Osteoarthritis and Cartilage (2008) **16,** 1245–1252

doi:10.1016/j.joca.2008.02.013

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Characterization of an ADAMTS-5-mediated cleavage site in aggrecan in OSM-stimulated bovine cartilage

M. Durigova M.Sc.†, P. Soucy B.Sc.†, K. Fushimi Ph.D., Dr.‡, H. Nagase Ph.D., Dr.‡, J. S. Mort Ph.D., Dr.†* and P. J. Roughley Ph.D., Dr.†

† *Shriners Hospital for Children, 1529 Cedar Avenue, Montreal, Quebec H3G 1A6, Canada*

‡ *Kennedy Institute of Rheumatology, Imperial College London, London W6 8LH, United Kingdom*

Summary

Objective: In a previous study, we identified a 50-kDa G3-containing aggrecan degradation product in bovine cartilage, released from the tissue after interleukin-1 (IL-1) stimulation in the presence of oncostatin M (OSM). Our objective was to purify, determine the N-terminal sequence of this fragment and verify whether this cleavage could be attributed to a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and ADAMTS-5 action *in vitro*.

Methods: Collected media from bovine cartilage explant cultures stimulated with IL-1 + OSM were subjected to anion-exchange chromatography. The N-terminal sequence of the fragment of interest in the purified fractions was determined by automated Edman sequencing. Fetal bovine aggrecan was digested with full-length recombinant ADAMTS-4 and ADAMTS-5 and resulting degradation products were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblotting using an anti-G3 antiserum and an anti-neoepitope antibody that had been generated to the new N-terminus of the G3 fragment.

Results: Characterization of the 50-kDa fragment showed that it possesses chondroitin sulfate (CS) and is the result of a cleavage within the C-terminal portion of the CS-2 domain, adjacent to the G3 region. Sequence analysis identified the cleavage region as TQRPAE^{2047}_^{2048}ARLEIE, suggesting an aggrecanase-derived product. Using an anti-neoepitope antibody specific for the additional cleavage site, it was shown that the product is generated *in vitro* upon digestion of aggrecan by ADAMTS-5 and, to a much lesser extent, by ADAMTS-4.

Conclusions: The abundance and rapid rate of release of this degradation product in organ cultures in the presence of OSM suggest that it could result from a unique aggrecan proteolysis mediated by aggrecanases.

Key words: Aggrecan, ADAMTS, Cytokine-mediated proteolysis, Oncostatin M, Interleukin-1.

Introduction

Aggrecan is the most abundant proteoglycan in the extracellular matrix (ECM) of articular cartilage, where it is bound non-covalently to a hyaluronan (HA) filament forming large, negatively charged macromolecular proteoglycan aggregates that are responsible for the hydration of the cartilage and therefore the capacity of the tissue to withstand the deformation and compressive forces to which it is exposed. Aggrecan consists of a 250-kDa core protein composed of three globular domains (G1, G2 and G3) and an extended region consisting of three sub-domains, which are heavily substituted with keratan sulfate (KS) and chondroitin sulfate (CS) glycosaminoglycan (GAG) side chains (Fig. 1, panel A)[1,2]. At the N-terminus, the G1 domain is responsible for the non-covalent binding of aggrecan to HA[3], an interaction that is further stabilized by a small glycoprotein termed the link protein (LP) which binds to both the G1 domain and HA. The G2 domain, connected to the G1 domain through a 140 residue interglobular domain (IGD), shares structural homology with the G1-HA binding region, but lacks the ability to bind to HA[3,4]. The G3 domain is located at the C-terminus of the molecule and is structurally different from both G1 and G2 domains. The extended region between the G2 and G3 domains is substituted by KS (KS-rich domain) and a very large number of CS (CS-1 and CS-2 domains) side chains.

Depletion of aggrecan from cartilage is a key-event in many arthritic diseases. This is the result of proteolytic cleavage along the aggrecan core protein by proteases that are present within the joint. Matrix metalloproteinases (MMPs), such as MMP-1, MMP-3, MMP-8 and MMP-13[5–9], as well as aggrecanases, members of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family, such as ADAMTS-1, ADAMTS-4 and ADAMTS-5[10–12], have been shown to cleave aggrecan at sites within the IGD that are specific for each enzyme family. Cleavage at these sites results in the loss of aggrecan fragments bearing the GAG side chains and therefore the loss of the osmotic properties of the tissue. Among these enzymes, ADAMTS-4 and ADAMTS-5 are considered to be the most likely candidates for aggrecan degradation under arthritic conditions, as they have been shown to be expressed in arthritic cartilage, to be localized in areas of aggrecan depletion, and to have the highest aggrecanolytic activities *in vitro*. Four ADAMTS-4 and

*Address correspondence and reprint requests to: Dr John S. Mort, Shriners Hospital for Children, Joint Diseases Laboratory, 1529 Cedar Avenue, Montreal, Quebec H3G 1A6, Canada. Tel: 1-514-282-7166; Fax: 1-514-842-5581; E-mail: jmort@shriners.mcgill.ca

Received 29 November 2007; revision accepted 17 February 2008.

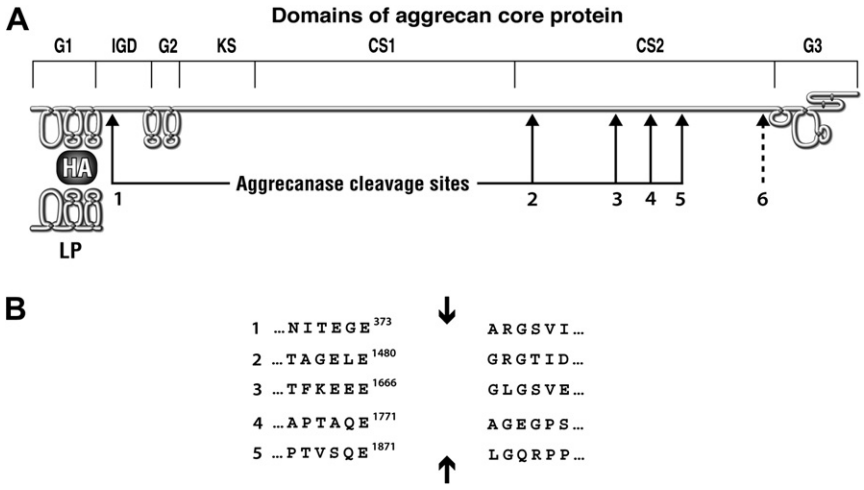

Fig. 1. Aggrecanase cleavage sites in bovine aggrecan. (A) Schematic representation of the bovine aggrecan core protein and its constituent domains: G1, IGD, G2 and G3, and the GAG-substituted domains: KS and CS-1 and CS-2 domains. Previously described aggrecanase cleavage sites are indicated by numbered arrows (1–5). Position of the additional aggrecanase-mediated cleavage site described in the present work is indicated by the dashed arrow (6). (B) Sites cleaved by aggrecanases. Cleavage site is indicated by an arrow and the position of the P1 glutamic acid residue in the bovine aggrecan sequence is also indicated.

ADAMTS-5-mediated cleavage sites have also been described within the CS-2 region of aggrecan[13,14], generating small G3-containing fragments whose size is the hallmark of aggrecanase action (Fig. 1, panel A). The aggrecanase-mediated cleavage sites all have Glu at the P1 position and a non-polar or uncharged residue such as Gly, Ala or Leu at the P1′ position. In bovine cartilage these cleavages are AGELE^{1480}–^{1481}GRGTI, FKEE^{1666}–^{1667}GLGSV, PTAQE^{1771}–^{1772}AGEGP, TVSQE^{1871}–^{1872}LGQRP (Fig. 1, panel B)[15] and, along with the cleavage site within the IGD, are highly conserved between various species[16].

Under arthritic conditions proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and oncostatin M (OSM) are known to stimulate the production and activation of aggrecanases[17–20], and are therefore responsible for the rapid degradation of aggrecan under these conditions. We have previously studied the effects of combinations of these catabolic cytokines on proteoglycan aggregate degradation in adult bovine articular cartilage[21]. Western blot analysis of deglycosylated culture media using an antibody directed against the C-terminal G3 domain of aggrecan has revealed the presence of an additional degradation product with an apparent molecular mass of ~50 kDa that was generated in the presence of OSM, either alone or in combination with the other cytokines. In this study we have purified the 50-kDa fragment from cytokine-stimulated bovine cartilage culture medium, identified the cleavage site within the aggrecan core protein, and determined that this G3-containing fragment is the product of ADAMTS action *in vitro*.

Methods

BOVINE CARTILAGE EXPLANT CULTURES

Bovine articular cartilage was obtained at a local abattoir from skeletally mature animals. Cartilage from metacarpophalangeal joints was cut into pieces (approximately 5 mm^3); pre-cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) buffered with 44-mM $NaHCO_3$, 20-mM HEPES, and containing 100-U/ml penicillin G sodium and 100-μg/ml streptomycin sulfate (Gibco), in 12-well culture plates at 100-mg tissue per 2-ml medium. The explants were then cultured for 4 days in DMEM containing 0.1-mg/ml BSA as a carrier and supplemented with a combination of human recombinant IL-1β (5 ng/ml) and OSM (10 ng/ml) (R&D)[21]. Media were collected at day 2 and day 4 of the culture.

KERATANASE AND CHONDROITINASE TREATMENTS

Aliquots of the conditioned culture medium were dialyzed against 10-mM sodium acetate, pH 6.0 (keratanase buffer) at 4°C and treated with 20-mU/ml keratanase II (Seikagaku) at 37°C overnight. Subsequently, the buffer was adjusted to 0.1-M Tris/HCl, 0.1-M sodium acetate, pH 7.3 and the samples were treated with 200-mU/ml chondroitinase ABC (MP Biomedicals) for 6 h at 37°C. At the end of the digestion period both enzymes were inactivated by incubation of the samples at 100°C for 5 min.

WESTERN BLOT

Keratanase/chondroitinase-treated samples were analyzed on Novex 4–12% gradient NuPAGE Bis–Tris gels (Invitrogen) under reducing conditions. Electrophoresis was performed at 180 V for 1 h 30 min and electroblotting to nitrocellulose membrane (Bio-Rad) was conducted for 2 h at 30 V. The membrane was blocked overnight in 5% skim milk (Carnation Instant milk powder) in Tris-buffered saline, pH 7.6 containing 0.1% Tween 20. Aggrecan degradation products were detected using rabbit polyclonal antibodies (1:1000), recognizing the G3 domain of aggrecan[22] or the new N-terminus (ARLEIE) of the G3-bearing aggrecan fragment described below. Binding of the primary antibody was detected using a secondary anti-rabbit Ig-biotinylated antibody (Amersham), followed by incubation with a streptavidin-biotinylated horseradish peroxidase (HRP) complex (Amersham), ECL Plus Western blotting detection reagents (Amersham), and exposure to Hyperfilm (Amersham).

FRAGMENT PURIFICATION BY ION-EXCHANGE CHROMATOGRAPHY

IL-1/OSM-treated cartilage culture media were pooled and subjected to DEAE Sepharose Fast Flow (Amersham) anion-exchange chromatography to purify aggrecan fragments. The sample was applied on a DEAE-Sepharose column (5 ml) equilibrated in 50-mM Tris, 0.15-M NaCl, pH 7.5. Fractions of 1 ml were collected at a flow rate of 20 ml/h. Bound aggrecan fragments were retrieved by step elution in 50-mM Tris, pH 7.5, containing 0.3-M and 1-M NaCl (10 ml). The protein content of the collected fractions was monitored by measuring absorbance at 280 nm. Aggrecan G3-containing fragments were identified by SDS/PAGE and immunoblotting (as described above). Fractions containing the 50-kDa G3-containing aggrecan fragment were then extensively dialyzed into 10-mM sodium acetate, pH 6.0 to reduce the NaCl concentration in the sample. The proteins were precipitated with four volumes of ethanol and the pellet was resuspended in a small volume of keratanase buffer for subsequent keratanase and chondroitinase treatments and western blot analysis as described above.