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ORIGINAL ARTICLE

Aggrecanase- and matrix metalloproteinase-mediated aggrecan degradation is associated with different molecular characteristics of aggrecan and separated in time *ex vivo*

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Abstract
Aggrecan is one of the first proteins to be depleted from articular cartilage in early osteoarthritis. We investigated the molecular differences between matrix metalloproteinase (MMP)- and aggrecanase-mediated aggrecan degradation, as a consequence of their distinct time-dependent degradation profiles. Cartilage degradation was induced by cytokine stimulation in bovine articular cartilage explants and quantified by a dye-binding assay and immunoassays. The size of degradation fragments was analysed by Western blot. Cytokine stimulation resulted in the early release of aggrecanase-mediated aggrecan degradation fragments. In contrast, MMP-mediated aggrecan degradation began only at day 16 and continued to day 21. Western blot analysis showed that glycosylated high-molecular-weight ^{374}ARGSVI fragments appeared at day 7, in contrast to deglycosylated low-molecular-weight ^{342}FFGVG fragments which were detected at day 21. Aggrecan degradation may be divided into two different pools, a high-molecular-weight aggrecanase-mediated pool, and a low-molecular-weight MMP-mediated pool. This may have implications for the development of intervention strategies for OA.

Keywords: *Proteoglycans; cartilage; osteoarthritis; ELISA; biomarkers; aggrecanases; MMPs*

Introduction

Cartilage is normally maintained by a balance between catabolic and anabolic processes. However, in joint degenerative diseases, such as osteoarthritis (OA), the rate of cartilage degradation exceeds the rate of formation, resulting in a net loss of cartilage matrix (Behrens et al. 1989, Felson & Neogi 2004). The extracellular matrix (ECM) consists mainly of collagen type II, which forms a fibrous network that entraps proteoglycans of which aggrecan is the most predominant (Hardingham & Fosang 1995, Kiani et al. 2002).

Aggrecan is one of the ECM proteins to be degraded in OA (Caterson et al. 2000), primarily by ADAMTSs (a disintegrin and metalloproteinase domain with thrombospondin motifs) (Glasson et al. 2005, Struglics et al. 2006) and matrix metalloproteinases (MMPs) (Fosang et al. 1996, Lark et al. 1997, Struglics et al. 2006). Aggrecanase activity leads to the release of glycosaminoglycans (GAGs), although it is not known if MMPs also play a role in aggrecan GAG loss (Sandy 2006, Struglics et al. 2006). As a consequence of aggrecan loss, the cartilage exhibits altered matrix properties such as loss of fixed negatively charged density of the tissue and thereby lower water content and elasticity (Roughley & Lee 1994). A deeper understanding of the molecular mechanisms involved in proteoglycan depletion may aid the development of efficient disease-modifying osteoarthritic drugs (DMOADs).

The core protein of aggrecan has three globular domains: G1, G2 and G3. G1 and G2 are separated by

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the interglobular domain (IGD), which is highly sensitive to proteolysis (Flannery et al. 1992, Hardingham & Fosang 1995). G2 and G3 are heavily saturated with highly negatively charged GAGs (Flannery et al. 1992, Hardingham & Fosang 1995) (Figure 1). The G2 domain is unique to aggrecan, and sandwich enzyme-linked immunosorbent assays (ELISAs) of aggrecan degradation have been developed against peptides with the G2 and the neoepitopes ^{342}FFGVG or ^{374}ARGSV (Karsdal et al. 2007, 2008) generated by MMPs (Flannery et al. 1992) and ADAMTS (Tortorella et al. 1999), respectively. The ADAMTS-specific ^{374}ARGSV site is located between the ^{342}FFGVG site and the G2 domain. The ^{342}FFGVG-G2 fragments assayed in the present study represent only MMP-mediated degradation, which has not already been processed by ADAMTSs. The ^{374}ARGSV-G2 represents fragments, which theoretically may have been pre-processed by MMPs at the ^{342}FFGVG site (Figure 1), although the available evidence suggests that this is very unlikely (Mercuri et al. 2000).

Studies with *ex vivo* cultures have shown that cytokine-stimulated cartilage releases collagen and proteoglycan fragments to the conditioned medium (Sondergaard et al. 2006a). These fragments can be detected by novel ELISA assays, which measure neoepitopes cleaved by specific proteases. These degradation patterns resemble the molecular mechanisms seen in the pathogenesis of OA (Caterson et al. 2000). Our group has previously shown that cytokine-stimulated cartilage releases products with specific neoepitopes from collagen type II and aggrecan (Karsdal et al. 2007, 2008, Rousseau et al. 2008, Sondergaard 2006a). Furthermore, we also showed that MMP-2 and -9 activity increased over time in cytokine-stimulated cartilage explants (Sondergaard 2006a), which is important when investigating the difference between aggrecanase- and MMP-mediated degradation of aggrecan.

Different research groups have suggested that articular cartilage aggrecan may be present in the tissue in two or more metabolic pools (Ilic et al. 1995, Lark et al. 1997, Lohmander et al. 1973, Mok et al. 1994, Struglics et al. 2006). However, currently, the temporal and spatial molecular degradation of aggrecan has not been clarified, and various contradictory theories and models have been proposed by different investigators (Fosang et al. 2000, Lark et al. 1997, Sandy 2006, Struglics et al. 2006). It has been proposed by Fosang et al. (2000) that aggrecan degradation by ADAMTSs and MMPs occurs in independent locations and with different kinetics. More recently Struglics et al. (2006) analysed human synovial fluids and concluded that ADAMTSs and MMPs act sequentially on the same substrate molecule. A review of the area by Sandy (2006) attempted to clarify these controversies. On the basis of available evidence, Sandy (2006) concluded that ADAMTSs and MMPs degrade different aggrecan structures. Specifically it was suggested that ADAMTSs act primarily on full-length or G1-G2-chondroitin sulfate1 aggrecan by a sequential process which removes the chondroitin sulfate-bearing domains and lastly at the Glu$^{373-374}$Ala bond in the IGD. In contrast it was proposed that MMPs degrade only the calpain (calcium-dependent cysteine protease) product

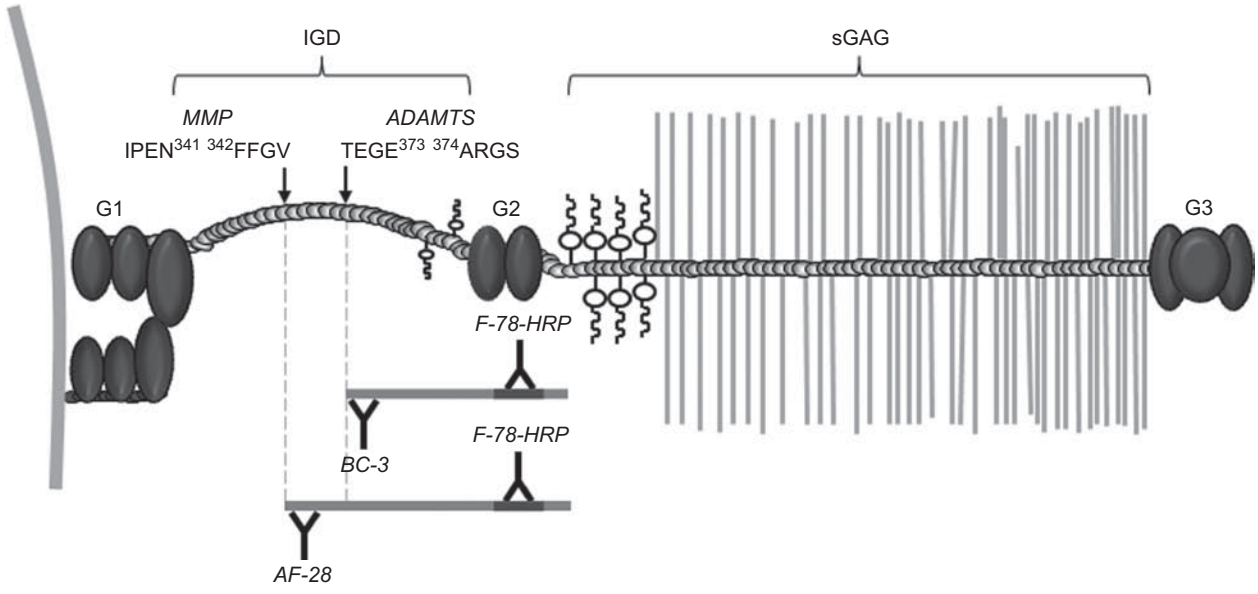

Figure 1. Protease-mediated neoepitopes in aggrecan. Aggrecan molecule with three globular domains G1, G2 and G3. The interglobular domain (IGD) is highly sensitive to proteolysis. Beneath the aggrecan molecule are the ^{342}FFGVG-G2 and ^{374}ARGSV-G2 fragments mediated by matrix metalloproteinase (MMP) and ADAMTS, respectively. The antibodies BC-3 and AF-28 recognize the neoepitopes and F-78 is the detection antibody binding to the G2 domain. The domain between G2 and G3 is heavily saturated with highly negatively charged glycosaminoglycans (GAGs).

G1-G2-keratan sulfate (Maehara et al. 2007, Oshita et al. 2004) by cleavage at the ^{342}FFGVG site. In this scenario the independent activities of ADAMTSs and MMPs are seen as being due to a protease-specific preference for different substrate structures. Furthermore, Lark et al. (1997) described three hypothetical models for pathways of proteolytic degradation of aggrecan, emphasizing the complex nature of the generation of various fragments from the same molecule.

To examine further the kinetics and molecular dynamics of aggrecanolysis in cartilage explant cultures, we investigated the degradation process by quantifying the time-dependent release of ADAMTS- and MMP-mediated aggrecan fragments by the ^{374}ARGSVI-G2 and ^{342}FFGVG-G2 ELISA assays, respectively. These new aggrecan fragments were categorized by size, via Western blot, to identify molecular differences regarding glycosylation of the core protein. Furthermore, we investigated the specificity pattern of MMP-9 and ADAMTS-4 for degrading native and deglycosylated aggrecan *in vitro* with respect to generating the aforementioned *ex vivo* neoepitopes.

Materials and methods

Tissue preparation

Bovine articular cartilage explants were harvested by using a scalpel to remove the articular cartilage without adherent calcified cartilage from stifle joints of 18-month-old bovine heifers. The cartilage explants (12 ± 2 mg) were washed in phosphate-buffered saline (PBS) and cultured in 96-well plates (Nunc, Slangerup, Denmark) in four replicas with 200 µl serum-free medium (Life Technologies, Naerum, Denmark) plus penicillin/streptomycin (Invitrogen, Taastrup, Denmark) per well in the absence or presence of tumour necrosis factor (TNF)-α (10 ng ml^{-1}) (R&D Systems, Abingdon, UK) and oncostatin M (OSM) (20 ng ml^{-1}) (Sigma Aldrich, Broendby, Denmark). These concentrations had been optimized in previous *ex vivo* model studies by Sondergaard et al. (2006a, b). As a negative control, cartilage was metabolically inactivated (MI) by three repeated freeze–thaw cycles in liquid N_2, and at 37°C in a water bath. The explants culture media were replaced every 2nd or 3rd day for 21 days. The conditioned media were kept at -20°C until tested for the presence of biochemical markers.

Biochemical markers of aggrecan degradation

MMP-mediated aggrecan degradation was quantified using the ^{342}FFGVG-G2 sandwich ELISA assay (Sumer et al. 2007), with AF-28 (Fosang et al. 1995) as the catching antibody. Quantification of the aggrecanase-mediated ^{374}ARGSV-G2 neoepitope was obtained in a similar way to the ^{342}FFGVG-G2 ELISA, except the catching antibody was BC-3 (Abcam, Cambridge, UK) to recognize the aggrecanase-mediated neoepitope ^{374}ARGSVI (Karsdal 2007). Both assays required the presence of the G2 domain on the fragments being assayed, with the detecting antibody being F-78. The efficacy of the antibodies was confirmed by blocking experiments in cytokine-stimulated explant media with the peptides ^{342}FFGVGEEDITVQT and ^{374}ARGSVILTVKPIF (Bachem, Weil am Rhein, Germany).

Detection of sulfated glycosaminoglycans

Sulfated glycosaminoglycan (sGAG) levels were measured in the conditioned medium using the alcian blue-binding assay according to the manufacturer's instructions (Euro-Diagnostica, Malmö, Sweden).

Western blot on supernatants

Conditioned medium from four replicates of MI, non-treated, or cytokine-stimulated cartilage explants from day 7 or 21 were either deglycosylated for 4 h with keratanase and chondroitinase lyase ABC (0.1 units/10 µg) (Sigma Aldrich) with gentle shaking at 37°C in 100 mM Tris/HCl and sodium acetate buffer at pH 6.5, or left untreated. Thirty microlitres of the deglycosylated or untreated sample was electrophoresed on 3–8% pre-casted Tris–acetate gradient gels under reducing conditions using Tris–acetate as running buffer (Invitrogen). After transferring the proteins to polyvinyl difluoride membranes overnight at room temperature (RT) at 120 mA in a 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid buffer with 5% ethanol, the membranes were blocked with 50 ml of 5% non-fat milk powder, 0.2% Tween-20 in PBS with shaking for 2 h at RT. Antibodies were applied in appropriate dilutions in 50 ml PBS with 5% bovine non-fat milk, 0.2% Tween-20 for 1 h at RT. BC-3 anti-^{374}ARGSVI cell supernatants (Caterson et al. 2000) diluted 1:50 or the anti-^{342}FFGVG monoclonal antibody AF-28 diluted 1:2250, giving a final concentration of 1200 ng ml^{-1} IgG (Fosang et al. 1995). After washing three times with PBS containing 0.2% Tween-20, the membranes were incubated for 1 h at RT with peroxidase-labelled rabbit antimouse antibody (1:5000) (Millipore, Copenhagen, Denmark) in PBS with 5% bovine non-fat milk powder, 0.2% Tween-20. After washing three times with PBS (0.2% Tween-20), the bands were visualized using an electrochemiluminescence developer system (Amersham, Hilleroed, Denmark).

ELISA for determining the total aggrecanase activity

The total aggrecanase activity in the conditioned medium from days 7–21 was measured in four replicates using a commercially available ELISA assay according to the manufacturer's instructions (MD Bioscience, Zurich, Switzerland).

Digestion of aggrecan fragments in the conditioned medium with ADAMTS-4

The conditioned medium from cytokine-stimulated cartilage explants from day 21 was diluted 1:50 in aggrecanase digestion buffer (50 mM Tris–HCI, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM leupeptin, 1 μM pepstatin, 1mM Pefabloc, 0.05% Tween-20) and digested for 15 min with shaking at 37°C, using a total concentration of 20 nM of ADAMTS-4 (Millipore). The digestions were stopped with 0.5 mM EDTA and the media tested using the ^{342}FFGVG-G2 and ^{374}ARGSV-G2 assays.

MMP-9 and ADAMTS-4 digestion on native and deglycosylated aggrecan

Purified bovine aggrecan (Sigma Aldrich) was dissolved in chondroitinase buffer (50 mM Tris, pH 8, 60 mM sodium acetate, 0.02% BSA) to a concentration of 2 mg ml^{-1}. One half of the solution was deglycosylated with chondroitinase ABC (0.01 U/10 μg) for 4 h at 37°C with shaking. The other half was left untreated but incubated along the deglycosylated half. Two hours before terminating the deglycosylation, the MMP-9 enzyme (Calbiochem, Herlev, Denmark) was activated in 4-aminophenylmercuric acetate (APMA) (1 mM) at 37°C for 2 h. The native and deglycosylated aggrecan were each digested with MMP-9 (1:100 enzyme/protein) and ADAMTS-4 (1:50 enzyme/protein). Digestion with MMP-9 continued for 3 days in a MMP buffer (100 mM Tris–HCl, 100 mM NaCl, 10 mM $CaCl_2$, 2 mM Zn-acetate, pH 8) while digestion with ADAMTS-4 took place over 22 h in a ADAMTS-4 buffer (75 mM Tris–HCl, 5 mM $CaCl_2.2H_2O$, 150 mM NaCl, pH 7.5) at 37°C, and was stopped with 0.5 mM EDTA. The MMP-9 and ADAMTS-4 digestions on native and deglycosylated aggrecan were examined using the ^{342}FFGVG-G2 and ^{374}ARGSV-G2 ELISA assays.

Statistics

Results are shown as mean + standard error of mean (SEM). Baseline was set at day 2 of culturing. Differences between mean values were compared using the Student's *t*-test for unpaired observations, assuming normal distribution where more than four replicates were used. Differences were considered statistically significant if $p < 0.05$.

Results

Depletion of sulfated glycosaminoglycans in cytokine-stimulated cartilage explants is mediated by aggrecanases

As aggrecan is one of the first proteins to deplete from articular cartilage in early OA, we investigated the time-dependent enzyme-mediated release pattern of aggrecan molecules from cytokine-stimulated bovine articular cartilage explants. The first cytokine-stimulated release of aggrecan fragments from bovine articular cartilage explants was caused by aggrecanase activity, as demonstrated by an increase in fragments detected by the ^{374}ARGSV-G2 assay. The maximum level of aggrecan fragments was detected at day 7 (Figure 2A) corresponding to an approximate sevenfold increase compared with non-stimulated bovine articular cartilage explants. After the peak at day 7, ^{374}ARGSVI-G2 concentrations were reduced to background levels.

To investigate if the reduction in ^{374}ARGSVI-G2 after day 7 resulted from depletion of the aggrecan protein as a substrate, we measured the MMP-mediated ^{342}FFGVG-G2 fragments. A time-dependent analysis showed that after day 16, increased ^{342}FFGVG-G2 fragments were released from cytokine-stimulated cartilage. At day 21, an increase of more than 1600% in these released fragments was observed in cytokine-stimulated cartilage compared with non-stimulated cartilage (Figure 2B). This indicted that aggrecan was present as a substrate during the entire culture period.

Karsdal et al. (2008) showed a total depletion of sGAGs, assessed by alcian blue staining from bovine cartilage explants as early as after 7 days after cytokine stimulation, while the non-stimulated cartilage retained the sGAGs in the ECM. Measurements of the time-dependent release of sGAGs to the conditioned medium (Figure 2C) showed a similar pattern to the aggrecanase-mediated aggrecan degradation. This indicated that the loss of sGAGs may be linked to the loss of aggrecanase-mediated aggrecan degradation.

Aggrecanase-mediated aggrecan degradation fragments are predominantly glycosylated whereas MMP-mediated aggrecan degradation fragments are predominantly deglycosylated

Due to the strong correlation between aggrecanase-mediated aggrecan degradation and sGAG-substituted molecules, we investigated aggrecan fragments in the early period (day 7) and late period (day 21) by Western blotting.

In the conditioned medium from cytokine-stimulated cartilage from day 7, a high-molecular-weight band – above 460 kDa – of the aggrecanase-mediated ^{374}ARGSVI

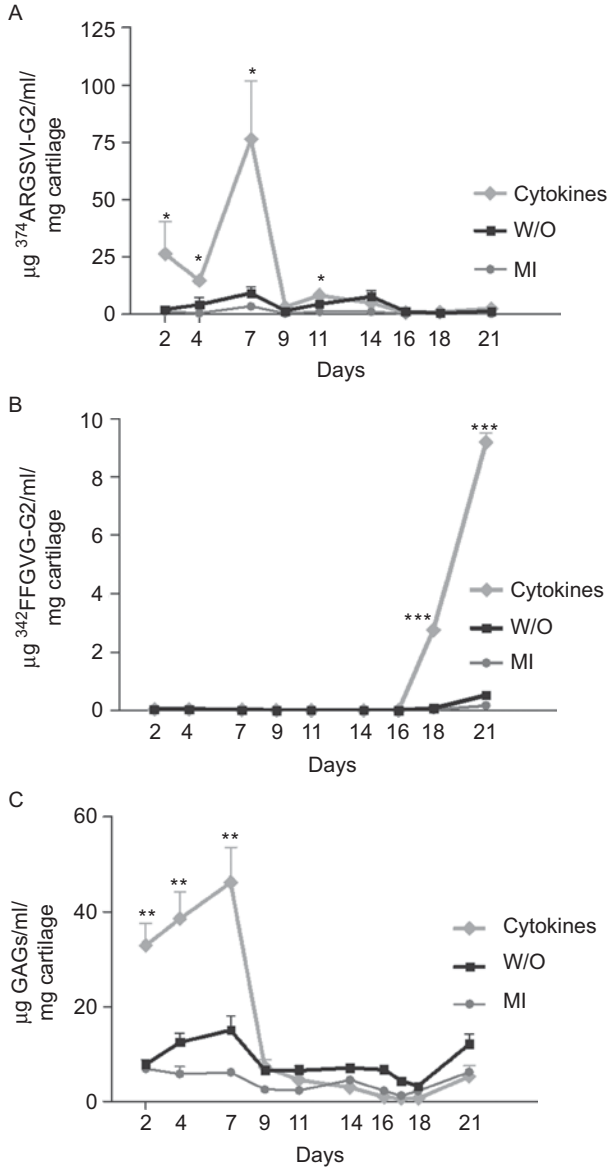

Figure 2. Quantification of biochemical markers. Bovine articular cartilage explants were isolated and cultured with the proinflammatory cytokines oncostatin M (OSM) (10 ng ml^{-1}) in combination with tumour necrosis factor (TNF)-α (20 ng ml^{-1}) (-◆-). The time-dependent release of aggrecanase-mediated ^{374}ARGSV-G2 (A), matrix metalloproteinase (MMP)-mediated ^{342}FFGVG-G2 fragments (B) or sulfated glycosaminoglycans (GAGs) (C) were quantified. Non-stimulated explants (-■-) were used as the vehicle. Metabolically inactivated (MI) explants (-●-) were used as a negative control to measure the non-chondrocyte-mediated release. The asterisks indicate significant difference compared with non-treated explants; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $n = 4$. W/O, non-stimulated controls).

neoepitope was recognized by the monoclonal antibody BC-3 (Figure 3A). This band was reduced to approximately 325 kDa upon deglycosylation (Figure 3B). In contrast, the low-molecular-weight smear of bands carrying the ^{342}FFGVG neoepitopes detected in the supernatant at day 21 with monoclonal antibody AF-28 (Figure 3C) was relatively insensitive to deglycosylation (Figure 3D). No bands were detected when BC-3 was used for staining of the conditioned media from day 21, or the monoclonal antibody AF-28 from day 7 (data not shown), which resemble the time-dependent analysis by the ^{374}ARGSV-G2 and ^{342}FFGVG-G2 assays in Figure 2A and B.

The calculated theoretic molecular weight difference between the neoepitopes ^{342}FFGVG-G2 and ^{374}ARGSV-G2 is approximately 3.6 KDa (32 aa). However, the molecular weight difference between these two neoepitopes when released as fragments to the conditioned media was far from this. The ^{374}ARGSV-G2 fragment was glycosylated and large, and the ^{342}FFGVG-G2 fragment was deglycosylated and small. This explains the correlation between the release patterns of sGAGs and aggrecanase-mediated aggrecan degradation. Furthermore, the Western blots clearly showed that the deglycosylated ^{374}ARGSV-G2 fragments were more than 200 KDa larger than the deglycosylated ^{342}FFGVG-G2 fragments. This indicates that we were not able to deglycosylate the ^{374}ARGSV-G2 fragments totally, and that the protein structure of the aggrecanase-mediated aggrecan fragments may be larger than the MMP-mediated ^{342}FFGVG-G2 fragments.

Lack of aggrecanase-mediated aggrecan degradation is not a consequence of lack of aggrecanase activity

In the late culture period, after day 16, we saw high levels of ^{342}FFGVG-G2 fragments and very low levels of ^{374}ARGSV-G2 fragments. To investigate if the lack of aggrecanase-mediated aggrecan degradation was caused by depletion of aggrecanase activity, we tested the conditioned medium at days 7 and 21. The concentration of aggrecanases released at day 7 from cytokines-stimulated cartilage did not show any increase compared with that from the non-stimulated cartilage. In contrast, the concentration of aggrecanases released at day 21 from cytokine-stimulated cartilage revealed an elevation of over 935% compared with non-stimulated cartilage (Figure 4). This reduced release of aggrecanases at day 7 is interesting, because of the high aggrecanase-mediated degradation of aggrecan at the same time. However, the aggrecanases might still be sited in the extracellular matrix, which is then released later due to the loss of sGAGs. It appeared that the low concentration of ^{374}ARGSV-G2 fragments at day 21 was not due to low aggrecanase activity.

The MMP-mediated deglycosylated aggrecan degradation released in the late period is not protected from further cleavage at the major aggrecanase site in the IGD in vitro

Aggrecanases were present in the conditioned medium at the end of the culture period. To investigate whether

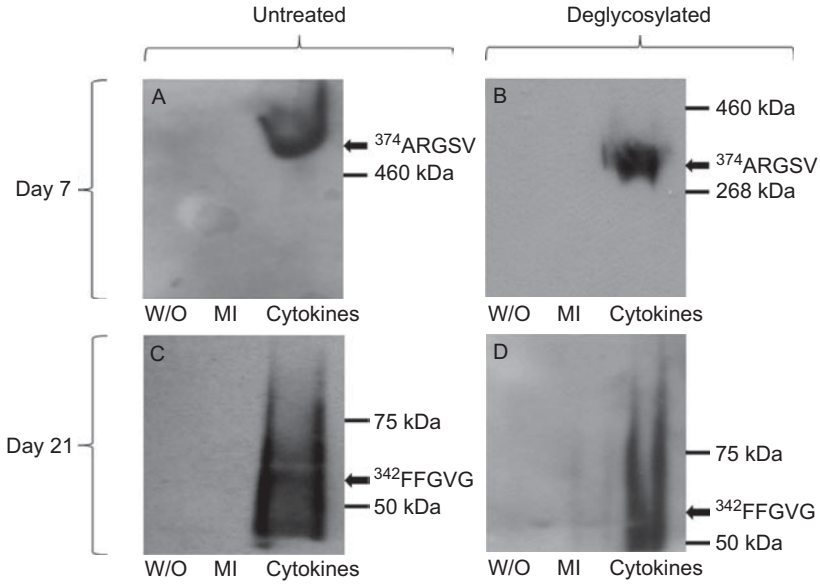

Figure 3. Western blot analysis of aggrecanase- and matrix metalloproteinase (MMP)-mediated fragments. Conditioned medium of four replicates of non-stimulated, metabolically inactivated (MI) or cytokines stimulated explants from day 7 or day 21 were separately pooled by days and either left untreated (A and C) or deglycosylated (B and D). Pooled supernatants were run in 3–8% precasted Tris–acetate gels under reducing conditions. The two uniform membranes were stained with either the monoclonal antibody BC-3 (1:50 dilution) staining for the aggrecanase-mediated ^{374}ARGSVI neoepitope (A and B) or the monoclonal antibody AF-28 (1200 ng ml^{-1}), staining for the MMP-mediated ^{342}FFGVG-sequence (C and D). Peroxidase-labelled rabbit antimouse antibody (1:5000 dilution) was used as a secondary antibody. A standard molecular weight marker was also run to determine the size of the detected fragments (not shown). W/O, non-stimulated controls).

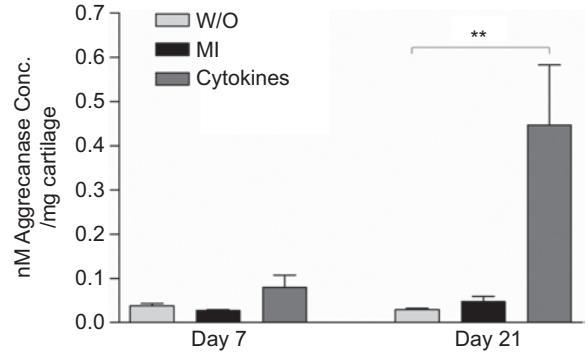

Figure 4. Quantification of aggrecanase activity in catabolically stimulated bovine explants. Bovine articular cartilage explants were isolated and cultured in the presence or absence of cytokines for 21 days. Non-stimulated explants were used as the vehicle. Metabolically inactivated (MI) explants were used as a negative control to measure the non-chondrocyte-mediated release. The mean value of aggrecanase activity in the conditioned medium was determined by measuring four different wells containing the same treatment. This was done for all three different treatments at days 7 and 21. The aggrecanase activity was significant increased ($p < 0.01$) in supernatants ($n = 4$) from cytokine-stimulated cartilage compared with the non-stimulated controls (W/O) at day 21.

the lack of ^{374}ARGSV-G2 fragments at day 21 was due to the inability of aggrecanases to process the ^{342}FFGVG-G2 fragments further, we added recombinant enzymes to the culture medium and measured further cleavage of the ^{342}FFGVG-G2 aggrecan fragments by quantitative measurements.

The conditioned medium from cytokine-stimulated explants from day 21 was digested *in vitro* with ADAMTS-4 and measured by the ^{342}FFGVG-G2 and ^{374}ARGSV-G2 assays. The aggrecanase degradation index (^{374}ARGSV-G2/^{342}FFGVG-G2) was calculated and the ADAMTS-4 digested media produced a 225% elevation compared with the index from non-ADAMTS-4-digested media (Figure 5). This indicates that the 373/374 cleavage site in the aggrecan fragments present at day 21 (only low-molecular-weight aggrecan) was functional, but only cleaved *in vitro*, not *ex vivo*.

Substrate affinities by MMPs and aggrecanases may be essential for the diverse generation of ^{342}FFGVG-G2 and ^{374}ARGSV-G2, and not the availability of aggrecan

But why were the aggrecanase-mediated aggrecan fragments not present *ex vivo* at day 21, when both aggrecanases and a functional aggrecanase cleavage site were present in the conditioned medium? To investigate the difference between *in vitro* and *ex vivo* cleavage in aggrecan, we focused on the importance of glycosylation, which was absent in the late culture period. We investigated the different proteolytic potentials for ADAMTS-4 and MMP-9 on either native or deglycosylated aggrecan *in vitro* and compared the results with the present *ex vivo* study. These proteolytic digestions were assessed by the ^{374}ARGSV-G2 and ^{342}FFGVG-G2 assays.

ADAMTS-4 digestion significantly increased the levels of ^{374}ARGSV-G2 on both native and deglycosylated aggrecan compared with the non-digested controls (Figure 6A). Furthermore, the ^{374}ARGSV-G2 levels were significantly higher for digested deglycosylated aggrecan compared with digested native aggrecan. MMP-9 digestion showed a similar degradation pattern to ADAMTS-4 digestion. The levels of ^{342}FFGVG-G2 were significantly increased on both native and deglycosylated aggrecan compared with the non-digested controls (Figure 6B). Furthermore, the ^{342}FFGVG-G2 levels were significantly higher for digested deglycosylated aggrecan compared with digested native aggrecan. This did not correlate with the detection of ^{374}ARGSV-G2 fragments at day 7 (high level) and day 21 (low level) in the *ex vivo* study (Figure 2A), but highlights the importance of distinguishing between *in vitro* and *ex vivo* results.

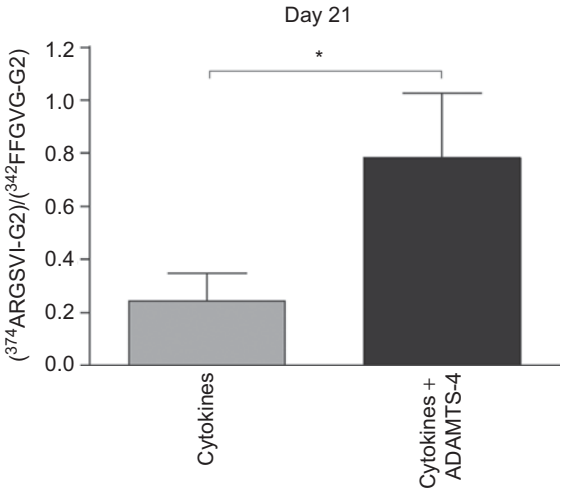

Figure 5. Further cleavage of released matrix metalloproteinase (MMP)-mediated ^{342}FFGVG-G2 fragments to ^{374}ARGSVI-G2 by ADAMTS-4. Supernatants from bovine articular cartilage explants cultured in the presence of cytokines for 21 days ($n=4$) were digested with 20 nM ADAMTS-4, diluted 1:50 and assessed by the immunoassays ^{374}ARGSV-G2 and ^{342}FFGVG-G2 quantifying aggrecanase- and MMP-mediated aggrecan fragments, respectively. The degradation index (^{374}ARGSV-G2)/(^{342}FFGVG-G2) was made for the ADAMTS-4-treated cytokine-stimulated supernatants (black bar) and the undigested (ADAMTS) cytokine-stimulated control (grey bar); *$p<0.05$.

Interestingly, the ^{374}ARGSV-G2 levels were approximately 50 times greater than the ^{342}FFGVG-G2 levels. This detectable difference between the two neoepitope assays measured from *in vitro* samples resembles the difference we were able to detect in the individual peaks at days 7 and 21 in the *ex vivo* samples (Figure 2A and B). This indicates that the *ex vivo* measurements were reliable. This confirms that the lower concentration level of ^{342}FFGVG-G2 compared with ^{374}ARGSV-G2 was not due to limited aggrecan as a substrate.

We were unable to conclude if glycosylation played an important role, based on the *in vitro* study. However, the substrate affinities of MMPs and aggrecanases may still be the essential factor for the diverse generation of ^{342}FFGVG-G2 and ^{374}ARGSV-G2 *ex vivo*.

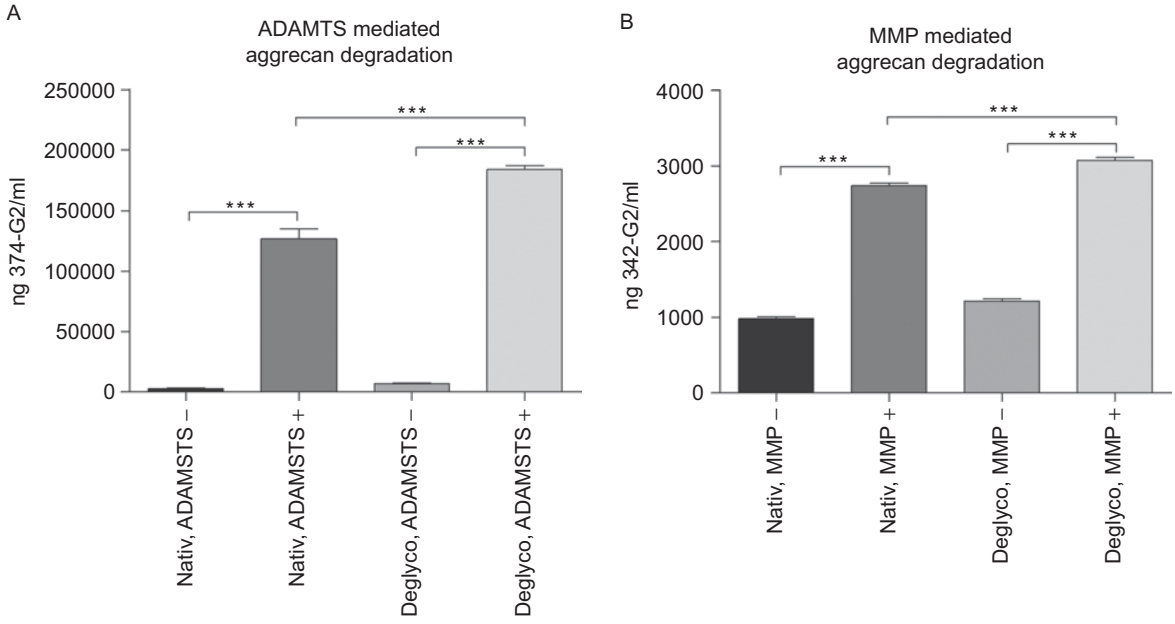

Figure 6. Matrix metalloproteinase (MMP)-9 and ADAMTS-4 digestion on native and deglycosylated aggrecan. Purified bovine aggrecan was either left untreated or deglycosylated. The native and deglycosylated aggrecan were each digested with MMP-9 and ADAMTS-4 and assessed by the ^{374}ARGSV-G2 (A) and ^{342}FFGVG-G2 (B) ELISA assays; *$p<0.001$; $n=4$.

Discussion

To elucidate the molecular mechanisms behind the destruction of the ECM, a large number of studies have reported the molecular nature of type II collagen and aggrecan fragments as they are released into the synovial fluid (Malfait et al. 2002, Sandy et al. 1992, Struglics et al. 2006) or into explant culture supernatants (Caterson et al. 2000, Fosang et al. 2000, Little et al. 1999, Sumer et al. 2007, Sztrolovics et al. 2002). While members of the MMP family have long been known to have the capacity to cleave aggrecan (Fosang et al. 1996, Struglics et al. 2006), only recently were the aggrecanases identified to have the same ability (Stanton et al. 2005, Struglics et al. 2006). Subsequently, the relative importance of MMPs and aggrecanases in aggrecanolysis has been debated.

In the present study we demonstrated that in cytokine-stimulated *ex vivo* cultures of articular bovine cartilage, both MMP- and aggrecanase-mediated aggrecan fragments were released into the supernatant, but not simultaneously. The catabolic stimulation of cartilage explants induced an early release of aggrecanase-mediated ^{374}ARGSVI-G2 fragments, and only after returning to background levels did we detect an increase in the MMP-mediated release of ^{342}FFGVG-G2 fragments. The peak at day 7 and subsequent drop in the release of aggrecanase-mediated aggrecan fragments in the *ex vivo* culture (Figure 2A) was not caused by depletion of the aggrecan as a substrate, because we detected MMP-mediated aggrecan degradation later in the culture period (Figure 2B), which verified the presence of the core proteins of aggrecan. Furthermore, our data suggested that the drop in aggrecanase-mediated aggrecan fragments was not caused by the lack of the aggrecanases, as a high level of their activity was detected in the late culture period (Figure 4).

As presented, we found both evidence of the presence of aggrecan as a substrate and aggrecanase activity in the late period of culture, but interestingly no ^{374}ARGSVI-G2 fragments. We further demonstrated that it was possible to generate ^{374}ARGSVI-G2 fragments from the MMP-mediated ^{342}FFGVG-G2 fragments, when treated with aggrecanases *in vitro*. This suggests that low concentrations of ^{374}ARGSVI-G2 fragments in the late culture period did not result from an inaccessible 373/374-cleavage site in the ^{342}FFGVG-G2 fragments. However, this does not explain why the cleavage only happens *in vitro* and not *ex vivo*. The ambiguity regarding the lack of ^{374}ARGSVI-G2 fragments in the late culture period resulted in further investigations. We detected an identical pattern of aggrecanase-mediated aggrecan degradation as occurred with the sGAG release. This link between ^{374}ARGSVI-G2 fragments and sGAGs was confirmed via Western blotting, showing highly glycosylated ^{374}ARGSVI-G2 fragments from the early culture period and deglycosylated ^{342}FFGVG-G2 fragments in the late culture period. These data suggest that two different pools of aggrecan molecules contain an N-terminal neoepitope in the IGD region (^{342}FFGVG and ^{374}ARGSVI), the G2 domain and a C-terminus of considerable difference. In particular, the C-terminus and glycosylation are obvious candidates to explain the discrepancy in the molecular sizes of the two aggrecan pools. The results from the present paper are summarized in Figure 7.

Our high- versus low-molecular-weight data support the theory described by Sandy (2006) that MMPs and aggrecanases act independently, and aggrecanases cleave intact aggrecan, whereas MMPs degrade aggrecan molecules which have already been C-terminally cleaved. Moreover, Lark et al. (1997) reported that MMPs are located in the damaged cartilage area, whereas aggrecanases are located in the non-damaged area. This is consistent with our study showing that aggrecanases are present in the beginning of the culture period and MMPs are present in the later culture period when the cartilage is further degraded.

Struglics et al. (2006) suggested that two distinct pathways were responsible for aggrecanolysis *in vivo:* first, the MMP-mediated ^{342}FFGVG-neoepitope in the pericellular matrix and secondly the aggrecanase-mediated ^{374}ARGSVI neoepitope in the inter-territorial matrix, on the same aggrecan molecule. This is not supported by the results in the present paper. However, the theory of the two distinct pathways is consistent with observations made in the present study, and is furthermore supported by identification of such aggrecan fragments in human synovial fluid spanning molecular sizes of 80–335 kDa (Struglics et al. 2006). Our Western blot data differ from those of Struglics et al. (2006) who found MMP-mediated high-molecular-weight fragments in the synovial fluid samples and in cartilage explants stimulated with MMPs. We only detected high-molecular-weight fragments from aggrecanase-mediated degradation (Figure 3).

One possible explanation for this interesting discrepancy is the differences between *ex vivo* and *in vivo* settings. In the current experiments we used a synchronic culture system, which presents a temporal predefined order of events following induction of catabolic activity against bovine cartilage (Karsdal 2007, 2008). This experimental setting is in contrast to OA patients who may have cartilage mal-metabolism ranging in severity at different distinct local places in the same joint. Enzymes are to some extent freely diffusible in the synovial fluid, so damaged cartilage areas mediated by extensive MMP activity may also 'share' some of these MMPs with other areas which have less cartilage damage, and which may primarily consist of aggrecanase activity. Such 'enzyme sharing' in the local environment may indeed generate the MMP-mediated high-molecular-weight fragments as described by Struglics et al. (2006).

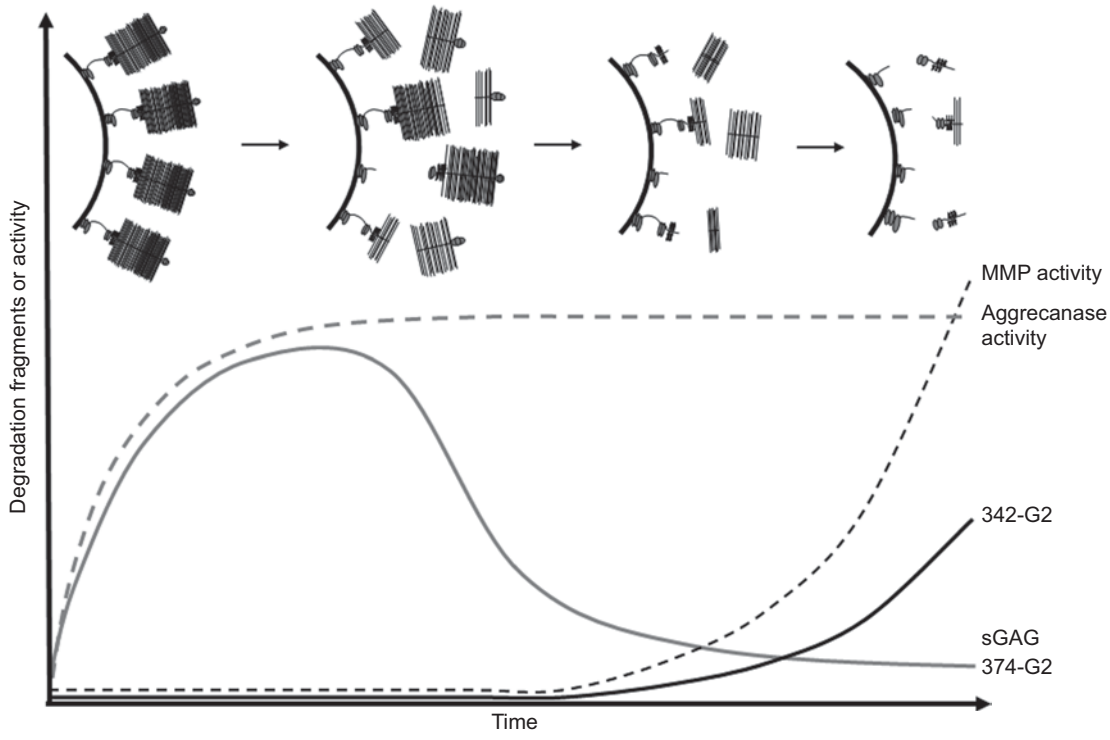

Figure 7. Schematic overview of aggrecanolysis in the explants model. The dotted lines represent protease activity of matrix metalloproteinase (MMPs) and aggrecanases over time. The other lines represent the degradation fragments released from the cartilage explants to the conditioned medium over time. Above the lines are cartoons of aggrecan attached to hyaluronic acid, which show the degradation and release of sulfated glycosaminoglycans (sGAGs) from the aggrecan molecule over time. Aggrecanases cleave the glycosylated aggrecan molecules in the early culture period, whereas MMPs degrade deglycosylated aggrecan molecules in the late culture period.

Importantly, Struglics et al. (2006) also suggested that aggrecanases cleaved MMP-mediated fragments in the highly glycosylated region (between G2 and G3), and not in the IGD. In addition, they presented data in agreement with Fosang et al. (2000), who clearly demonstrated that aggrecanases were unable to cleave ^{342}FFGVG fragments at the NITEGE$^{373–374}$ARGSVI site (Fosang et al. 2000). Our data support these observations and conclusions *ex vivo*, albeit not *in vitro*, as we observed that ADAMTS-4 was capable of processing the MMP-mediated ^{342}FFGVG-G2 fragments further in the IGD (Figure 5). However, a high concentration of aggrecanases was used to post-cleave the fragments released into the supernatant, and thus our data suggest that these fragments released *ex vivo* were protected from aggrecanase-mediated cleavage at the NITEGE$^{373–374}$ARGSVI site. The molecular mechanism behind this protection *ex vivo* is currently debated, but it may be the lack of glycosylation of the MMP-mediated aggrecan fragments. We support the suggestion by Tottorella et al. (2000) that aggrecanases may favour highly glycosylated forms of aggrecan, or depend on the protein sequence upstream of the ^{374}ARGSVI site for proper recognition and processing *ex vivo*. In fact, another group reported that aggrecanase activity is promoted by the presence of glycosylated aggrecan (Pratta et al. 2000).

All the aforementioned biomarkers were measured in conditioned medium synthesized by *ex vivo* cleavage. We designed an *in vitro* experiment to investigate the differences of *in vitro* and *ex vivo* cleavage in aggrecan. This enabled us to investigate the different proteolytic affinities for ADAMTS-4 and MMP-9 on native or deglycosylated aggrecan, which we assessed by the ^{374}ARGSV-G2 and ^{342}FFGVG-G2 assays. The measurements showed significant increased levels for ^{374}ARGSV-G2 and ^{342}FFGVG-G2 fragments when stimulated with ADAMTS-4 and MMP-9, respectively. *In vitro* cleavage showed that ^{374}ARGSV-G2 levels were approximately 50 times greater than the ^{342}FFGVG-G2 levels. This difference between the two neoepitope assays measured in the *in vitro* samples resembled the difference we detected in *ex vivo* samples (Figure 2A and B). This suggests that the approximately ten times lower levels of ^{342}FFGVG-G2 compared with ^{374}ARGSV-G2 levels measured from the *ex vivo* cleavage, were not due to limited aggrecan as a substrate, and therefore the measurements *ex vivo* represent a reproducible difference between the neoepitopes. Interestingly, the *in vitro* measurements showed that ADAMTS-4 and MMP-9 both show a preference to degrade deglycosylated aggrecan rather than native aggrecan. This is somewhat different from the *ex vivo* measurements, which indicated that ADAMTS-4 prefer to degrade glycosylated aggrecan

and MMP-9 favoured degrading deglycosylated aggrecan (Figures 2 and 3). This highlights the need to distinguish between *in vitro* and *ex vivo* results.

In conclusion, this study highlights the importance of the separate and distinct proteolytic processing of aggrecan, which varies over time. If the current findings are to be useful in a clinical setting, it may be favourable to target activity or expression of aggrecanases early in the OA process, whereas at later stages, after the depletion of sGAGs, it may be more efficacious to target expression or activity of MMPs.

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Declaration of interest

All authors declare that the affiliation declares full disclosure. In addition, Drs Karsdal and Qvist own stock in Nordic Bioscience.

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