SITES OF CLEAVAGE OF AGGRECAN BY THE RECOMBINANT HUMAN AGGRECAN-DEGRADING-METALLO-PROTEASE (ADMP), "AGGRECANASE"

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RELEVANCE TO MUSCULOSKELETAL DISEASE

Joint destruction in arthritic diseases involves degradation of articular cartilage aggrecan. These studies characterize the cleavage sites and temporal sequence of cleavage of the aggrecan core protein by the novel cartilage protease, "aggrecanase."

INTRODUCTION

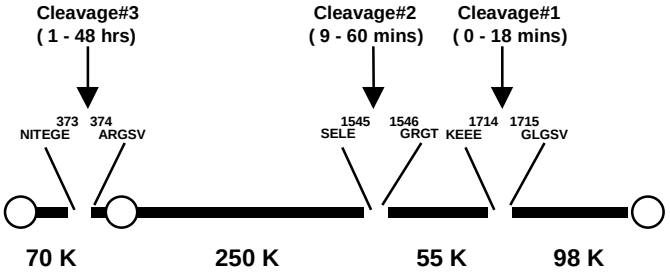
Aggrecan is the major proteoglycan of cartilage and provides this tissue with its mechanical properties of compressibility and elasticity. In cartilage degradation associated with diseases such as osteoarthritis, aggrecan is one of the first matrix components to undergo measurable loss. Two major sites of proteolytic cleavage have been identified within the interglobular domain (IGD) of the aggrecan core protein, one between amino acids ASN^{341}-Phe^{342} which is cleaved by MMPs and the other between Glu^{373}-Ala^{374} which is attributed to "aggrecanase." Aggrecanase is novel aggrecan-degrading protease thought to play a role in the cartilage destruction that occurs in arthritic diseases. This enzymatic activity is characterized by cleavage, between amino acid residues Glu^{373}-^{374}Ala. However, demonstration of the ability of aggrecanase to cleave at additional proposed sensitive sites within the C-terminus of the aggrecan core protein awaited isolation and purification of this protease. Here we use the recombinant human "aggrecan-degrading-metallo-protease" (ADMP) representing "aggrecanase" to evaluate cleavage of both bovine and human aggrecan.

METHODS

Enzyme digestion — Digestions were carried out in 100 ul of 50 mM Tris/HCl buffer, pH 7.5, containing 100 mM NaCl and 10 mM $CaCl_2$. Isolated bovine or human aggrecan (500 nM) was incubated with rADMP at 37°C for the various times**.** Following incubation, the reactions were quenched with EDTA. ***N-terminal sequencing*** — Aggrecan fragments digested with ADMP were N-terminally sequenced at Argo Bioanalytica Inc. (Morristown, NJ) ***Neoepitope Antibodies***—Monoclonal neoepitope antibodies BC-3 [1] and AF-28 [2] were from Dr. Bruce Caterson (University of Wales) and Dr. Amanda Fosang (University of Melbourne). Polyclonal neoepitope antibodies to the peptide sequences ASTASELE and PTTLKEEE on the C-terminus of aggrecan fragments generated by cleavage at the Glu^{1545}-Gly^{1546} bond and at the Glu^{1714}-Gly^{1715} bond, respectively, were prepared by Quality Controlled Biochemicals (Hopkinton, MA). ***Analysis of aggrecan products*** — For analysis of fragments by Western blot, aggrecan was enzymatically deglycosylated with chondroitinase ABC (0.1 units/10 ug of aggrecan) and with keratanase (0.1 units/10 ug of aggrecan) and keratanase II (0.002 units/10 ug of aggrecan) for 3 hours at 37°C in buffer containing 50 mM sodium acetate, 0.1 M Tris/HC1, pH 6.5. ***Western blot analysis*** — 20 ug of glycosaminoglycan from each sample was loaded on a 4-12% Tris/glycine gel and separated by SDS PAGE under reducing conditions. The separated proteins were transferred to PVDF membranes and immunolocated with neoepitope antibodies.

RESULTS AND DISCUSSION

Recombinant ADMP cleaves preferentially at two sites within the chondroitin-sulfate rich region of the aggrecan core protein, between G2 and G3. This cleavage occurs prior to the cleavage within the IGD at the Glu^{373}-^{374}Ala bond that produces BC-3-reactive fragments with the N-terminus ARGS. Cleavage occurred first at the $KEEE^{1666}$-^{1667}GLGS bond to produce both a small C-terminal fragment and a 375 kDa fragment, which retains an intact G1. This was followed by cleavage at the $SELE^{1545}$-^{1546}GRGT bond to produce a 55 kDa C-terminal fragment and a G1-containing fragment of 320 kDa. Cleavage of this 320 kDa fragment within the IGD at the Glu^{373}-^{374}Ala bond then occurred to release the 250 kDa C-terminal, BC-3-reactive fragment from the G1 domain. However, ADMP did not cleave aggrecan at the Asn^{341}-^{342}Phe bond under any conditions tested. This is consistent with the profile of endogenous aggrecanase generated in chondrocyte cultures stimulated with IL-1 or retinoic acid where aggrecanase is apparently capable of cleaving at the Glu^{373}-Ala^{374} bond in the complete absence of any detectable cleavage at the MMP site [3].



The rate of cleavage at the Glu^{1714}-^{1715}Gly site was very rapid, being detected immediately upon addition of the enzyme and complete in as little as 18 min. Cleavage at the Glu^{1545}-^{1546}Gly site was also very rapid, with cleavage being detected by 9 min and complete by 1 h. In contrast, cleavage at the BC-3 site Glu^{373}-^{374}Ala was first detected at 1 h and was not complete until 48 h (Figure). Examination of aggrecan cleavage in IL-1-stimulated bovine articular cartilage cultures demonstrated that cleavage occurs at the same sites as that produced by recombinant ADMP. Thus, our data demonstrate that recombinant ADMP 1) cleaves within the interglobular domain of aggrecan at the Glu^{373}-^{374}Ala bond and not at the Asn^{341}-^{342}Phe bond, 2) that initial cleavage occurs within the C-terminal domain of aggrecan to produce a truncated form of the molecule which is then released from the G1 domain by cleavage at the Glu^{373}-^{374}Ala bond, and 3) that cleavage *in situ*, occurs at the same sites as that produced by recombinant ADMP, suggesting that this protease is responsible for cartilage aggrecan cleavage.

REFERENCES

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