INHIBITION OF ADAM-TS4 AND ADAM-TS5 PREVENTS AGGREGAN DEGRADATION IN OSTEOARTHRITIC CARTILAGE

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Abstract

Osteoarthritis is a degenerative joint disorder, characterized by breakdown of articular cartilage. Degradation of aggrecan, which together with type II collagen provides cartilage with its unique characteristics of compressibility and elasticity, is an early and sustained feature of osteoarthritis. The present work was set up to identify the enzyme(s) responsible for aggrecan breakdown in osteoarthritis. We found that the two cartilage aggrecanases, ADAM-TS4 and ADAM-TS5 are present in osteoarthritic cartilage and that they are responsible for aggrecan degradation, without the participation of MMPs. This is based on (1) neoepitopes found on aggrecan fragments in OA cartilage explants in vitro; (2) aggrecan fragments detected in synovial fluid of OA patients; (3) the observation that an aggrecanase-inhibitor, BB-16, blocked aggrecan degradation in OA cartilage in vitro, whereas the MMP-inhibitor XS309 did not; (4) the presence of mRNA and protein for ADAM-TS4 and ADAM-TS5 in OA cartilage. These results suggest that ADAM-TS4 and ADAM-TS5 represent a potential target for the treatment of osteoarthritis.

Key words (not in title): osteoarthritis, aggrecanase, aggrecanase-inhibitors, neoepitopes.
Introduction

Osteoarthritis (OA) is the most common joint disorder in the world. According to the World Health Organisation, OA affects 190 million people worldwide, thereby representing a major cause for pain and disability, especially in the aging population.

The main pathologic features are loss of articular cartilage, accompanied by hypertrophy in the subchondral bone and the joint margin. The pathogenesis of OA is poorly understood, but a major feature is the loss of aggrecan from the cartilage matrix (1). The key components of the cartilage ECM are type II collagen and aggrecan, which make up to 90% of the dry weight of healthy cartilage. Aggrecan hydrates the collagen network and thus provides cartilage with its properties of compressibility and elasticity. Maintenance of aggrecan content in articular cartilage is therefore critical to the function of the tissue. Aggrecan monomers consist of a 250-kDa-protein core with chondroitin sulfate and keratan sulfate glycosaminoglycan (GAG) side chains attached to it, resulting in a molecule of 1 to 2 million Da molecular mass. The amino terminal region of the aggrecan core protein contains two globular domains, G1 and G2, which are separated by an interglobular domain (IGD) that spans about 150 amino residues. The G2 region is followed by a long GAG attachment region and by the C-terminal globular domain G3 (2,3). Aggrecan monomers interact with hyaluronan through their G1 domain and thus form large aggregates containing 10-100 aggrecan monomers on a hyaluronan backbone.

Aggrecan depletion in arthritic cartilage has been ascribed to increased proteolytic cleavage of the core protein. Two classes of enzymes present in articular cartilage may be involved in the breakdown of aggrecan. First, there are the matrix
metalloproteinases (MMPs), which cleave aggrecan at the Asn$^{341}$-Phe$^{342}$ bond in the IGD. MMPs present in cartilage include MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, and MT1-MMP (MMP-14), all of which are capable of cleaving aggrecan at the Asn$^{341}$ and Phe$^{342}$ bond in vitro (4-7). A second class of enzymes, for a long time known as an activity given the name “aggrecanase”, cleaves the aggrecan core protein at another site in the IGD, between Glu$^{373}$ and Ala$^{374}$ (8-11). Interestingly, aggrecan fragments in inflammatory and OA synovial fluid appear to be generated by cleavage at this second site between Glu$^{373}$ and Ala$^{374}$ (12,13). Two cartilage aggrecanases, aggrecanase 1 or ADAM-TS4 (14) and aggrecanase 2 or ADAM-TS5 (15) have recently been identified and cloned. Both enzymes belong to the a disintegrin and metalloprotease with thrombospondin motifs (ADAM-TS) family of zinc metalloproteases that consist of an N-terminal propeptide domain, a metalloproteinase domain, a disintegrin-like domain, and a varying number of thrombospondin type 1 motifs, the sequence of which is the conserved motif in thrombospondin 1 and 2 (16).

Both ADAM-TS4 and ADAM-TS5 cleave aggrecan within the IGD at the aggrecanase site between residues Glu$^{373}$-Ala$^{374}$ at concentrations as low as 50 pM of enzyme, but at these concentrations do not cleave at the MMP site between residues Asn$^{341}$-Phe$^{342}$. Aggrecan fragments released upon IL-1 treatment of bovine cartilage contain the $^{374}$ARGS-neoepitope, suggesting that aggrecanase-activity is responsible for the aggrecan cleavage occurring in this system (17,18). MMP-8 and MT1-MMP are also capable of cleaving aggrecan at the Glu$^{373}$-Ala$^{374}$ bond to generate $^{374}$ARGS-containing fragments (19,20), suggesting that other metalloproteinases, other than ADAM-TS4/ADAM-TS5, may generate this neoepitope. However, cleavage at the
Glu$^{373}$-Ala$^{374}$ bond requires very high concentrations of MMP-8, therefore suggesting that MMP-8 may not represent cartilage aggrecanase (21).

We have recently demonstrated that human recombinant ADAM-TS4 and ADAM-TS5 cleave aggrecan preferentially at 4 additional sites located in the chondroitin sulfate-rich region, between G2 and G3 at the Glu$^{1545}$-Gly$^{1546}$, Glu$^{1714}$-Gly$^{1715}$, Glu$^{1819}$-Ala$^{1820}$ and Glu$^{1919}$-Leu$^{1920}$ bonds (22) (and Tortorella et al, submitted) (Fig.1). These sites correspond with fragments previously reported to be released during IL-1$\alpha$-treatment of bovine articular cartilage (9,18). Neoeptipe Ab to the aggrecan fragments generated by cleavage at the ADAM-TS4/ADAM-TS5-preferred sites provide a specific tool for confirming the involvement of these enzymes in aggrecan breakdown. Consistent with other models of aggrecan catabolism (23,24), it was thus established that aggrecan fragments released during IL-1 treatment of bovine articular cartilage are the result of cleavage at all the ADAM-TS4/ADAM-TS5 sites, suggesting that these are the enzymes involved in aggrecan degradation in this model (25).

Despite several reports in the literature implicating aggrecanase-activity in human OA (12,13,26,27), no evidence has been reported to demonstrate that ADAM-TS4/ADAM-TS5 are responsible for aggrecan breakdown in OA and more importantly, whether or not the breakdown in OA cartilage can be blocked. The current study was undertaken to address these issues.
Methods

Materials  - DMEM, FCS and penicillin/streptomycin were purchased from BioWhittakers (Verviers, Belgium). Keratanase, keratanase II and chondroitinase ABC were from Seikagaku Kogyo (Tokyo, Japan). The monoclonal neoeptope Ab BC-3 that recognizes the new N-terminus ARGS on aggrecan fragments produced by cleavage at the Glu\textsuperscript{373}-Ala\textsuperscript{374} bond (28), was a gift from Dr. C. Hughes (University of Wales, Cardiff, UK). Neoepitope Ab AF-28 that recognizes the new N-terminus FFGVG on aggrecan fragments produced by cleavage at the Asn\textsuperscript{341}-Phe\textsuperscript{342} bond (29), was a gift from Dr. A. Fosang (University of Melbourne, Parkville, Australia). ADAM-TS1, ADAM-TS4, and ADAM-TS5 were cloned and expressed in Drosophila S2 cells as described (14,22). Full length MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13 and the catalytic domain of ADAM-17 were cloned and expressed in E. coli at DuPont Pharmaceuticals. MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16) and MT4-MMP (MMP-17) were a gift from Dr. D. Pei (University of Minnesota, Minneapolis, MN). The hydroxamic acids XS309 ([3S-\(\text{3R}^*\),2\(-2\text{R}^*\), 2\(-\text{R}^*\text{,S}^*\)]-hexahydro-2\(-2\text{-}([2\text{-}((\text{hydroxyamino})\text{-}1\text{-}methyl\text{-}2\text{-}oxyethyl})\text{-}4\text{-}methyl\text{-}1\text{-}oxypentyl}\text{-}N\text{-}\text{methyl}\text{-}3\text{-}pyridazinecarboxamide) and BB-16 (2S,2R,6S-3-aza-4-oxo-10-oxa-5-hexyl-2-(methylcarboxamido)-(10)paracyclophe-6-N hydroxycarboxamide) were synthesized at DuPont Pharmaceuticals. The IC\textsubscript{50} values of these compounds against MMP-1, -2, -3, -8, -9 and -14 were calculated using the fluorogenic peptide substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH\textsubscript{2}, at a concentration of 1 \(\mu\text{M}\), and against ADAM-TS4 and ADAM-TS5 were determined using 500 nM purified bovine nasal aggregcan as described (22). XS309 is a nanomolar inhibitor of a large number of MMPs, but is inactive at <10\(\mu\text{M}\) in blocking ADAM-TS4/ADAM-TS5. In contrast, BB-16 is a nanomolar inhibitor of both MMPs
and ADAM-TS4/ADAM-TS5. Both small molecules, XS309 and BB-16, have been tested for stability both in buffer and in human plasma at 37 °C over a 24-hour time period. Integrity of the compound was monitored by bioassay and traditional liquid chromatography mass spectrometry (LC-MS). In the bioassay, both compounds were added to either buffer containing 50 mM Tris, 150 mM NaCl, 10 mM CaCl, pH 7.2, or human plasma at several concentrations, including 1000, 300, 100, 30, 10, 3, and 1 at 37 °C for up to 8 hours. Following the incubations, an equal volume of acetone was added to the samples, and total compound was collected by passing the material through the Microcon 96 Filtrate Assembly (Millipore). The compounds were then tested for their ability to inhibit MMP-3. No change in $K_i$ value was detected, indicating no breakdown of either compound over an 8-hour period. Stability of the compounds was also tested by LC-MS. In this study, both compounds were spiked in either buffer or plasma at a single concentration of 1 µM for 24 hours at 37 °C. Following the incubations, integrity of the compounds was assessed by LC-MS. No breakdown products of either compound were detected.

**Digestion of aggrecan** – All protein digestions were carried out in 100 µl of 50 mM Tris/HCl buffer, pH 7.5, containing 100 mM NaCl and 10 mM CaCl$_2$. Purified bovine aggrecan (500 nM) was incubated with MMPs at a concentration of 100 nM, with recombinant human ADAM-TS1 at 25 nM or with ADAM-TS4 and ADAM-TS5 at a concentration of 2 nM, at 37 °C for 24h. The reactions were stopped with EDTA and products analyzed by immunolocation in a Western blot analysis.
**Aggrecan neoepitope Ab** - Polyclonal neoepitope Ab to human aggrecan fragments generated by cleavage at the Glu_{1545}-Gly_{1546} (Glu_{1480}-Gly_{1481} for bovine aggrecan), Glu_{1714}-Gly_{1715}, Glu_{1819}-Ala_{1820} and Glu_{1919}-Leu_{1920} bonds were developed at DuPont Pharmaceuticals and found to be highly reactive and specific for the respective neoepitopes, as previously described (22).

**ADAM-TS Ab** - Polyclonal Ab for ADAM-TS4 and ADAM-TS5 were prepared to the peptide sequences ILTSIDASKP (residues 502-511), VMAHVDPEEP (residues 502-511) and DAKQCASLNG (residues 481-490), respectively, as previously described (25).

**RT-PCR for ADAM-TS4 and ADAM-TS5** – RT-PCR for ADAM-TS4 and ADAM-TS5 was performed as previously described (25).

**Cartilage explant culture** – Human OA cartilage was obtained from knees or hips at the time of joint replacement. Control healthy cartilage was obtained at autopsy within 24 hours post mortem from the knees of human donors. Only visually intact cartilage was used (labelled “normal” cartilage). All donors were 20 to 89 yr-old and had died of trauma or cerebro/cardiovascular accidents, and none of them had received long-term treatment with steroids or cytostatic drugs. Cartilage was allowed to equilibrate for 3 days in DMEM supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cartilage was then cut into 3x3 mm explants, weighing approximately 15 mg each and 8 replicates per treatment were incubated in 96-well plates in 200 µl of serum-free DMEM, containing antibiotics as above, for 48 or 72
hours. Following the incubation, 20 µl of conditioned media from each of the 8 wells/treatment were pooled prior to analysis.

*Extraction of aggrecan and its catabolites from cartilage* – At the end of the culture period, proteoglycans were extracted from the cartilage explants for 5 days at 4 °C with 4 M guanidinium hydrochloride (GuCl), 50 mM sodium acetate buffer, pH 6.8, containing the following protease-inhibitors: disodium EDTA (0.01M), 6-aminohexanoic acid (0.1M), benzamidine hydrochloride (0.05M), and PMSF (2mM).

*Glycosaminoglycan assay* – Sulfated GAG levels in the culture media were determined by the dimethylmethylene blue (DMMB) assay, as previously described (30).

*Deglycosylation of aggrecan and aggrecan catabolites* - Proteoglycans and their breakdown products were digested with keratanase (0.001 units per µg GAG), keratanase II (0.01 units per µg GAG), and chondroitinase ABC (0.001 units per µg GAG) and prepared for gel electrophoresis, as previously described (25).

*Western blot analysis* - Twenty µl of the pooled media for each treatment were analyzed by SDS-PAGE on 4-12% polyacrylamide gels under reducing conditions. The separated proteins were then transferred to PVDF membranes and immunolocalized with a 1:1000 dilution of one of the neoepitope Ab, as described (25).
Detection of ADAM-TS4 and ADAM-TS5 in cartilage matrix - Freshly obtained cartilage from the femoral head was extracted as described above, in a total volume of 9 ml. Cesium chloride (CsCl) was then added to a $d$ of 1.55 g/ml and a gradient was established by centrifugation at 40,000 rpm for 48h at 4 °C. Nine equal fractions per tube were taken, ranging in $d$ from 1.2 g/ml (fraction 1) to greater than 1.55 g/ml (fraction 9). These fractions were then dialysed exhaustively against water, and deglycosylated for Western Blot analysis.

Synovial fluid analysis – Synovial fluid (SF) was collected from the knees of 11 patients with knee OA, at various stages of disease. Clinical and radiological diagnosis of OA was made according to the classification criteria of the American College of Rheumatology (31). Fluids were spun to remove cells and frozen at -20°C for storage and shipment. One ml of SF was added to 1 ml of 8 M GuCl with 50 mM sodium acetate, pH 6.8, in the presence of protease inhibitors (0.1 mM PMSF, 5 mM EDTA, 5 mM iodoacetamide, 1 µg/ml pepstatin), and gently mixed for 8 h at 4 °C. CsCl was then added to a $d$ of 1.55 g/ml and gradients were established by centrifugation at 40,000 rpm for 48 h at 4 °C. Four equal parts per tube were taken, yielding fractions D1 (bottom, highest $d$) to D4 (top, lowest $d$). These fractions were then deglycosylated for Western blot analysis, as described above.
Results

MMPs do not cleave aggrecan at the aggrecanase-preferred sites

In order to determine whether neoepitope Ab that detect cleavage at the aggrecanase-sensitive sites within the C-terminal region of the aggrecan core protein may serve as specific tools for monitoring ADAM-TS4/ADAM-TS5-activity in cartilage, we assessed the ability of these sites to be cleaved by other proteases. We evaluated MMP-1, -2, -3, -8, -9, -13, and -14 (MT1-MMP), which have been shown to be present in the cartilage ECM, for the ability to cleave at these sites in comparison with their ability to cleave at the MMP site. We also evaluated MMP-15 (MT2-MMP), -16 (MT3-MMP), -17 (MT4-MMP), ADAM-17 (TACE), and ADAM-TS1. All of the MMPs and ADAM-17 were tested at a concentration of 100 nM, while ADAM-TS1 was tested at 25 nM, and ADAM-TS4/ADAM-TS5 at 2 nM. Bovine nasal aggrecan (500 nM) was incubated with each enzyme for 24h at 37°C and neoepitopes produced were assessed by Western blot analysis. Results are summarized in Table 1. As expected, all the MMPs tested, with the exception of MT4-MMP, cleaved aggrecan between residues Ser$^{341}$-Phe$^{342}$ to generate DIPES$^{341}$ (human = DIPEN$^{341}$) and $^{342}$FFGVG neoepitopes (Fig 2a). However, none of the MMPs under these experimental conditions (a relatively high enzyme to substrate ratio) were able to produce any of the aggrecanase-derived neoepitopes with the exception of MMP-8, which cleaved aggrecan in the IGD between residues Glu$^{373}$-Ala$^{374}$ to generate a $^{374}$ARGS aggrecan fragment of approximately 180 kDa (Fig. 2b). However, MMP-8 did not cleave any the C-terminal bonds that are readily hydrolyzed by ADAM-TS4 and ADAM-TS5 (Table 1 and Fig 2c). ADAM-TS1, at a concentration of 25 nM, did not cleave aggrecan at the MMP- or aggrecanase-
sensitive sites (Table 1). The catalytic domain of ADAM-TS1 shares 66% identity with ADAM-TS4 and 60% with ADAM-TS5, but did not cleave aggregan at any of the aggreganase-sensitive sites or in fact at any site in aggregan. Recently, Kuno et al. reported that human recombinant ADAM-TS1 did cleave bovine nasal aggregan between residues Glu<sup>1871</sup>-Leu<sup>1872</sup>, but the ratio of enzyme and the substrate used was about 20-times more than we employed for ADAM-TS5 and ADAM-TS4 (32). Buttner and colleagues reported that MT1-MMP cleaved rAgg1mut, a recombinant substrate composed of the complete IGD sequence of aggregan, at the Glu<sup>373</sup>-Ala<sup>374</sup> site (20). However, in the present study we found that MT1-MMP cleaved native aggregan exclusively at the MMP site between residues Asn<sup>341</sup>-Phe<sup>342</sup>, but not at any of the aggreganase sensitive sites, suggesting that the action of MT1-MMP on native aggregan is different from that of rAgg1mut.

**OA cartilage explants release aggregan fragments that contain aggreganase-generated, but not MMP-generated neoepitopes.**

Aggrecan extracted from cartilage was analyzed by Western blot for MMP-generated IPEN<sup>341</sup> and aggreganase-generated TEGE<sup>373</sup>-containing products. Both the IPEN<sup>341</sup> and the TEGE<sup>373</sup> epitopes were detected in normal and OA cartilage, with no apparent change in OA cartilage (Fig.3a). The presence of a 64-kDa fragment with the IPEN<sup>341</sup> C-terminus and a 70-kDa fragment with the C-terminus TEGE<sup>373</sup> in non-arthritic cartilage was a reproducible finding in cartilage from several individuals, as has been reported previously (26,27,33). We could detect these neoepitopes in cartilage from a donor as young as 7 weeks old (data not shown). This suggests that these fragments are retained in the matrix through the interaction of the G1 domain with hyaluronan and thus accumulate in cartilage during development and during the
lifetime of the individual (26,33). Quantifying these neoepitopes by Western Blot analysis and observing any change in disease will therefore be very difficult, and hence they will not be useful in providing an accurate parameter of aggrecanase- or MMP-activity in OA.

Therefore, we examined aggrecan fragments diffusing from normal and OA cartilage explants during a given culture period, using neoepitope Ab that recognize various aggrecan fragments lacking a G1 domain. Age-matched normal and OA cartilage explants were cultured for 72h and supernatants were then analyzed for GAG-content, which is a measure of the loss of degraded aggrecan into the culture media. More GAG was released into the media from OA than normal healthy cartilage over a 72-h culture period (Fig.3b). We detected no 342FFGV or 374ARGS neoepitopes in the supernatants of normal cartilage explants under the experimental conditions employed (Fig. 3b). Culture media of OA explants contained no detectable MMP-generated 342FFGV, but in contrast several different sized aggrecan fragments with the N-terminus 374ARGS were present (Fig. 3b). Since the AF28 Ab is about 10 times more reactive than the BC3 Ab (unpublished observations), it is unlikely that we are missing MMP-generated fragments.

To confirm that aggrecan fragments diffusing from OA cartilage are the result of cleavage by ADAM-TS4/ADAM-TS5, we looked for fragments generated by cleavage at the additional ADAM-TS4 and ADAM-TS5-preferred sites shown in Fig.1 that are not cleaved by other MMPs. The most preferred cleavage site of ADAM-TS4 and ADAM-TS5 is between Glu1714-Gly1715, generating the C-terminal KEEE1714 and the N-terminal 1715GLGS neoepitope (22) (and Tortorella et al, submitted). Using anti-KEEE1714, we detected a 375-kDa fragment in the OA culture media (Fig.3c), most likely representing the fragment with the 374ARGS N-terminus.
A larger fragment bearing this epitope was just detectable, probably representing the G1-containing fragment (Fig. 3c). Anti-\textsuperscript{1715}GLGS detected a 140-kDa fragment, likely representing a fragment containing G3 (Fig. 3c). We then evaluated media for aggrecan products resulting from cleavage of aggrecan at the other ADAM-TS4 and ADAM-TS5 preferred sites, one between residues Glu\textsuperscript{1545}–Gly\textsuperscript{1546}, one between Glu\textsuperscript{1819}–Ala\textsuperscript{1820}, and one between Glu\textsuperscript{1919}–Leu\textsuperscript{1920} (Fig. 3c). Using the anti-SELE\textsuperscript{1545} antibody, we detected a 250-kDa fragment in the OA culture media, most likely the fragment with the \textsuperscript{374}ARGS amino terminus. Again, the bigger fragment containing the G1 domain was less prominent. Anti-\textsuperscript{1820}AGEG detected a 120-kDa fragment and finally, anti-\textsuperscript{1920}LGQR detected a 98-kDa reactive fragment in the OA cartilage culture medium. The size of the detected fragments is consistent with fragments that are generated when recombinant human ADAM-TS4 and ADAM-TS5 cleave isolated aggrecan monomers (22). Dramatically lower levels of all the aggrecanase-generated fragments were detected in the supernatants from age-matched healthy cartilage (Fig. 3c).

OA cartilage matrix also contained all these ADAM-TS4/ADAM-TS5-generated neoepitopes (Fig. 3d). As anticipated, the larger fragments bearing the KEEE\textsuperscript{1667} or the SELE\textsuperscript{1545} epitopes were mainly retained in the matrix, and therefore these are most likely the G1-containing fragments. The \textsuperscript{374}ARGS-containing fragments were barely detected in the matrix (Fig. 3d), as they have lost the G1 domain that binds hyaluronan. This is also true for fragments with the N-terminus \textsuperscript{1820}AGEG and \textsuperscript{1920}LGQR. None of the aggrecanase-generated fragments were detected in healthy control cartilage.

In total, we studied OA cartilage from more than 15 donors, aged 50 to 85. In most cases, the cartilage was taken from the femoral head at the time of joint
replacement. Although all these patients needed hip replacement, the condition of the cartilage was very variable. Two samples were taken from the femoral condyles, one at the time of total knee replacement and one in a patient who underwent amputation of the lower limb because of osteomyelitis. One sample was a patch of fibrillated cartilage on the medial condyle, taken post mortem. We found that detection of aggrecanase-generated aggrecan fragments was a reproducible finding. Fig. 4 shows aggrecan fragments eluting out of 10 different OA samples.

**Aggrecan degradation in OA cartilage explants is blocked by an “ADAM-TS inhibitor”, but not by an “MMP inhibitor”**.

To confirm that neoepitopes released into the culture medium from OA cartilage was due to aggrecan cleavage occurring during the 72-h culture period and was mediated by ADAM-TS4 and ADAM-TS5, inhibition studies were performed using two different synthetic inhibitors containing a hydroxamic acid group. XS309 is a potent inhibitor of the known MMPs that is ineffective in blocking ADAM-TS4 and ADAM-TS5 at concentrations below 10 µM (referred to as “MMP inhibitor”) and BB-16 is potent in inhibiting both MMPs and ADAM-TS4/ADAM-TS5 (referred to as “ADAM-TS inhibitor”) (25).

Explants from normal and OA cartilage were cultured for 72 h in the presence of various concentrations of either XS309 or BB-16. At the end of the culture period, GAG levels in the culture media were determined by DMMB assay (Fig. 5a). The ADAM-TS inhibitor, BB-16, dose-dependently suppressed the loss of GAG from OA cartilage, with the 10 µM concentration suppressing GAG levels to the base level released by healthy cartilage (Fig. 5a). The MMP inhibitor, XS309, did not block GAG release unless used at 30 µM, a concentration at which this compound also
inhibits ADAM-TS4 (14,25). GAG release into the culture media was found to correlate with the presence of the $^{374}$ARGS neoepitope (Fig.5b). In a more extensive dose-response experiment, it was found that BB-16 blocked the release of aggrecan fragments bearing the $^{374}$ARGS neoepitope in a dose-dependent manner, with an IC$_{50}$ of less than 0.03 $\mu$M (Fig.5c). Inhibition experiments with BB-16 were reproduced in at least 10 independent experiments performed on OA cartilage from different donors. These findings represent the first demonstration that MMPs are not involved in aggrecan catabolism in OA cartilage.

**ADAM-TS4 is induced in OA cartilage whereas ADAM-TS5 is constitutively expressed in both normal and OA cartilage.**

RT-PCR was used to analyse freshly obtained human normal and OA cartilage for the presence of ADAM-TS4 and ADAM-TS5 mRNA (Fig.6). The former was not found in normal cartilage but was present in OA cartilage, suggesting that ADAM-TS4 is induced in disease. ADAM-TS5 mRNA was present in both normal and OA cartilage, suggesting that this enzyme is constitutively expressed, as described for bovine cartilage (25,34). However, more detailed analysis, where samples are matched in terms of age of the donor, gender, and joint location, is required before it can be concluded that this is a typical result.

mRNA results correlated with protein expression as assessed by Western blot analysis. For this purpose, OA cartilage matrix was extracted in 4 M GuCl and fractionated on a CsCl gradient under dissociative conditions. Both ADAM-TS4 (Fig.7a) and ADAM-TS5 (Fig.7b) were found in the low $d$ fractions, but some enzyme could be detected in the 1.6 $g/ml$ $d$ fraction, which is the fraction containing aggrecan. This may illustrate the tight interaction of the enzyme with its substrate, even under
stringent denaturing conditions. For ADAM-TS4 the main bands detected were at 98 kDa, 64 kDa, and 30 kDa (Fig. 7a). Based on expression of recombinant human ADAM-TS4 in insect cells, these bands most likely represent the zymogen, the active form of the enzyme in which the propeptide has been removed, and a proteolytic fragment, respectively (14). For ADAM-TS5, two major bands were detected, a broad band at 70 kDa (ADAM-TS5 has several glycosylation sites, which may explain the broadness of the band), and a C-terminally truncated form at 30 kDa. No band was seen corresponding to the predicted molecular weight of the zymogen. In a different experiment, age-matched normal and OA cartilage were compared. ADAM-TS4 was only detected in OA cartilage, even though the healthy cartilage was taken from a very old donor (89 yr-old) while ADAM-TS5 was found in both OA and healthy cartilage matrix (data not shown).

Aggrecan fragments in OA synovial fluids contain aggrecanase-generated neoepitopes.

Synovial fluids (SF) of OA patients were fractionated on a CsCl gradient and analyzed for the presence of neoepitopes. Most aggrecan fragments were found in the high d fractions D1 and D2. Fig. 8 shows the analysis of the SF of a patient with early OA (43 yr-old). No MMP-generated 342FFGV-containing fragments were detected in any of the fractions D1-D4, whereas the aggrecanase-generated neoepitopes 1820AGEG, 1715GLGS, SELE1545, KEEE1819, and 1920LGQR were all present on aggrecan fragments of the predicted size (55 kDa, 98 kDa, 120 kDa, 140 kDa, 250 kDa and 300 kDa). In this sample, no 374ARGS-containing fragments were detected. Eight additional OA SF samples were analyzed and variable levels of the aggrecanase-
generated neoepitopes were detected in fraction D1 (Fig. 9). The MMP-generated 343FFGV epitope was not detected in any of the SF analyzed.
Discussion

The present findings collectively suggest that aggrecan degradation in OA cartilage is mediated by the cartilage aggrecanases ADAM-TS4 and/or ADAM-TS5, without the participation of MMPs. This is based on (1) neoepitopes found on aggrecan fragments diffusing from OA cartilage explants in vitro; (2) aggrecan fragments detected in SF of OA patients; (3) the observation that aggrecanase-inhibitors such as the hydroxamic acid BB-16 block aggrecan degradation in OA cartilage in vitro, whereas MMP-inhibitors such as XS309 do not; (4) the presence of ADAM-TS4/ADAM-TS5 mRNA and protein in OA cartilage.

These findings bear important implications for the development of treatments for OA. Protection of aggrecan may provide the key to cartilage protection, as maintenance of aggrecan content in cartilage matrix is critical to the function of the tissue. Different animal models of OA, including spontaneous arthritis or arthritis induced by immobilization or joint destabilization, show that aggrecan depletion is an early feature of the pathology, irrespective of the etiopathogenesis (35-39). Likewise, in human OA, proteoglycan loss is an early feature of the disease (1). It has been hypothesized that aggrecan protects the collagen fibrillar network from proteolytic attack by collagenases due to steric and charge hindrance by the long negatively charged GAG chains attached to its core. Recently, we found evidence for this “protection” theory when we observed no detectable levels of collagen release in IL-1 stimulated bovine articular cartilage explants until after 14 d of culture when most of the aggrecan had been depleted from the ECM, consistent with other researchers (40). In contrast, live explants that were depleted of aggrecan by pretreatment with chondroitinase ABC, showed release of collagen in as little as 24 h following IL-1 stimulation (Tortorella and Pratta, unpublished data).
Until now, the development of new drugs for cartilage protection has focused on MMP-inhibitors, with as yet no apparent clinical benefit (41,42). Our findings suggest that ADAM-TS4/ADAM-TS5 will provide a better target, and blockade of these enzymes may prove beneficial in OA. Clinical evaluation of a hydroxamic acid that selectively inhibits aggrecanase is warranted, as this would avoid the potential multiple side effects of broad spectrum MMP-inhibitors (43-45).

The present work suggests that, contrary to existing evidence, it is possible to stop aggrecan breakdown in OA cartilage ex vivo, irrespective of the stage of disease. For the inhibitor studies described here (n > 10), we used a varied array of OA cartilage, from patches of early degenerative changes present on the femoral condyle to the few remnants of cartilage left on an eburnated femoral head at the time of joint replacement. In every case it was found thataggrecan degradation could be abrogated by the in vitro addition of BB-16, suggesting that aggrecanase-mediated aggrecan breakdown is both an early and a sustained feature of OA. This observation suggests that the inhibition of ADAM-TS4/ADAM-TS5 will provide a valuable target for the development of new cartilage-protective drugs. A major roadblock in clinical trials for OA, however, is the lack of good surrogate markers to monitor cartilage aggrecan breakdown. As demonstrated in synovial fluid, neoepitope antibodies to aggrecanase-generated aggrecan fragments provide a means of monitoring activity of ADAM-TS4 and ADAM-TS5. Analysis of 8 different SF samples revealed that a fragment containing the $^{1820}$AGEG N-terminus was most readily detected. Based on the preferential rate of cleavage at this site (22), one would predict to find a higher ratio of this epitope in comparison with other neoepitopes, for example $^{373}$ARGS, and this is indeed what we found for all but one SF sample (SF3, Fig.9). Equally important, neoepitope antibodies recognizing the ADAM-TS4/ADAM-TS5 preferred sites of
cleavage within the C-terminal region of aggrecan are specific for the aggrecanases, as no MMP was able to cleave at these sites, including MMP-8 that does cleave at the classical aggrecanase site between residues Glu$^{373}$-Ala$^{374}$. We are currently exploring the use of neoepitope antibodies as markers for cartilage degradation in OA.

Future studies will focus on which aggrecanase, ADAM-TS4 or ADAM-TS5, is critical for aggrecan catabolism in OA. In addition to monitoring the increase in ADAM-TS4/ADAM-TS5 message and protein, it will be equally important to study the activation of these proteinases as an alternative mechanism for inducing aggrecan catabolism in disease (46).
Acknowledgements

The work described herein was mostly performed at the Kennedy Institute of Rheumatology, London, UK. The authors would like to thank Michael Pratta at DuPont Pharmaceuticals for development of the anti-NITEGE and anti-DIPEN antibodies. We are indebted to Dr. Andrew Wallace, Orthopaedic Surgeon at the Charing Cross Hospital, London, UK, for hip and knee replacement specimens, to Dr. J. Billiet and colleagues, Algemeen Ziekenhuis Sint-Jan, Bruges, Belgium and to Professor G. Verbruggen and colleagues, Dept.of Rheumatology, University of Ghent, Belgium for providing us with normal cartilage.
References


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Figure Legends

Fig. 1. Schematic representation of ADAM-TS4/ADAM-TS5 and MMP-cleavage sites in the human aggrecan core protein. In bovine aggrecan, cleavage at the Glu\textsuperscript{1480}-Gly\textsuperscript{1481} bond generates the C-terminal neoepitope GELE rather than the SELE neoepitope of the human aggrecan fragment with a single amino acid substitution of G for S in the P4 position. In addition, the IPEN C-terminal neoepitope generated by cleavage at the Asn\textsuperscript{341}-Phe\textsuperscript{342} bond in the IGD of human aggrecan is IPES in bovine aggrecan with a single amino acid substitution of N for S at the P1 position.

Fig. 2. Cleavage of aggrecan by metalloproteinases. MMPs (100 nM), ADAM-17 (100 nM), ADAM-TS1 (25 nM), ADAM-TS4 and ADAM-TS5 (2 nM) were incubated with 500 nM of bovine aggrecan for 24 h at 37°C. Following the incubation, the products were analyzed for (a) DIPES, (b) ARGS and (c) GELE neoepitopes by Western blot analysis.

Fig. 3. Cleavage of aggrecan in osteoarthritic cartilage. (a) Age-matched normal (N - age: 62 yr) and osteoarthritic (OA – age: 66 yr) cartilage explants were cultured for 72 h. Cartilage matrix was analyzed for the aggrecanase-generated TEGE\textsuperscript{373} neoepitope (right panel) and the MMP-generated IPEN\textsuperscript{341} neoepitope (left panel). (b) Media were analyzed for the aggrecanase-generated \textsuperscript{374}ARGS (right panel) and the MMP-generated \textsuperscript{342}FFGV (left panel). GAG-levels in the media are shown for \( n = 5 \) wells ± SD. In an independent experiment, media (c) and cartilage matrix (d) were analyzed for all the ADAM-TS4/ADAM-TS5-generated neoepitopes. N= 72-yr-old and OA = 72-yr-old.

Fig. 4. Cleavage of aggrecan in osteoarthritic cartilage in 10 donors. OA cartilage explants from 10 donors were incubated for 72 h. Culture media were analyzed for the presence of the aggrecanase-generated neoepitopes \textsuperscript{374}ARGS, SELE\textsuperscript{1545}, or \textsuperscript{1820}AGEG. The age of the donors is shown on top of the blots.

Fig. 5. Inhibition of aggrecan degradation in osteoarthritic cartilage by hydroxamic acid metalloproteinase inhibitors. Normal (N) or OA cartilage
explants were cultured for 72 h in the presence of XS309 (open bars) or BB-16 (hatched bars) and media were analysed for (a) GAG-levels \( n = 5 \) wells per group \( \pm \) SD) and (b) the presence of the \(^{374}\text{ARGS-neoepitope}\); (c) OA cartilage was cultured for 72 hours in the presence of a wide concentration range of BB-16, and media were analysed for the \(^{374}\text{ARGS-neoepitope}\) by Western Blot analysis and the amount of product was determined by scanning densitometry.

**Fig. 6. Expression of ADAM-TS4 and ADAM-TS5 message in normal (N) and OA cartilage.** RT-PCR for ADAM-TS4, ADAM-TS5, and GAPDH in a sample of freshly isolated normal (N – age: 56 yr) and OA (age: 76 yr) cartilage. Full length cDNAs for ADAM-TS4 and ADAM-TS5 were included as positive controls.

**Fig. 7. Expression of ADAM-TS4 and ADAM-TS5 protein in OA cartilage.** Western Blot analysis for (a) ADAM-TS4 and (b) ADAM-TS5 in CsCl fractionated cartilage extract of a patient with OA.

**Fig. 8. Aggrecan neoepitopes in OA synovial fluid.** Synovial fluid of a 43-yr-old male with early OA was fractionated on a CsCl gradient and fractions D1 (high \( d \)) to D4 (low \( d \)) were screened for aggrecan fragments containing MMP- or aggrecanase-generated neoepitopes.

**Fig. 9. Aggrecan neoepitopes in OA synovial fluids.** Analysis of CsCl fraction D1 of 8 synovial fluid (SF) samples for FFGV, AGEV, ARGS and SELE containing fragments by Western blot analysis. SF1 = late OA, 75 yr-old; SF2 = late OA, 86 yr-old; SF3 = early OA, 43 yr-old; SF4 = early OA, 81 yr-old; SF5 = late OA, 78 yr-old; SF6 = late OA, 66 yr-old; SF7 = early OA, 80 yr-old; SF8 = early OA, 55 yr-old.
Table 1. Cleavage of aggrecan by metalloproteinases*.

<table>
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<tr>
<th>Protease</th>
<th>MMP Epitopes</th>
<th>ADAM-TS4 and ADAM-TS5 Derived Epitopes</th>
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<tr>
<td></td>
<td>IPES FFGV</td>
<td>TEGE ARGS KEEE GLGS GELE AGEG LGQR</td>
</tr>
<tr>
<td>MMP-1</td>
<td>+ +</td>
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</tr>
<tr>
<td>MMP-2</td>
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* MMPs (100 nM), ADAM-17 (100 nM), ADAM-TS1 (25nM) or ADAM-TS4 and ADAM-TS5 (2 nM) were incubated with 500 nM of aggrecan for 24 h at 37 °C. Following the incubation, the products were analyzed for MMP-generated neoepitopes and aggrecanase-generated neoepitopes by Western blot analysis. ‘+’ indicates presence and ‘-’ indicates absence of neoepitope-reactive bands.
Figure 1

ADAM-TS4/ADAM-TS5 Cleavage Sites

70 kDa                   250 kDa          55 kDa       20 kDa   22 kDa    98 kDa

TEGE^{373} 374 ARGS

G1  G2  G3

70 kDa  250 kDa  55 kDa  20 kDa  22 kDa  98 kDa

IPEN^{341} 342 FFGV
IPES^{341}

MMP Cleavage site

Figure 1
Figure 2a
Figure 2b
Figure 2c
Figure 3a
Figure 3b

GAG release into medium: 0.48 ± 0.05 (microgram/mg) 0.98 ± 0.1

FFGV

MW  N  OA
320 kDa
250 kDa
98 kDa
64 kDa
50 kDa
36 kDa
30 kDa

ARGS

MW  N  OA
0.48 ± 0.05 0.98 ± 0.1
Figure 3c
Figure 3d
Figure 4
Figure 5a

Inhibitor [micromolar]

GAG release [µg/mg]
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<th>10</th>
<th>30</th>
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<tbody>
<tr>
<td></td>
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<td>98 kDa</td>
<td>64 kDa</td>
<td>50 kDa</td>
<td>36 kDa</td>
<td>30 kDa</td>
<td>normal cartilage</td>
<td>OA cartilage</td>
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</table>

**BB-16 [microM]**

**XS309 [microM]**

Figure 5b
Figure 5c

MW     10        3        1       0.3     0.1    0.03    0.01   0

BB16 [microM]

250 kDa
98 kDa
64 kDa

IC50

Density
Figure 6
Figure 7b
Cesium chloride fractions

Figure 8
Figure 9
Inhibition of Adam-TS4 and Adam-TS5 prevents aggrecan degradation in osteoarthritic cartilage
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