ADAMTS4 (AGGREGANASE-1) CLEAVES AT BOTH THE AGGREGANASE SITE (GLU373-ALA374) AND THE MATRIX METALLOPROTEINASE SITE (ASN341-PHE342) IN THE AGGREGAN INTERGLOBULAR DOMAIN

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INTRODUCTION

In diseases characterized by cartilage degradation, like rheumatoid arthritis and osteoarthritis, increased aggrecan release from the cartilage occurs early and before the bulk of the collagen network is attacked. Proteolytic cleavage of aggrecan within the interglobular domain (IGD) separates the GAG-rich region from the HA-anchored G1 domain resulting in GAG release from the cartilage matrix to the synovial fluid. Biomechanical tests on cartilage discs have shown it is the proteolytic attack at this IGD site that is primarily responsible for the detrimental loss of the mechanical properties of cartilage. If uncontrolled, this process ultimately leads to complete loss of cartilage tissue from the bone end and joint dysfunction.

Identification of the proteinases responsible for this “destructive” cleavage of aggrecan has therefore been a major focus of experimentation in arthritis-related research.

In this regard, two major cleavage sites that occur in vivo have been identified in the IGD of human aggrecan. One is the matrix metalloproteinase (MMP)/Cathepsin B-sensitive site at VDIPEN341*F342FG, where “*” represents the scissile bond. The second site in the IGD lies at NITEGE373*A374RGs (where “s” represents the scissile bond). Because of the high degree of structural homology between the IGD and the interglobular domain (IGD) of aggrecan, we have encountered reports that indicate the MMP/Cathepsin B-sensitive site at VDIPEN341*F342FG is a preferred site for the generation of aggrecan fragments.

A review of the literature on this subject shows there is now a general consensus that cleavage at the aggrecanase site (Glu373-Ala374) is due to ADAMTS activity whereas cleavage at the matrix metalloproteinase site (Asn341-Phe342) is due to MMP or perhaps Cathepsin B activity. Indeed, it has become common practice in many laboratories to use neoepitope antibodies recognizing aggrecan fragments with VDIPEN341 and F342FG at terminal sequences to monitor MMP activity in situ and likewise antibodies to NITEGE373 and A374RGS to monitor aggrecanase (ADAMTS) activity.

With the availability of recombinant ADAMTS4 with high specific activity, we have now re-examined the activity of ADAMTS4 against the human aggregan IGD. Remarkably we have now shown that this enzyme cleaves at both IGD sites and further this dual specificity is also seen with recombinant G1-G2 substrate.

EXPERIMENTAL PROCEDURES

Human Aggrecan Digests

To analyze the sites cleaved within human aggrecan, rADAMTS4 (kindly provided by Genetics Institute, Boston, MA) or MMP3 (kindly provided by Merck) was incubated with normal mature human aggrecan, (from Dr Peter Roughley) at 37ºC in digestion buffer (20 mM Tris, pH7.5, 100 mM NaCl, 10 mM CaCl2). These samples were analyzed by Western analysis using antibodies specific for the aggrecan G1 domain (G1), and the affinity purified C-terminal neoepitopes generated by cleavage after Asn341 (VDIPEN) and Glu373 (NITEGE) (from Dr. John Mort).

**Inhibitor Studies**

To test inhibition by TIMP-1 and TIMP-2, rADAMTS4 (54 nM) or MMP3 (44 nM) was incubated with TIMP-1 (kindly provided by Merck) and TIMP-2 (kindly provided by Dr. Gillian Murphy) at either 125, 250, 750 nM. For incubations containing EDTA (10 nM) or MMP inhibitor II, (100 or 500 nM, Calbiochem), the inhibitor was added to either 10 nM ADAMTS4 or 44 nM MMP3. All digests were analyzed by Western analysis.

**Digests of Recombinant G1-G2**

Recombinant G1-G2 substrates, either wildtype, A374RG to NVY mutant, or ΔE340NFF deletion mutant, were expressed and purified as described previously. For digests, rADAMTS4 (381 nM) was incubated with recombinant G1-G2 substrate (1.4 uM) in digestion buffer at 37 degrees C for 16 hours. The samples were then analyzed by Western analysis. The antibodies used were specific for the aggrecan G1 domain, the C-terminal neoepitopes generated by cleavage at Asn341 (affinity purified anti-VDIPEN from A. Fosang), or cleavage at Glu373 (affinity purified anti-NITEGE from J. Mort), and the N-terminal neoepitope generated by cleavage at Phe342 (monoclonal antibody, anti-FGG from A. Fosang).

RESULTS

rADAMTS4 cleaves human aggrecan at the VDIPEN341-F342FG site

A timecourse analysis of rADAMTS4 digestion of human aggrecan reveals that while the hallmark aggrecanase product, G1-NITEGE, is primarily generated, rADAMTS4 can also cleave at the VDIPEN341-F342FG site. These G1VDIPEN fragments, previously thought to be MMP-derived products, are generated by rADAMTS4 as early as 2 hours.

The MMP-like activity of rADAMTS4 is not due to contaminating MMPs or Cathepsin B

Inhibition studies using MMP inhibitor II, TIMP-1 and TIMP-2 reveal that these inhibitors do not prevent the generation of G1-VDIPEN by rADAMTS4 even at concentrations known to inhibit MMPs, such as MMP3. Instead, these inhibitors actually showed a slight activation of the rADAMTS4 aggrecanase activity including the formation of the G1-VDIPEN product. Additionally, EDTA completely inhibited all of the rADAMTS4 aggrecanase activity including cleavage at the VDIPEN341-F342FG site.

rADAMTS4 cleaves at VDIPEN341-F342FG independently of cleaving the NITEGE373-A374RG site

Wild type and mutant recombinant aggrecan G1-G2 protein was used to analyze the ability of rADAMTS4 to cleave at the VDIPEN341-F342FG and NITEGE373-A374RG sites. Both the G1-NITEGE and G1-VDIPEN fragments were formed from rADAMTS4 digestion of the wildtype substrate indicating that both IGD sites are cleaved in the recombinant G1-G2 substrate. To test whether rADAMTS4 can cleave at these two sites independently, two G1-G2 mutants were tested in which one of the two sites was eliminated by mutagenesis. When the mutant substrate containing deletions in the VDIPEN341-F342FG site was digested with rADAMTS4, only the G1-NITEGE fragment was generated. This supports previous work showing that rADAMTS4 can cleave at this aggrecanase site independent of cleavage at the MMP site.

SUMMARY

The results presented show for the first time that rADAMTS4 can cleave aggrecan at both the aggrecanase and MMP sites within the IGD. This dual specificity of ADAMTS4 is similar to what has been observed with atrolysin C, MTI-MMP and MMP8. This work, however, contradicts what has previously been reported for the specificity of ADAMTS4 on aggrecan degradation. With regard to the likely physiological relevance of this finding, analysis of the VDIPEN-generating ability of ADAMTS4 relative to equal concentrations of MMP3 suggests that ADAMTS4 exhibits about 10% of the activity shown by MMP3. This surprising result implies that ADAMTS activity alone may be responsible for generating both G1-NITEGE and G1-VDIPEN species present in human cartilage. Additionally, the many conclusions based on these antisera concerning the relative importance of aggrecanases and MMPs in cartilage aggrecanalysys may now require re-evaluation.

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