low molecular weight AF-28 band was seen in samples digested with ΔMT1-MMP, with and without MMP-2, but not in samples digested with MMP-2 only. This fragment with its F_{342}FGVG... N-terminus has been labelled fragment 5. The most likely interpretation of the data is that fragment 5 represents the N-terminal portion of fragment 2, which is cleaved by ΔMT1-MMP to produce fragment 3 (Fig. 2). To determine whether ΔMT1-MMP was able to hydrolyse the E-A bond, Western blots were carried out with monoclonal BC-3, specific for the ARGSVI... N-terminus on aggrecanase-derived fragments. No bands were detected with monoclonal BC-3 (data not shown).

3.2. *N-terminal sequence analysis of the ΔMT1-MMP digestion product*

N-terminal sequencing of fragment 3 was done in order to identify which peptide bond in the IGD was cleaved by ΔMT1-MMP to produce fragments 3 and 5. The analysis revealed that the N-terminus of fragment 3 was TVxxPDVELPLP, corresponding to ΔMT1-MMP cleavage at $Q_{354}\downarrow T_{355}$. This novel cleavage site in the aggrecan IGD has not been reported for other MMPs or any proteinase Fig. 3.

4. Discussion

The localisation of MT1-MMP to areas of articular cartilage undergoing resorption in OA tissue has highlighted the potential role of this enzyme in joint pathology [18]. In addition to the involvement of aggrecanase in arthritic disease [9,10,31], there is also significant involvement of one or more MMPs in aggrecan degradation. MMP inhibitors can specifically block release of aggrecan from resorbing cartilage [32–34] but more compelling evidence for MMP involvement is that N- and C-terminal aggrecan neo-epitope sequences have been found in cartilage by sequencing [1] and immuno-

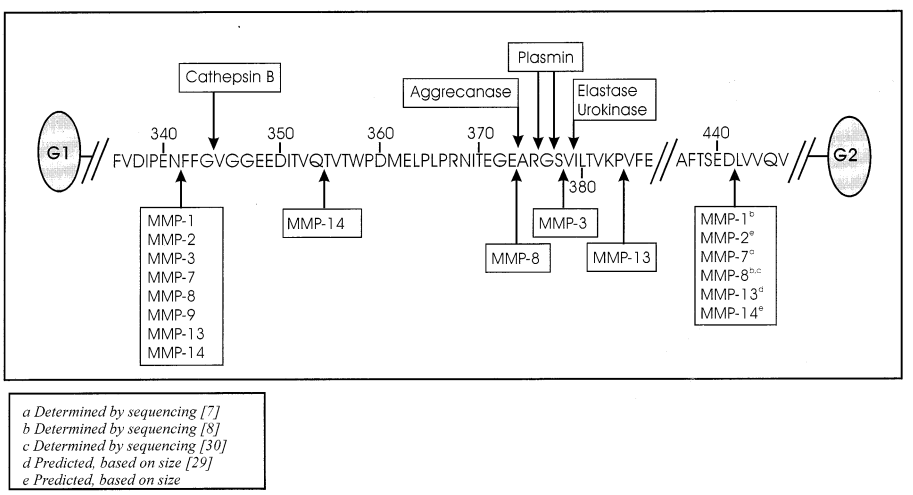

Fig. 3. MMP and proteinase cleavage sites in the aggrecan IGD. A portion of the proteinase-sensitive amino acid sequence (human [39]) located between the G1 and G2 domains of aggrecan is shown and includes the known cleavage sites for MMPs, plasmin, urokinase, cathepsin B and elastase.

localisation [2,4,5] experiments, and in synovial fluids by immunoassay [3]. Since MT1-MMP shares the same sequence specificity for cleavage at $N_{341}\downarrow F_{342}$ as other members of the MMP family, it must be considered as one of the candidate mediators of aggrecan degradation.

The cleavage at $Q_{354}\downarrow T_{355}$ by MT1-MMP occurred 13 amino acids C-terminal to the major $N_{341}\downarrow F_{342}$ site. Detection of threonine in the first sequencing cycle shows that T_{355} is not substituted with keratan sulphate or other *O*-linked oligosaccharides in pig laryngeal aggrecan. However, T_{357} at cycle three was not detected, suggesting that this amino acid may be substituted with keratan sulfate as has been shown for aggrecan from bovine articular cartilage [35].

Cleavage at $Q_{354}\downarrow T_{355}$ appears to be more substantial than the secondary cleavages at $S_{377}\downarrow V_{378}$, $E_{373}\downarrow A_{374}$ and $P_{384}\downarrow V_{385}$ by MMP-3 [30], MMP-8 [30] and MMP-13 [29], respectively. High in vitro concentrations of MMP-3, MMP-8 and MMP-13 were required to achieve cleavage at these minor sites, and the products were present in correspondingly minor amounts. Furthermore cleavage at the minor sites occurred subsequent to complete cleavage at $N_{341}\downarrow F_{342}$. In contrast, for DMT1-MMP digestions, a significant proportion of fragment 3 was produced concomitantly with production of G1 and the 85-kDa G2 fragment, and with only partial cleavage of the native G1-G2 substrate (Fig. 1a, lane 2), suggesting that there is no requirement by MT1-MMP for a prior cleavage at $N_{341}\downarrow F_{342}$. Büttner et al. using an artificial recombinant substrate rAgg1 [36], have also shown that compared with MMP-3 and MMP-8, cleavage at the $N_{341}\downarrow F_{342}$ site by MT1-MMP was significantly less [37].

Although the molecular identity of aggrecanase is unknown, MMP-8 [30] and the snake venom metalloproteinase atrolysin C [38] have been shown to cleave at the $E_{373}\downarrow A_{374}$ aggrecanase site in the IGD. We were unable to detect aggrecanase products in MT1-MMP digests of G1-G2, by sequencing or by BC-3 Western blotting, in contrast to Büttner et al. who found that MT1-MMP generated BC-3 epitope following cleavage of the rAgg1 substrate [37]. The discrepancy in the results may reflect the source of recombinant enzymes or the nature of the substrates.

Whether MT1-MMP cleaves aggrecan in vivo is not known. Some AF-28 fragments detected in human synovial fluids are of a size similar to fragment 5 [3]. Because these small AF-28 fragments lack the ...$ITEGE_{373}$ C-terminus, an enzyme other than aggrecanase must be cleaving in close proximity to the aggrecanase site. Further work is needed to determine whether cleavage by MT1-MMP at $Q_{354}\downarrow T_{335}$ generates fragments of this type in human disease.

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