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

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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.
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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

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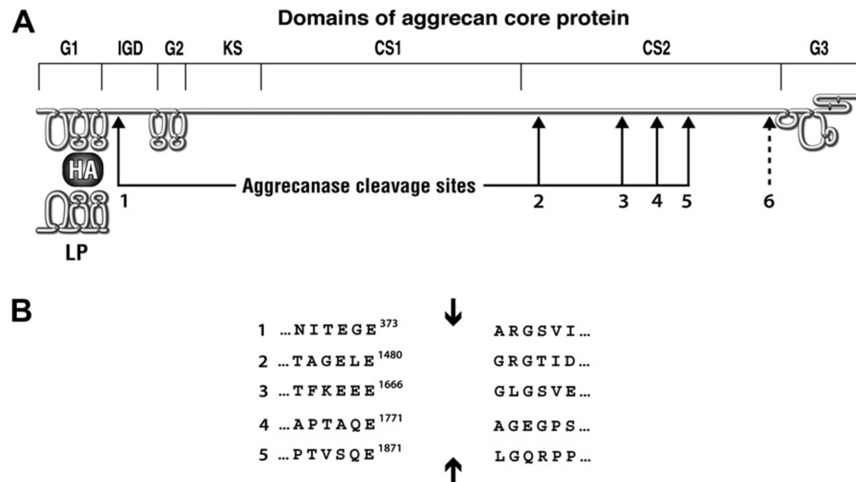

Fig. 1. Aggrecanase cleavage sites in bovine aggrecan. (A) Schematic representation of the bovine aggrecan core protein and its constituent domains: G1, IGD, G2 and G3, and the GAG-substituted domains: KS and CS-1 and CS-2 domains. Previously described aggrecanase cleavage sites are indicated by numbered 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.

N-TERMINAL SEQUENCE DETERMINATION

Fractions containing the 50-kDa G3-containing aggrecan degradation product were subjected to SDS/PAGE using 4–12% Novex gels (Invitrogen) and the products transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by electroblotting as described above. The membrane was stained with 0.1% Coomassie Blue R-250 in 50% methanol, then briefly destained in 50% methanol and washed with water. Bands corresponding to the degradation product of interest were excised and analyzed by automated Edman degradation sequencing (carried out at the Sheldon Biotechnology Centre, McGill University).

GENERATION OF ANTI-NEOEPITOPE ANTIBODIES

An anti-neoepitope antibody to the new N-terminus of the 50-kDa G3-containing aggrecan fragment was generated as described previously[23] by immunization of a rabbit (carried out at the McGill University Animal Resource Centre) with a synthetic peptide, ARLEIE*GGC*, coupled to keyhole-limpet haemocyanin (KLH). The residues indicated in italics represent a linker region, consisting of a cysteine residue, to permit the conjugation to KLH, and two glycine spacer residues. The resulting antiserum was assayed for selectivity by competitive enzyme-linked immunosorbant assay (ELISA), as described previously[23], using the immunizing peptide as well as a test-peptide that differs from the immunizing peptide by a substitution of the first two N-terminal residues (AR → TH). The specificity of the anti-neoepitope antibody was also verified in immunoblotting experiments against intact aggrecan.

EXTRACTION OF FETAL BOVINE AGGRECAN

Fetal bovine cartilage from the epiphyses of metacarpal bones was obtained from a local abattoir. Proteoglycan was extracted with 4-M guanidinium chloride as described previously[24]. Filtered extracts were adjusted to a density of 1.5 g/ml by addition of cesium chloride, while the concentration of guanidinium chloride was maintained at 4 M. The extract was then subjected to density-gradient centrifugation at 100,000 g_{av} at 10°C for 48 h. Fractions of density >1.55 g/ml were pooled, desalted by dialysis, freeze-dried and stored at 4°C until subsequent use for aggrecanase digestion.

DIGESTION OF BOVINE AGGRECAN WITH RECOMBINANT ADAMTS-4 AND ADAMTS-5

Recombinant full-length aggrecanases were expressed in insect cells and purified as described previously and the concentrations of active ADAMTS-4 and ADAMTS-5 determined by titration with the N-terminal domain of tissue inhibitor of metalloproteinases-3 (TIMP3)[25,26]. All aggrecan digestion assays were carried out in 100-μl 50-mM Tris–HCl buffer, pH 7.5, containing 125-mM NaCl and 5-mM $CaCl_2$ (aggrecanase buffer) containing 200 μg of purified fetal bovine aggrecan. The samples were incubated with ADAMTS-4 or ADAMTS-5 at 5 nM for 24 h at 37°C and the reaction was stopped by heating the samples at 75°C for 10 min. In addition, a time-course study of ADAMTS-5 digestion was carried out using 1 nM of the enzyme, with digestion being stopped after 1, 3, 6 and 18 h. For analysis, samples were then precipitated by adding four volumes of cold ethanol, incubated overnight at −20°C and centrifuged. The pellet was washed with cold ethanol, dried and resuspended in keratanase buffer for subsequent keratanase and chondroitinase treatments and western blot analysis, as described above.

Results

RELEASE OF G3-CONTAINING FRAGMENTS INTO CULTURE MEDIUM

Culture media of bovine cartilage explants treated with IL-1β in combination with OSM over a 4-day culture period were analyzed by SDS/PAGE and immunoblotting. After 2 days of culture in the absence of cytokines, intact high molecular weight aggrecan was detected in the culture media, with very little sign of proteolytic degradation (Fig. 2, lane 1). No further degradation was observed after 4 days of culture. However, in the presence of IL-1β and OSM, several degradation products could be detected (Fig. 2, lanes 2 and 3). The sizes of the larger fragments are compatible with aggrecanase cleavage within the CS-2 region of aggrecan. An additional 50-kDa fragment was also detected. Its size and detection by the anti-G3 antibody suggest that it could consist of a "free" G3 domain.

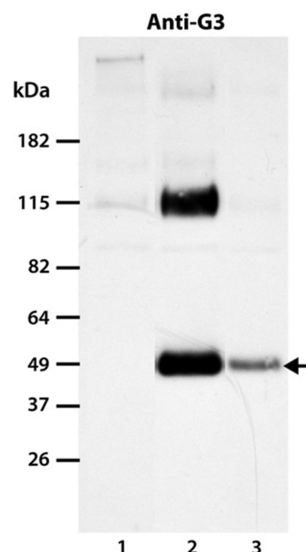

Fig. 2. Release of G3-containing fragments from bovine cartilage explant cultures. Bovine cartilage explants were cultured in the presence of IL-1β and OSM over a 4-day period. Culture medium was collected at day 2 and day 4, treated with keratanase II and chondroitinase ABC, analyzed by SDS/PAGE and immunoblotting using an antibody recognizing the G3 domain of aggrecan. Material released from equal amounts of cartilage explants was loaded onto the gel. Lane 1, control day 2; lane 2, IL-1β + OSM day 2; lane 3, IL-1β + OSM day 4. Migration positions of molecular weight markers are indicated on the left. Migration position of the additional G3-bearing fragment is indicated by an arrow.

PURIFICATION OF G3-CONTAINING FRAGMENTS BY ION-EXCHANGE CHROMATOGRAPHY

G3-containing fragments were purified by anion-exchange chromatography from 76 ml of pooled culture medium from bovine explants treated with IL-1β and OSM. Aggrecan fragments containing the G3 domain, including the 50-kDa fragment, remained bound to the column and were eluted with increasing NaCl concentrations (Fig. 3, panels A and B), indicating their anionic character and the presence of GAG chains. In addition, a 30-kDa G3-containing product was also detected (Fig. 3, panel B), although in lower abundance than the 50-kDa fragment.

AMINO ACID SEQUENCE ANALYSIS OF G3-CONTAINING FRAGMENT

To establish the N-terminal amino acid sequence of the 50 kDa fragment, fraction 98 (Fig. 3, panel B) was fractionated by SDS/PAGE, transferred to PVDF membrane, and identified with Coomassie blue (Fig. 4). The 50-kDa band was excised and subjected to protein sequencing. A single sequence was obtained: ARLEIESSSP, which corresponds to residues 2047–2056 in the published protein sequence of bovine aggrecan (accession number: NP_776406). This indicated that cleavage has occurred within the CS-2 region of aggrecan between Glu^{2047} and Ala^{2048}. The amino acid sequence surrounding the cleavage site is TQRPAE–ARLEIE. This could be a putative cleavage site for aggrecanases, which are considered to be glutamyl-endopeptidases[13].

DIGESTION OF AGGRECAN WITH AGGRECANASES

To verify that such a cleavage can be reproduced *in vitro* by aggrecanases, fetal bovine aggrecan was digested by

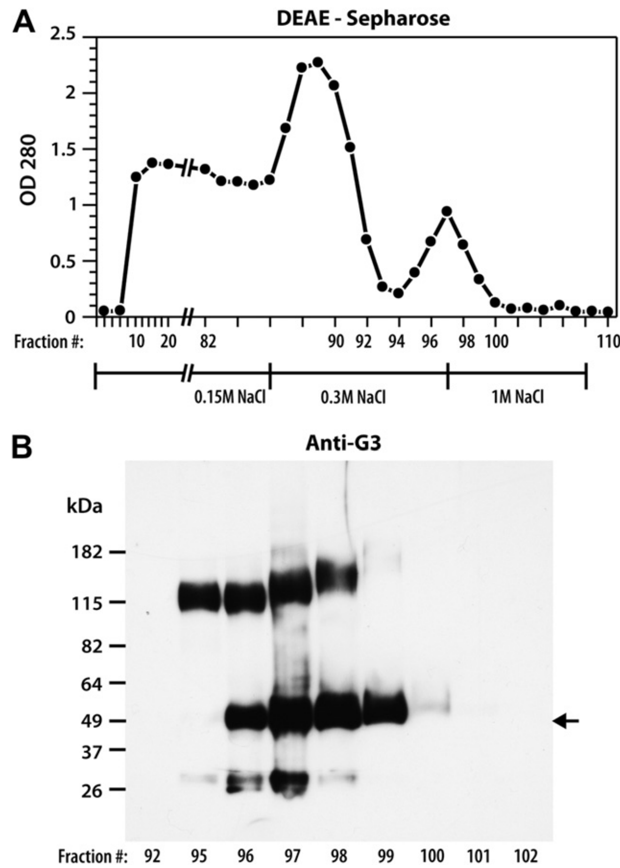

Fig. 3. Purification of G3-containing aggrecan fragments by ion-exchange chromatography. (A) Culture media from day 2 and day 4 of IL-1β/OSM-treated explant cultures were pooled and loaded onto a DEAE-Sepharose column pre-equilibrated in 50-mM Tris, 0.15-M NaCl, pH 7.5. Bound aggrecan material was retrieved by step elution with 0.3-M and 1-M NaCl. Protein content in the collected 1-ml fractions was estimated by measuring absorbance at 280 nm. (B) Aliquots of the fractions were deglycosylated and analyzed by SDS/PAGE and western blotting using anti-G3 antibody. Migration positions of molecular weight markers are indicated on the left. Migration position of the G3-bearing fragment of interest is indicated by an arrow.

recombinant full-length ADAMTS-4 and ADAMTS-5, and G3-containing fragments were analyzed by immunoblotting. Fetal bovine aggrecan was used as a substrate because of its high content of intact aggrecan molecules bearing the G3 domains. Treatment with ADAMTS-4 (Fig. 5, panel A, lane 2) or ADAMTS-5 (Fig. 5, panel A, lane 3) resulted in the generation of several G3-containing products that were absent in the undigested aggrecan (Fig. 5, panel A, lane 1). The sizes of these degradation products were compatible with aggrecanase-mediated cleavages within the CS-2 region of aggrecan. ADAMTS-4 generated two products that have been previously shown to be due to cleavages at Glu^{1771}–Ala^{1772} and Glu^{1871}–Leu^{1872}. ADAMTS-5 also generated these degradation products, with cleavage at Glu^{1871}–Leu^{1872} being predominant. Both aggrecanases were also able to generate the 50-kDa G3-containing fragment, though in vastly different abundance. ADAMTS-5 readily cleaves at this site, whereas cleavage by ADAMTS-4 could only be detected when excessive amounts of digest were used (data not shown). Medium from day 4 of bovine explant cultures stimulated with IL-1β and OSM (Fig. 5, panel A, lane 4), exhibits an equivalent aggrecan degradation pattern to that of fetal bovine aggrecan digested with ADAMTS-5.

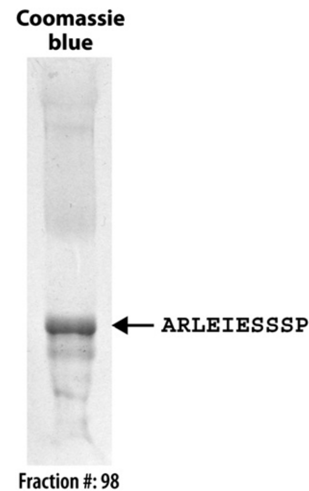

Fig. 4. Amino acid sequence analysis of G3-containing aggrecan fragment. Fraction number 98 [see Fig. 3(B)] containing the G3 product of interest was treated with keratanase II and chondroitinase ABC. The sample was run on a 4–12% Bis–Tris Novex gels and transferred to a PVDF membrane. The Coomassie Blue-stained band of interest (marked by an arrow) was excised and analyzed by automated Edman degradation sequencing. The determined N-terminal sequence is indicated.

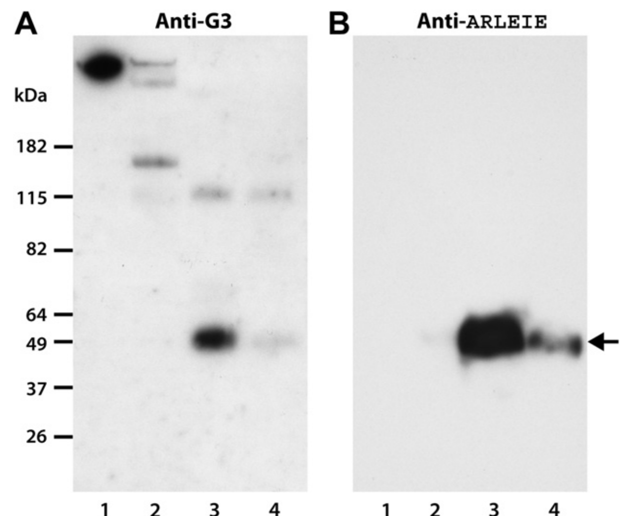

Fig. 5. Digestion of fetal bovine aggrecan with aggrecanases. Purified fetal bovine aggrecan (2 mg/ml) was digested with recombinant full-length human ADAMTS-4 or ADAMTS-5 (5 nM) for 24 h at 37°C. The samples were then treated with keratanase II and chondroitinase ABC, then analyzed by SDS/PAGE and immunoblotting using either an anti-aggrecan G3 antibody (A) or an anti-neoepitope antibody directed against the new N-terminus of the G3 fragment (B). Lane 1, undigested aggrecan; lane 2, aggrecan digested with ADAMTS-4; lane 3, aggrecan digested with ADAMTS-5; lane 4, culture media from IL-1β/OSM-treated bovine explants collected at day 4. Migration positions of molecular weight markers are indicated on the left. Migration position of the G3-bearing fragment of interest is indicated by an arrow.

In order to verify that the 50-kDa G3-containing aggrecan fragment was the product of aggrecanase activity at Glu^{2047}–Ala^{2048}, immunoblotting was repeated using an anti-neoepitope antibody recognizing the new N-terminal amino acid sequence of the 50-kDa fragment, ARLEIE. The ARLEIE epitope was not detected in undigested aggrecan (Fig. 5, panel B, lane 1). However, upon digestion by ADAMTS-5, abundant ARLEIE-G3 product was detected (Fig. 5, panel B, lane 3). In contrast, a large amount of ADAMTS-4-digested material was necessary to detect the 50-kDa ARLEIE-G3 product with the anti-neoepitope antibody (data not shown). This confirms that full-length ADAMTS-5 is more active than ADAMTS-4 at cleaving the Glu^{2047}–Ala^{2048} bond. As expected, the 50-kDa fragment was also detected by the anti-neoepitope antibody in the culture media of IL-1β and OSM-treated adult bovine cartilage explants (Fig. 5, panel B, lane 4).

To determine whether the cleavage at the Glu^{2047}–Ala^{2048} bond represents a preferential or a terminal cleavage, a time-course study of digestion of fetal bovine aggrecan by ADAMTS-5 was performed (Fig. 6). G3-bearing degradation products resulting from the cleavages within the CS-2 region of aggrecan were detected after 1 h of digestion (Fig. 6, lane 2). These degradation products accumulate over time, with cleavage following Glu^{1871} being predominant after 1, 3 and 6 h of digestion (Fig. 6, lanes 2–4). However, as the ^{1872}Leu-G3 fragments accumulate, they are progressively converted into the described 50-kDa G3-containing products (Fig. 6, lanes 3–4), and after 18 h of digestion the 50-kDa fragment is the major degradation product (Fig. 6, lane 5). Thus, the ^{2048}Ala-G3 fragment represents a terminal cleavage product of aggrecan by ADAMTS-5.

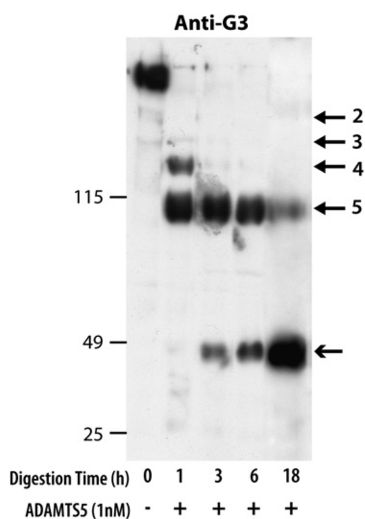

Fig. 6. Digestion of fetal bovine aggrecan by ADAMTS-5: a time-course study. Purified aggrecan (2 mg/ml) was digested with recombinant human ADAMTS-5 (1 nM) for 1, 3, 6 or 18 h at 37°C. The samples were then treated with keratanase II and chondroitinase ABC, and analyzed by SDS/PAGE and immunoblotting using an anti-aggrecan G3 antibody. Lane 1, undigested aggrecan; lane 2, aggrecan digested with ADAMTS-5 for 1 h; lane 3, aggrecan digested with ADAMTS-5 for 3 h; lane 4, aggrecan digested with ADAMTS-5 for 6 h; lane 5, aggrecan digested with ADAMTS-5 for 18 h. Migration positions of molecular weight markers are indicated on the left. Migration position of the G3-bearing fragment of interest is indicated by an arrow.

ANALYSIS OF CS AND KS PRESENCE OF G3-CONTAINING FRAGMENT

The ability of the 50-kDa fragment to bind to the DEAE resin suggests that it is substituted with GAG chains. The presence of such GAG chains was confirmed by treating the fetal bovine aggrecan ADAMTS-5 digests with either chondroitinase ABC and/or keratanase II; followed by SDS/PAGE and immunoblotting (Fig. 7). Non-deglycosylated samples, whether they were treated with or without ADAMTS-5, could not be detected by the anti-G3 or anti-ARLEIE antibody (Fig. 7, panels A and B, lanes 1 and 2). Similarly, keratanase treatment alone of the ADAMTS-5-digested aggrecan did not allow the detection of aggrecan degradation products by either antibody (Fig. 7, panels A and B, lane 3). In contrast, treatment with chondroitinase (Fig. 7, panel A, lanes 4 and 5) allowed the detection of 115-kDa and 50-kDa fragments by the anti-G3 antibody and the 50-kDa fragment by the anti-ARLEIE antibody (Fig. 7, panel B, lanes 4 and 5). This indicates that the 50-kDa G3 fragment contains at least one CS chain. A similar experiment using chondroitinase and/or keratanase treatment of culture media collected from IL-1/OSM-treated bovine cartilage explants verified that chondroitinase treatment is essential to visualize the 50-kDa G3-bearing fragment by immunoblotting (data not shown).

Discussion

The results presented in this and a previous study[21], have shown that OSM in combination with IL-1β, is capable of stimulating bovine aggrecan degradation not only at the previously described aggrecanase-sensitive cleavage sites within the IGD and CS-2 domains of aggrecan, but also at an additional site within the CS-2 region. This was a terminal cleavage, and resulted in the release of a 50-kDa G3-bearing fragment. Analysis indicates that the fragment is

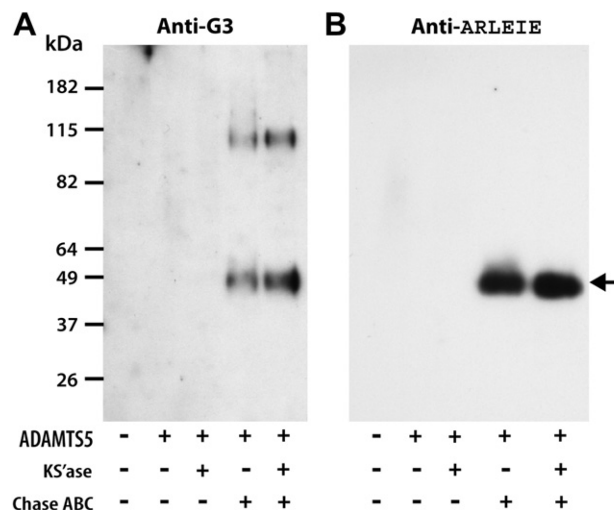

Fig. 7. Analysis of CS and KS presence of G3-containing aggrecan fragment. ADAMTS-5 digests of fetal bovine aggrecan were treated with either keratanase II or chondroitinase ABC, or both enzymes. Sample were then analyzed by SDS/PAGE on 4–12% Bis–Tris Novex gels and immunoblotting using either an anti-aggrecan G3 antibody or an anti-neoepitope antibody directed against the new N-terminus of the G3 fragment. Migration positions of molecular weight markers are indicated on the left. Migration position of the G3-bearing fragment of interest is indicated by an arrow.

generated by cleavage near the C-terminus of the CS-2 domain, and that it possesses at least one CS chain.

In addition to the predominant 50-kDa G3 fragment, a minor 30-kDa fragment was also detected. Neoepitope analysis indicates that the 30-kDa fragment possesses the same N-terminal amino acid sequence as the 50-kDa fragment (unpublished data). Although this fragment is not produced by ADAMTS-4 or ADAMTS-5 treatment of fetal bovine cartilage aggrecan, additional studies have shown that ADAMTS-5 can generate such a fragment from adult bovine aggrecan (unpublished data). Thus, the 30-kDa is probably derived from the C-terminally processed aggrecan that may be present in mature cartilage.

In the bovine aggrecan gene, the CS-2 domain of aggrecan is encoded by exon 12, whereas the downstream exons code for the various sub-domains of the G3 domain[27]. The N-terminal sequence of the 50-kDa G3 fragment verified that the cleavage site is located within the region encoded by exon 12, close to the C-terminus of the CS-2 domain of aggrecan (Fig. 1). Further analysis of the bovine aggrecan sequence shows that a potential sequence for CS chain substitution (^{2060}Ser–Gly) is present within the 50-kDa fragment, so accounting for its anionic character and sensitivity to chondroitinase.

Generation of an aggrecan fragment with the same N-terminal amino acid sequence as the 50-kDa fragment has been previously reported from the culture media of bovine ligament explants[28]. It seems that in the ligament this fragment is a naturally occurring catabolite of aggrecan as no cytokine stimulation was necessary for the fragment to be released from the tissue. An aggrecan fragment of similar size has also been previously observed and reported as an m-calpain generated product[29], and evidence has been presented for the role of this protease in cartilage degradation[30]. However, in cartilage, aggrecan turnover, both under normal and pathological conditions, is thought to be mediated mainly by aggrecanases (ADAMTS-4 and ADAMTS-5), as products derived from cleavages by these enzymes can be detected in synovial fluids of patients suffering from OA, joint injury or inflammatory joint diseases[31]. The 50-kDa G3 fragment described in this study is generated by cleavage following a glutamic acid residue. Such cleavage is not characteristic of m-calpain action, but rather implicates aggrecanases. It was suggested that aggrecanase action accounted for generation of the G3 fragment from the cultured bovine ligament[28], though there was no direct evidence to support this proposal. Use of an anti-neoepitope antibody specific to the N-terminus of the 50-kDa G3 fragment confirms that the observed cleavage can be mediated by both ADAMTS-4 and ADAMTS-5 *in vitro*. However, these enzymes do not cleave this region to the same extent, with full-length recombinant ADAMTS-5 cleaving much more readily than ADAMTS-4. This is not surprising as previous studies have shown that recombinant full-length ADAMTS-4 and ADAMTS-5 used in these studies exhibit different levels of activity, with ADAMTS-5 being up to 20–30 times more efficient at cleaving aggrecan than ADAMTS-4[26,32].

Variations in the activity of the aggrecanases in cleaving aggrecan have been previously reported. These enzymes have variable activities within the different cleavage regions of the aggrecan core protein and while cleavage at the Glu^{373}–Ala^{374} site within the IGD is considered to be the signature of aggrecanase activity, it has been shown that cleavage within the CS-2 region is more favored[33–35]. Moreover, the presence of CS chains has been described as an important feature in promoting aggrecanase activity, as both ADAMTS-4 and ADAMTS-5 have been shown to interact with GAG side chains through their ancillary C-terminal domains[36,37]. Furthermore, the activities of both ADAMTS-4 and ADAMTS-5 can be regulated by C-terminal processing, either by autocatalysis or the action of other proteases[38,39]. Previous studies have suggested the presence of truncated forms of ADAMTS-4 lacking the spacer domain or part of the Cys-rich domain in pig cartilage following IL-1 treatment[38,40]. Such truncated forms of ADAMTS-4 exhibit lower aggrecanase activities in the CS-2 region[25]. Similarly, truncated forms of ADAMTS-5 have been detected in human synovial tissue[41], and studies have shown that ADAMTS-5 lacking the spacer domain exhibits reduced aggrecanase activities in both the IGD and the CS-2 regions[26].

The presence of the 50-kDa aggrecan fragment in OSM-treated bovine cartilage suggests that cleavage at Glu^{2047}–Ala^{2048} could occur *in vivo* during cartilage catabolism, when OSM is present within the joint. OSM is a cytokine associated with mononuclear cells and is believed to play an important role in the degradation of the main ECM components, aggrecan and collagen type II, in the inflammatory joint[42–44]. Interestingly, bovine chondrocytes treated with IL-1 express both OSM and OSM receptor mRNA (unpublished data), suggesting that bovine chondrocytes could produce this cytokine as well as readily respond to its presence within the joint. Our previous data suggest that OSM might play a role in enhancing the effect of other catabolic cytokines such as IL-1 and TNF-α, resulting in excessive disruption of the ECM scaffold through degradation of HA in addition to enhanced aggrecanolysis[21]. Previous studies have shown that OSM alone is capable of inducing ADAMTS-4 and MMP-13 expressions by human articular chondrocytes[45]. Furthermore, in combination with TNF-α, OSM has been shown to stimulate ADAMTS-5 mRNA expression in bovine nasal and human articular chondrocytes[46]. High levels of aggrecanase production induced by OSM could therefore account for the observed extensive aggrecan degradation characterized in this study. Preliminary studies involving PCR analysis of bovine cartilage explants treated with IL-1 + OSM or IL-1 alone over a 2- or 4-day culture period have indicated that ADAMTS-5 mRNA levels are elevated when OSM is present, supporting a potential role for ADAMTS-5 in the generation of the 50-kDa aggrecan product (unpublished observations). In addition, it is possible that OSM influences aggrecanase C-terminal truncation, so altering the affinity of the different forms of the enzymes for the aggrecan.

The four well characterized aggrecanase cleavage sites located within the CS-2 region of aggrecan are very well conserved among species. However, the additional C-terminal cleavage site reported in this study shows variation between the species (Fig. 8). The N-terminal region of the

	P1	P1′
Bos taurus	...TQRPAE	ARLEIE...
Homo sapiens	...TQRPAE	THLEIE...
Canis lupus familiaris	...TQRPAE	AHLEIE...
Mus musculus	...TRYPTE	TLQEIE...
Rattus norvegicus	...TQHPTE	TLQEIG...

Fig. 8. Sequence comparison between different species at the C-terminal aggrecanase cleavage site. Bovine, human, dog, mouse and rat aggrecan amino acid sequences at the cleavage site were aligned. P1 indicates the first amino acid upstream of the scissile bond; P1′ represents the first amino acid downstream of the scissile bond. Amino acids are represented by their respective single letter code.

scissile bond is fairly well conserved between the species, with the glutamic acid residue at the P1 position being 100% conserved. Recent studies have described a seven amino acid putative cleavage motif for ADAMTS-4 action in aggrecan[47], and it has been suggested that an extended region downstream of the scissile bond (P′ amino acids) is involved in substrate recognition[47,48]. Such downstream sequence exhibits the most variation between species (Fig. 8). In addition, the presence of CS-attachment motifs in the proximity of the cleavage site could also have an impact on aggrecanases action. Sequence comparison revealed that the potential CS-substitution motifs, located upstream and downstream of the C-terminal cleavage site, are well conserved between the species (data not shown) and could therefore facilitate aggrecanase action. However, whether the presence of sequence variations impairs or enhances aggrecanase action in these species still needs to be established.

Conflict of interest

The authors have no conflict of interest.

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