ADAMTS-5 DEFICIENCY DOES NOT BLOCK AGGREGANOLYSIS AT PREFERRED CLEAVAGE SITES IN THE CHONDROITIN SULPHATE-RICH REGION OF AGGREGAN

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Running Title: ADAMTS-5 is not soley responsible for CS-2 domain cleavage of aggrecan

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Summary

In the mouse, proteolysis in the aggrecan interglobular domain is driven by ADAMTS-5, and mice deficient in ADAMTS-5 catalytic activity are protected against aggrecan loss and cartilage damage in experimental models of arthritis. Here we show that despite ablation of ADAMTS-5 activity, aggrecanolysis can still occur at two preferred sites in the chondroitin sulphate-rich region. Retinoic acid was more effective than IL-1α in promoting cleavage at these sites in ADAMTS-5-deficient cartilage. These results suggest that cleavage at preferred sites in the chondroitin sulphate-rich region is mediated by ADAMTS-4 or an aggrecanase other than ADAMTS-5. Following retinoic acid or IL-1α stimulation of cartilage explants, aggrecan fragments in medium and extracts contained SELE\textsuperscript{1279} or FREE\textsuperscript{1467} C-terminal sequences. Some SELE\textsuperscript{1279} and FREE\textsuperscript{1467} fragments were retained in the cartilage, with intact G1-domains. Other SELE\textsuperscript{1279} fragments were released into the medium and co-migrated with \textsuperscript{374}ALGS neoepitope, indicating they were aggrecanase-derived fragments. In contrast none of the FREE\textsuperscript{1467} fragments released into the medium co-migrated with \textsuperscript{374}ALGS neoepitope, suggesting that, despite their size, these fragments were not products of aggrecanase cleavage in the interglobular domain. ADAMTS-5, but not ADAMTS-1, -4, or -9, was upregulated 8-fold by retinoic acid and 17-fold by IL-1α treatment. The data show that whereas ADAMTS-5 is entirely responsible for cleavage in the interglobular domain, cleavage in the chondroitin sulphate-rich region is driven either by ADAMTS-4, which compensates for loss of ADAMTS-5 in this experimental system, or possibly by another aggrecanase. The data show that there are differential aggrecanase activities with preferences for separate regions of the core protein.

Introduction

A feature of joint pathology in arthritis is destruction of articular cartilage. The major structural components of cartilage are type II collagen and the large aggregating proteoglycan, aggrecan. In healthy cartilage, type II collagen and aggrecan confer strength and compliance that enables this tissue to resist compressive forces. In arthritic diseases, the progressive degradation of aggrecan and type II collagen leads to cartilage erosion. Aggrecan has two globular domains, G1 and G2 at the N-terminus, and a third globular domain, G3 at the C-terminus. An extended sequence between the G2 and G3 domains is heavily substituted with keratan sulphate and chondroitin sulphate chains, organised into a keratan-sulphate-rich region and chondroitin sulphate-1 (CS-1) and chondroitin sulphate-2 (CS-2) domains. An interglobular domain (IGD) of approximately 150 amino acids separates G1 from G2.

The aggrecan IGD is highly sensitive to proteinases. Proteolysis in the IGD releases the entire chondroitin sulphate and keratan sulphate-rich regions essential for the biomechanical properties of aggrecan, and is therefore thought to be the most detrimental for cartilage function. In pathology, proteolysis is driven mainly by aggrecanases, but there may also be some involvement of matrix metalloproteinases (MMPs) in late stage disease (1-3). “Aggrecanase” was first identified as a novel activity that cleaved the aggrecan core protein at the
E$^{373} \downarrow 374$ A bond in the IGD (4-6) and at four specific sites in the CS-2 domain (5,6). The products of this cleavage were found in synovial fluids from osteoarthritic, joint injury and inflammatory joint disease patients (7,8). The aggrecanases are members of the ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondin motifs) family, designated ADAMTS-4 (aggrecanase-1)(9) and ADAMTS-5 (aggrecanase-2)(10). Several other ADAMTS enzymes have weak activity against aggrecan in vitro, including ADAMTS-1 (11), ADAMTS-8 (12), ADAMTS-9 (13) and ADAMTS-15, however ADAMTS-4 and -5 are the most efficient aggrecanases (12-14). The ADAMTS proteins are a family of zinc-dependent enzymes within the metzincin family of metalloproteinases.

In addition to the catalytic domain that is maintained in a latent form by an N-terminal prodomain, members of the ADAMTS family also contain a disintegrin domain, one or more thrombospondin motifs, a cysteine-rich domain and a spacer domain of variable length (15,16). ADAMTS-5 has recently been identified as the major aggrecanase in mouse cartilage (17,18).

Aggrecan cleavage at the E$^{373} \downarrow 374$ A bond in the IGD is the signature activity of the aggrecanases and is widely reported in humans and animals as a marker of aggrecanase activity. However, cleavage at E$^{373} \downarrow 374$A is not the preferred action of these enzymes. In vitro, recombinant ADAMTS-4 and -5 preferentially cleave aggrecan in the CS-2 domain (19,20). The two most preferred cleavage sites in bovine aggrecan are at KEEE$^{1666} \downarrow 1667$GLGS, followed by GELE$^{1480} \downarrow 1481$GRGT. Thereafter, further cleavages occur at TAQE$^{1771} \downarrow 1772$AGEG and VSQE$^{1871} \downarrow 1872$LGQR in the CS-2 region and at NITEGE$^{373} \downarrow 374$ARGS in the IGD. A similar hierarchy of cleavage preferences is shown by native aggrecanases in cell culture (21,22).

We recently published that ablation of ADAMTS-5 protects against aggrecan loss and cartilage erosion in a mouse model of inflammatory arthritis (18). This study was complimentary to a study by Glasson et al (17) who showed that the ADAMTS-5 deficient mouse was also protected against aggrecan loss and cartilage erosion in a surgically-induced model of arthritis, more like human osteoarthritis. Here, we present new data on aggrecanase cleavage at preferred sites in the mouse CS-2 domain that were not examined in our earlier study. We find that unlike cleavage in the IGD, cleavage in the CS-2 domain at FREEE$^{1467}$ and SELE$^{1279}$ is not blocked in the ADAMTS-5 Δcat mouse. Our results highlight the recent realisation that an accurate readout of aggrecanolysis requires analysis of both IGD and CS-2 domain cleavage.

**Experimental Procedures**

**Generation of ADAMTS-4 and ADAMTS-5 Δ-cat mice**

The generation of ADAMTS-4 and -5Δcat mice by Cre-mediated excision of floxed exons encoding the catalytic sites has been described previously (18). In the present study, cartilage taken for in vitro experiments was harvested from mice in which the Cre transgene, previously present on one allele of chromosome X, was out-bred. Cre was removed to avoid the possibility of Cre-mediated recombination between cryptic pseudo-loxP sites and the effects reported in vitro (23) and in vivo (24). Age-matched mice from wildtype breeders on a C57/129 mixed background were used as controls for mutant mice from Δcat breeders.

**Cartilage explant cultures**

Femoral head (hip) cartilage was harvested from 3 week old mice. These explants contain a small amount of growth plate cartilage as reported previously (25) but no bone, periosteum or synovium. Remnants of ligament (ligament teres femoralis) are rarely present. Hypertrophic chondrocytes and mineral are present in a central region of calcified cartilage that separates the growth plate from the articular surface. However, because the femoral head does not become vascularised, it does not form a true secondary centre, marrow cavity, or bone. The explants were initially cultured at 37°C with 5% CO$_2$ in DMEM containing 10% FCS, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine and 20 mM HEPES. After 2 days, the explants were washed in serum-free medium and placed in a 48-well culture dish with fresh serum-free medium containing either 10$^{-3}$M retinoic acid (Sigma) or 10ng/mL human recombinant IL-1α (Peprotech, NJ, USA) and cultured for a further 3 days. Medium and explants were collected at day 0, 1, 2 and 3 after changing to serum-free medium. Aggrecan and aggrecan fragments were extracted from the explants for 48 h at 4°C with 4M guanidine hydrochloride (GuHCl), 50mM sodium acetate, pH 5.8, 10mM EDTA and 0.1M caproic acid. Residual, non-extractable aggrecan remaining in the cartilage was recovered by digesting overnight at 60°C with 3.5 U/ml papain.
in buffer containing 0.1M sodium acetate, pH 5.5, 5 mM cysteine, 5mM EDTA.

Quantitation of aggrecan
The 4M GuHCl cartilage extracts were dialysed against ultra pure water containing 10mM EDTA at 4°C. The concentration of sulphated glycosaminoglycan (as a measure of aggrecan) in extracts, medium and papain digests was determined using the 1,9-dimethylmethylene blue assay (26). Total aggrecan was calculated as the sum of glycosaminoglycan in the conditioned media, GuHCl extracts and papain digests. The cumulative release of aggrecan into the conditioned media was expressed as a percentage of total aggrecan.

Western Blotting
Prior to SDS-PAGE, aliquots of conditioned media and dialysed extracts were digested for 6 hours at 37°C with 0.01 units Chondroitinase ABC (Seikugaku, Japan) per 10ug glycosaminoglycan in 0.1M Tris acetate pH 6.5, containing proteinase inhibitors E-64 (10ug/ml), AEBSF (0.5mM), pepstatin (5ug/ml) and EDTA (10mM). The total amount of aggrecan (medium + extract + residue) was calculated for each sample. We found that total aggrecan was a more accurate denominator than tissue wet weight for normalising samples for Western blot analysis because the weight of a single mouse hip is so small (between 0.5–1.0mg). The volume of medium or extract loaded onto the gel was determined empirically for each neoepitope. Samples analysed for 374ALGS, and NVTEGE 373 neoepitopes were electrophoresed on 7.5% SDS-polyacrylamide gels containing 2M urea, under reducing conditions and then transferred to PVDF membranes (Millipore, USA). Membranes were probed with antibodies that recognise neoepitopes created by aggrecanase activity and include anti-NITEGE 373 (27), anti-FREEE 1467, anti-SELE 1279 and anti-374ALGS. The anti-NITEGE 373 antibody recognised the mouse NVTEGE 373 neoepitope after aggrecanase cleavage. After immunodetection with anti-NITEGE 373 or anti-374 ALGS, the blots were stripped with Re-blot Plus (Chemicon International, USA) and reprobed with monoclonal antibody 2B6 that recognises stubs of chondroitin-4-sulphate chains on aggrecan after digestion with chondroitinase ABC. Samples analysed for SELE 1279 and FREEE 1468 neoepitopes were electrophoresed on 5% gels. Primary antibody binding was detected using HRP-conjugated secondary antibodies (Dako, Glostrup, Denmark) and enhanced chemiluminescence (ECL-plus, Amersham, Buckinghamshire, UK). Western blot membranes for direct comparison with each other were developed together. In most cases, blots were developed with ECL reagent until the most strongly-detected band in the series approached saturation.

Generation of the anti-374 ALGS, anti-FREEE 1467 and anti-SELE 1279 neoepitope antibodies
Synthetic peptides with the sequence ALGSVILTAGCC, CGGPTTFREEE and CGGATTSSELE (Auspep, Australia) were conjugated to ovalbumin using bromoacetic acid N-hydroxysuccinimide ester (28). Polyclonal rabbit antiseraum was raised against the ovalbumin-conjugated peptides at the Institute for Medical and Veterinary Science (IMVS; Adelaide, Australia) and screened against the same peptide immunogens conjugated to bovine serum albumin. Polyclonal rabbit antiseraum against the identical ovalbumin-conjugated 374 ALGS immunogen was also raised by Chemicon International (CA, USA). The anti-374ALGS, anti-FREEE 1467 and anti-SELE 1279 IgGs were purified from rabbit sera by protein-A affinity chromatography. The titre and specificity of the purified anti-374 ALGS IgG from IMVS and Chemicon was the same.

RNA isolation and Real-time RT-PCR analyses
Total RNA was extracted from ∆cat and wildtype cartilage using the RNeasy kit (Qiagen) and isolated according to the manufacturer’s instructions. Reverse transcription was done on 100ng total RNA using MuLV reverse transcriptase (Applied Biosystems) and random hexamer primers. The reaction was done for 1 h at 42°C, after which the enzyme was inactivated by incubation for 5 min at 99°C. Taqman® real-time RT-PCR was used to measure the mRNA levels of ADAMTS-1, ADAMTS-4, ADAMTS-5 and ADAMTS-9. Primer and probe sets for the detection of each ADAMTS mRNA (18) were designed and synthesised using the ABI “Primer-by-Design” service and reactions were done using the ABI Prism 7700 Sequence Detection System (Perkin Elmer, Boston, MA, USA). Multiplex PCR was done with primer/probe sets to the target (ADAMTS) and reference (18s rRNA) in the same reaction. mRNA as quantitated using the comparative C_T method (29), using 18S rRNA as the reference. Preliminary experiments showed that the efficiencies of target and reference
amplifications were similar in all cases, thus validating the use of this protocol. The relative amount of mRNA expression in samples taken at days 1, 2 or 3, compared with samples taken immediately ex vivo (day 0), was determined by the formula \(2^{\Delta \Delta Ct}\), where \(\Delta Ct\) was calculated by subtracting the \(Ct\) value for day 0 from the \(Ct\) value for day 1, 2 or 3. The experiment was done once with pooled cartilage from 5 - 7 mice for each genotype, at each time point, and it required 70 mice.

Results

For the present study, we generated new lines of \(\Delta cat/\Delta cat\) and control mice that lacked the \(Cre\) transgene. Heterozygous, \(Cre\)-negative ADAMTS-4-\(\Delta cat\) breeders on a mixed C57/129 background produced litters of normal size and gender distribution (390 pups from 55 litters) with the expected Mendelian ratio of wildtype, heterozygous and homozygous \(\Delta cat\) pups (24%, 49% and 27% respectively). Heterozygous \(Cre\)-negative ADAMTS-5-\(\Delta cat\) breeders also produced litters of normal size and gender distribution (450 pups from 60 litters) with the expected ratio of wildtype, heterozygous and homozygous \(\Delta cat\) pups (28%, 48% and 24% respectively).

Phenotypic analysis

Homozygous ADAMTS-4- and ADAMTS-5-\(\Delta cat\) mice were phenotypically normal with no morphological differences between mutant mouse lines and wildtype mice in heart, kidney, lung, liver, spleen, thymus and small intestine (data not shown). Given the urogential phenotype of the ADAMTS-1 null mouse (30,31), we also examined the kidney, ovary and testis of the ADAMTS-5-\(\Delta cat\) mouse (32). Glasson et al have published the normal histology of tissues in the ADAMTS-4-\(\Delta cat\) mouse (32).

Aggrecanase activity and ADAMTS-4 protein are present in mouse growth plate (17,32,33), so ablation of the ADAMTS catalytic sites might have been expected to disturb or delay normal skeletal growth. We found that the average body weights for wildtype, ADAMTS-4 and ADAMTS-5-\(\Delta cat\) mice were indistinguishable at all ages, indicating that neither ADAMTS-4, nor ADAMTS-5 catalytic deficiency, interferes with mouse growth and development (data not shown). There were no detectable differences in tibiofemoral growth plates at any age between 3 days and 6 months. The width of tibial growth plates did not vary with genotype, and there was no apparent accumulation of aggrecan, as assessed by toluidine blue staining (data not shown). In combination, the growth curves and the growth plate histology indicate that ADAMTS-4 and -5 have negligible roles in aggrecan resorption during endochondral ossification.

Retinoic acid and IL-1α induce aggrecanase-mediated aggrecan loss from mouse cartilage explants

Previously we examined the effect of IL-1α on the release of aggrecan from mouse cartilage after 3 days in culture (18). In the present study, we examined the kinetics of aggrecan loss by analysing samples on each of days 1, 2 and 3 of culture following treatment with retinoic acid, and compared them with IL-1α-treated samples. The results show that for both retinoic acid and IL-1α, the rate of aggrecan loss from wildtype cartilage is non-linear. The rate of loss is greatest on day 1, and progressively less on days 2 and 3 (Figure 1 a,b). Aggrecan loss from ADAMTS-4- and ADAMTS-5-\(\Delta cat\) cartilage is also non-linear, and is reduced after the first day in culture. Aggrecan loss from ADAMTS-4-\(\Delta cat\) cartilage was approximately 15% less than from wildtype cartilage following treatment with either agent. In contrast, aggrecan loss from ADAMTS-5-\(\Delta cat\) cartilage was substantially less than from wildtype cartilage, and was ~49% less than wildtype in retinoic acid-treated cultures and ~56% less than wildtype in IL-1α-treated cultures. IL-1α promoted a greater release of aggrecan than retinoic acid for all genotypes.

The role of ADAMTS-4 and -5 in retinoic acid and IL-1α induced aggrecanolysis

To further examine the role of ADAMTS-4 and ADAMTS-5 in driving in vitro aggrecanolysis, we next examined aggrecanase cleavage at preferred sites in the CS-2 domain and compared it with IGD cleavage. Cleavage sites in the mouse IGD and CS-2 domains are highly conserved but not identical to other species (Figure 2b,c). Three new neoepitope antibodies, specific for the mouse sequences ALGS, SEL and FREE (Figure 2a), were generated in rabbits. To confirm the neoepitope specificity of the antibodies, aggrecan present in 4M GuHCl extracts of wildtype mouse cartilage was incubated at 37°C, with or without recombinant human ADAMTS-4 (p40; Chemicon...
International, CA, USA), and analysed by Western blotting (Figure 2 d-f). In each case, the antibodies failed to detect epitope in undigested cartilage extracts, but detected fragments of the predicted size in samples digested with ADAMTS-4. The results in Figure 2 and subsequent figures confirm the neoepitope specificity of the antibodies used in this study.

Retinoic acid and IL-1α induce ADAMTS-5 cleavage in the aggrecan IGD
Cleavage at the E↓374 A bond in the IGD was examined by Western blotting medium samples containing the 374ALGS neoepitope (Figure 3b,d), and cartilage extracts containing the NVTEGE↓373 neoepitope present on the G1 domain (Figure 3a,c). In retinoic acid treated cultures, neither NVTEGE↓373 nor 374ALGS epitopes were detected on days 1 or 2 of culture, but were present by day 3 in both wildtype and ADAMTS-4-Δcat cartilage. No IGD neoepitopes were detected in ADAMTS-5-Δcat cartilage treated with retinoic acid, in this system (Figure 3 a,b). IL-1α treatment of wildtype and ADAMTS-4-Δcat cartilage stimulated IGD cleavage commencing on day 1 of culture and increasing up to day 3, however IGD fragments were not detected in cultures of ADAMTS-5-Δcat cartilage (Figure 3c,d). Over-exposing the Western blots did not reveal NVTEGE↓373 or 374ALGS neoepitopes in either retinoic acid or IL-1α-treated ADAMTS-5-Δcat cartilage. Stripping the blots and reprobing with antibody 2B6 confirmed that samples were loaded in each lane (data not shown). These results strongly suggest that ADAMTS-5 is the major aggrecanase responsible for cleavage in the IGD of mouse aggrecan in response to treatment with both retinoic acid and IL-1α.

ADAMTS-5 deficiency does not block aggrecanase cleavage in the CS-2 domain of aggrecan
Next we examined cleavage at E↓1279↓1280 G and E↓1467↓1468 G in the CS-2 domain using anti-SELE↓1279 and anti-FREEE↓1467 antibodies respectively. We analysed media and cartilage extracts and found that retinoic acid and IL-1α treatment generated SELE↓1279 and FREEE↓1467 epitopes in cartilage from all genotypes including ADAMTS-5-Δcat cartilage (Figure 4). The epitopes were present on days 2 and 3 of culture and were detected in both the media and extracts. These results are consistent with our previous study (18) showing limited CS-2 domain cleavage at E↓1572↓1573 A in ADAMTS-5-Δcat explants.

IL-1α stimulated release of SELE↓1279 and FREEE↓1467 fragments from wildtype and ADAMTS-4-Δcat cartilage (Figure 4f,h,i). However, these fragments were barely detectable in media from ADAMTS-5-Δcat cartilage (Figure 4f,h,i). In contrast, ADAMTS-5-Δcat cartilage stimulated with retinoic acid released SELE↓1279 and FREEE↓1467 fragments into the culture medium in proportions that appeared by Western blotting to be equivalent to wildtype and ADAMTS-4-Δcat cartilage (Figure 4b,d). Collectively these data suggest that one or more aggrecanases, other than ADAMTS-5, cleaves in the CS-2 domain. With retinoic acid stimulation, CS-2 domain cleavage is equally efficient in each of wildtype, ADAMTS-4-Δ-cat and ADAMTS-5-Δ-cat cartilage. With IL-1α stimulation, CS-2 domain cleavage is substantially reduced in ADAMTS-5-Δ-cat cartilage (Figure 4e,f,h,i), but not completely blocked (Figure 4h,i). Unstimulated epiphyseal chondrocytes from ADAMTS-5-Δ-cat mice, cultured as non-adherent cell clusters, also released trace amounts of SELE↓1279 fragments (34).

The SELE↓1279 and FREEE↓1467 bands detected in the medium were doublets, whereas the SELE↓1279 and FREEE↓1467 bands in cartilage extracts were always single bands (Figure 4). We postulated that the slow-migrating SELE↓1279 and FREEE↓1467 fragments have intact G1 domains. To confirm this, replicate media samples were electrophoresed in adjacent lanes on gels and probed with anti-G1 domain, anti-SELE↓1279 and anti-FREEE↓1467 antibodies (Figure 5, lanes 1-3). As expected from their size and retention in the cartilage matrix, slow migrating FREEE↓1467 and SELE↓1279 bands co-migrated with anti-G1-immunoreactivity. The G1-FREEE↓1467 and G1-SELE↓1279 bands detected in this study therefore correspond with fragments 4 and 5 respectively, reported in the rat and human (21,22). A G1-containing band that may correspond with fragments a or b (21,22) was also detected.

The faster migrating SELE↓1279 band co-migrated with 374ALGS-immunoreactive bands (Figure 5, lanes 4,6), and corresponds with fragment 13 reported previously in rat and human (21,22). Alignment of the 374ALGS-positive and SELE↓1279-positive bands in Figure 5 confirms that 374ALGS-SELE↓1279 fragments are generated by retinoic acid in wildtype cartilage (Figure 4b). However it also raises questions about how the absence of 374ALGS fragments in ADAMTS-5-Δcat cartilage (Figure 3b).
can be reconciled with the presence of postulated $^{374}$ALGS-SELE$^{1279}$ fragments in the ADAMTS-5-Δcat mouse (Figure 4b). One possibility is that the fast-migrating SELE$^{1279}$ band in ADAMTS-5-Δcat cartilage does not have an $^{374}$ALGS N-terminus, but rather an N-terminus from a cleavage nearby that produces a fragment of indistinguishable size. However, it is much more likely that ADAMTS-5-Δcat cartilage stimulated with retinoic acid does contain $^{347}$ALGS-SELE$^{1279}$ fragments. This would suggest that, in the ADAMTS-5-Δcat genotype, the level of $^{347}$ALGS-SELE$^{1279}$ fragments is below the detection limit for the anti-$^{374}$ALGS antibody but within the detection limit for the anti-SELE$^{1279}$ antibody, in this system. The anti-SELE$^{1279}$ signal in Figure 4b appears relatively uniform in all genotypes, despite evidence in Figure 3b that $^{374}$ALGS-SELE$^{1279}$ fragments in ADAMTS-5-Δcat cartilage are undetectable. This apparent discrepancy highlights the limitations of Western blotting for quantitative analyses. We have previously demonstrated that there can be as much as a 10-fold difference between two different antibodies for their ability to detect equimolar amounts of antigen (35). Thus, whereas Western blot signals detected with the same antibody might provide limited information on relative amounts of antigen, Western blot signals detected with two different antibodies cannot be used to quantitatively compare antigens.

We were surprised to find that the faster migrating FREE$^{1467}$ band did not co-migrate with an $^{374}$ALGS-reactive band (Figure 5, lanes 4,5), suggesting either that it too, was present in low abundance, or that aggrecanase was not responsible for cleaving at the N-terminus of FREE$^{1467}$ fragments. The slow migrating SELE$^{1279}$ and FREE$^{1467}$ bands present in the medium had the same migration on 5% SDS gels as the single SELE$^{1279}$ and FREE$^{1467}$ bands retained in the cartilage, in both retinoic acid and IL-1α-treated cultures (data not shown).

To determine whether the fast-migrating FREE$^{1467}$ band had an N-terminus derived from MMP cleavage, we probed with an anti-$^{342}$FFGVG antibody (data not shown). No $^{342}$FFGVG immunoreactivity co-migrating with the smaller FREE$^{1467}$ band was detected, although $^{342}$FFGVG bands were present in a positive control containing mouse aggrecan digested with MMP-8. The data suggest that FREE$^{1467}$ fragments are processed differently to SELE$^{1279}$ fragments in the mouse, and differently to FREE$^{1467}$ fragments in other species (21,22). At present it is not clear which enzyme family is responsible for generating the N-terminus of FREE$^{1467}$ fragments, or whether $^{342}$FFGVG and/or $^{374}$ALGS neoepitope might be lost due to aminopeptidase activity.

**Increased expression of ADAMTS-5 mRNA in cartilage explants treated with retinoic acid or IL-1α**

Taem real-time RT-PCR was used to measure the levels of ADAMTS-1, -4, -5 and -9 mRNA following stimulation of cartilage cultures with retinoic acid or IL-1α. Expression levels on days 1, 2 and 3 were calculated relative to the level of RNA extracted at day 0. Retinoic acid treatment increased ADAMTS-5 mRNA expression in cartilage from wildtype, ADAMTS-4-Δcat and ADAMTS-5-Δcat mice at each time point (Table 1). The level of expression was increased by an average of 8-fold by day 3 and there was no change in mRNA expression of ADAMTS-1, -4, and ADAMTS-9 at any time point (data not shown). IL-1α treatment also increased expression of ADAMTS-5 mRNA in cartilage from wildtype, ADAMTS-4-Δcat and ADAMTS-5-Δcat mice on each of days 1-3 (Table 1). The level of expression was increased by an average of 17-fold by day 3. There was no change in mRNA expression of ADAMTS-1, ADAMTS-4, and ADAMTS-9 at any time point (data not shown). IL-1α increased ADAMTS-5 mRNA expression approximately 2-fold more than retinoic acid.

**Discussion**

Historically, “aggrecanase activity” has been regarded as a single entity with specificity for all sites along the aggrecan core protein. However it is now apparent that this is not the case, and that there are differential aggrecanase activities with preferences for separate regions of the core protein. Our present data indicate that although ADAMTS-5 is the major aggrecanase responsible for IGD cleavage, in vitro in the mouse, ADAMTS-5 may not have a major role in CS-2 domain cleavage, particularly in response to retinoic acid. Our data show that when ADAMTS-5 activity is ablated, one or more aggrecanases continues to cleave at SELE$^{1279}$↓$^{1280}$GRGT and FREE$^{1467}$↓$^{1468}$GLGS, confirming and extending our previous observations of cleavage at the TAQE$^{1572}$↓$^{1573}$AGEG site in the CS-2 domain (18). This interpretation of our data is supported by earlier studies showing that
glucosamine inhibits aggrecanase cleavage in the CS-rich region more efficiently than it inhibits cleavage in the IGD (36), and more recently by studies showing that full length recombinant ADAMTS-4 has abundant aggrecan-releasing activity, yet very little IGD-cleaving activity (37,38). Thus it appears that more than one aggrecanase cleaves aggrecan in vitro, and a measure of each different activity is needed to obtain an accurate readout of total aggrecanase activity.

We propose that total aggrecanase activity (X) measured by release of aggrecan, is the sum of IGD cleavage (Y) + CS-2 domain cleavage (Z). Our present study with mouse cartilage suggests that there is a positive correlation between X and Y with respect to ADAMTS-5, since ablation of ADAMTS-5 activity markedly reduces aggrecan loss from explant cultures (X) and it also blocks cleavage in the IGD (Y). However in vitro studies with full length recombinant ADAMTS-4 indicate that for this enzyme, there is a negative correlation, between X and Y, and a positive correlation between X and Z (37). Roughley et al also note that recombinant ADAMTS-4 is less efficient at IGD cleavage than ADAMTS-5 (39).

There are two possible interpretations of our findings with retinoic acid stimulation. One is that ADAMTS-4 cleaves in the CS-2 domain in the absence of ADAMTS-5 activity. The other possibility is that a third aggrecanase, with specificity for the CS-2 domain, is present in cartilage. It is entirely possible that ADAMTS-4 compensates for ADAMTS-5 deficiency in the ADAMTS-5-Δcat mouse. If this were the case, then the converse interpretation, that ADAMTS-5 cleaves in the CS-2 domain in the absence of ADAMTS-4 must also be invoked to account for the fragments present in the ADAMTS-4-Δcat mouse. Thus, ADAMTS-4 and -5 might both have moderate activity against CS-2 domain cleavage sites that can be upregulated if compensatory cleavage is required. Although ADAMTS-5 mRNA was upregulated by retinoic acid and IL-1α, ADAMTS-4 mRNA expression was not increased by either of these modulators. However, there is evidence to suggest that in chondrocytes, ADAMTS-4 activity might be regulated at the translational, rather than transcriptional level (40).

The second possibility, that there may be a third aggrecanase, is more intriguing. To date there has been no reason to suspect that there may be a third aggrecanase activity and there are no obvious candidates for an ADAMTS enzyme with sufficient activity to function as an aggrecanase. ADAMTS-1, -8, -9, -15 have weak aggrecan-degrading activities in vitro, that are orders of magnitude less than ADAMTS-4 and ADAMTS-5 (11-14). However the relative activities of these proteinases against aggrecan in vivo is unknown. Their low activity in vitro could be due to the expression system used to make them, or the extent of N- or C-terminal processing which directly affects activity (37,38). There are also reports of a membrane-bound aggrecanase whose activity is distinct from ADAMTS-4 and -5 however its identity is unknown (41).

The present study shows that in ADAMTS-5-Δcat cartilage, CS-2 domain fragments are readily detected after retinoic acid treatment but only weakly detected after IL-1α treatment. Trace amounts of SELE1279 fragments were also detected in cultured epiphyseal chondrocytes from ADAMTS-5-Δcat cartilage, and it is interesting that these fragments were present, albeit at very low levels, without stimulation of the cell clusters (34). The extent and pattern of CS-2 domain cleavage might therefore depend on the source of chondrocytes and the type of stimulus. Further work in cell and explant cultures systems is needed to characterise these differences. Similarly, further studies to quantitate the fragments generated under various experimental conditions are also needed, once quantitative assays for each fragment have been developed.

The faster-migrating SELE1279 band in the medium contained the 374 ALGS neoepitope at its N-terminus, confirming it is an aggrecanase cleavage product. However, the absence of an 373 ALGS N-terminus on FREE1467 fragments in the medium was a surprise since 373 ARGS-FKEEE1714 fragments are found in human synovial fluids (22) and 373 ARGS-FREE1459 fragments are found in rat chondrosarcoma cultures (21) (Figure 2b,c). Multiple experiments failed to detect co-migrating FREE1467 and 373 ALGS fragments giving us confidence that these fragments were not detectable in the mouse explant system. We were also unable to detect 342 FFGVG fragments co-migrating with FREE1467, however we are less confident that these fragments may not, in fact, be
generated, and then lost. Western blot analysis of medium and cartilage extracts from the 3 day cultures revealed DIPEN\textsuperscript{341} neoeptiope present uniformly in all genotypes, however the corresponding \textsuperscript{342}FFGVG epitope that is generated in equimolar amounts with DIPEN\textsuperscript{341} was not detected in the medium, even though we have shown previously that the anti-\textsuperscript{342}FFGVG antibody detects significantly lower molar amounts of antigen than the anti-DIPEN\textsuperscript{341} antibody (35). The lack of detectable \textsuperscript{342}FFGVG neoeptiope raises the possibility that it may be destroyed in mouse explant culture, possibly by the action of an aminopeptidase. It therefore remains uncertain whether FREEE\textsuperscript{1467} fragments released from cartilage are products of MMP as well as ADAMTS activity, and whether cleavage may occur at a non-canonical site. Proteolysis by MMPs at the \textsuperscript{N341}↓\textsuperscript{342}F bond in the IGD does not appear to contribute to glycosaminoglycan loss during early phases of experimental arthritis in mice. However MMP cleavage at \textsuperscript{N341}↓\textsuperscript{342}F correlates with late stage cartilage damage in mouse models of arthritis (1-3), and it may also be involved in the baseline turnover of aggrecan \textit{in vitro} (35) and \textit{in vivo} (42). The products of \textit{in vitro} proteolysis at both the MMP and the aggrecanase sites have been found in humans (43-46), and in mice with experimental arthritis (247-49).

The doublet of SELE\textsuperscript{1279} and FREEE\textsuperscript{1467} bands released into explant medium contains a slow migrating band the same size as the SELE\textsuperscript{1279} and FREEE\textsuperscript{1467} bands retained in the cartilage. Because the slow-migrating bands have intact G1 domains, we postulate that enzymes other than proteinases might be responsible for the release of these fragments (50,51). We (52) and others (53,54) have detected concomitant release of hyaluronan, link protein and aggrecan G1 fragments into conditioned medium of cytokine-treated cartilage explant cultures, consistent with the hypothesis that hyaluronidases may contribute to depolymerisation of the aggrecan aggregate (51). Our present data confirms previous work suggesting that proteolytic and non-proteolytic mechanisms of degradation proceed together (50,51), and also that the non-proteolytic mechanism(s) may be more responsive to treatment with retinoic acid given the pattern and distribution of the SELE\textsuperscript{1279} and FREEE\textsuperscript{1467} bands seen in Figure 4.

The ratio of fast:slow migrating SELE\textsuperscript{1279} and FREEE\textsuperscript{1467} bands is variable between samples and reflects the dynamic nature of aggrecan catabolism. Intermediates such as SELE\textsuperscript{1279} and FREEE\textsuperscript{1467} are created, then lost during further processing. In human synovial fluids, G1-SELE\textsuperscript{1545} and G1-KEEE\textsuperscript{1714} fragments are transient intermediates and G1-NITEGE\textsuperscript{373} is the only stable terminal product (22). Dynamic processing, together with the non-quantitative nature of Western blotting, is likely to account for the sample-to-sample variation in the ratio of SELE\textsuperscript{1279}:FREEE\textsuperscript{1467} bands, and the ratio of fast:slow migrating SELE\textsuperscript{1279} and FREEE\textsuperscript{1467} bands.

One important conclusion from this study and others (17,18,32) is that ablation of ADAMTS-4 and ADAMTS-5 apparently has no impact on normal skeletal growth or development, suggesting that aggrecan resorption from the growth plate can proceed effectively without these enzymes. ADAMTS-1 null mice have no skeletal abnormalities (25), and MMPs are not required for aggrecan resorption from growth cartilage since mice resistant to MMP cleavage in the IGD develop normally without growth plate deformities (55). These findings lend further support to the hypothesis that there might be non-proteolytic mechanisms for resorbing aggrecan aggregates that are entirely adequate for endochondral ossification in the mouse (50,51).

Aggrecanase cleavage in the IGD releases the entire chondroitin sulphate and keratan sulphate-rich regions that are essential for weight-bearing, and cleavage in the IGD is therefore assumed to be the most detrimental for cartilage function. There is an argument that cleavage in the CS-2 domain may be less critical for cartilage function, since 1) a significant proportion of glycosaminoglycan is retained in the CS-1 and KS rich regions following cleavage in the CS-2 domain, and 2) most aggrecan in adult tissues lacks the G3 domain and varying portions of the CS-rich region (56), suggesting that processing in the CS-2 domain may be part of normal maturation and ageing (57). It will be important to determine not only the identity of the CS-2-degrading enzymes, but also whether CS-2 domain cleavage contributes to cartilage erosion \textit{in vivo}. It will also be important to identify what distinguishes CS-2 domain aggrecanases from IGD aggrecanases; they may be different gene products, or may be the same gene products but with
different activities depending on the extent to which they are truncated from their C-termini (37,38).
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Abbreviations used: CS-2 chondroitin sulphate-2 domain; IGD interglobular domain; MMP, matrix metalloproteinases; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif

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Legend to Figures

Figure 1. Kinetics of aggrecan release from cartilage explants stimulated with retinoic acid or IL-1α. The release of aggrecan from cartilage explants stimulated with retinoic acid (a) or IL-1α (b) over 3 days of culture was measured by the dye-binding assay. The results are pooled data from two identical experiments, with total replicates of n = 5 or 6 (a) or n = 7 (b). The data are the mean of wildtype (■), ADAMTS-4-Δcat (●) and ADAMTS-5-Δcat (▲) mice.

Figure 2. Antibodies recognising ADAMTS neoepitopes in mouse aggrecan. The schematic shows cleavage sites in the IGD and CS-2 domains of mouse aggrecan examined in this study (a). Aggrecanase cleavage sites in the IGD (b) and CS-2 domain (c) of mouse, human, bovine and rat aggrecan are aligned. The keratan sulphate attachment region between the G2 and CS-1 domains of human and bovine aggrecan is not present in mouse aggrecan. Some keratan sulphate substitution, represented by straight lines, is present in the mouse IGD (AJ Fosang, unpublished data). Aggrecan present in extracts of mouse femoral head cartilage was incubated with (+) or without (-) recombinant human ADAMTS-4 and analysed by Western blotting for 374ALGS (d), SELE1279 (e), or FREEE1467 (f) neoepitopes.

Figure 3. Retinoic acid and IL-1α induce ADAMTS-5 cleavage in the aggrecan IGD. Aggrecan fragments derived from aggrecanolyis in the IGD following stimulation with retinoic acid (a,b) or IL-1α (c,d) were examined by Western blotting. NVTEGE374 neoepitope extracted from cultured cartilage was detected with anti-NITREGE374 antibody (a,c). Fragments released into the medium were detected with anti-374ALGS antibody (b,d). Gel loading was normalised to total glycosaminoglycan content. The blots were exposed to X-ray film until the most strongly-detected band in the series approached saturation. The blots from one experiment are shown and are representative of two separate experiments.

Figure 4. Ablation of ADAMTS-5 activity does not block cleavage in the CS-2 domain. Aggrecan fragments derived from aggrecanolyis in the CS-2 domain following stimulation with retinoic acid (a-d) or IL-1α (e-i) were examined by Western blotting. Fragments released into the medium (b,d,f,h,i) or recovered in 4M GuHCl extracts (a,c,e,g) were detected with anti-SELE1279 (a,b,e,f) or anti-FREEE1467 (c,d,g,h,i) antibodies. Gel loading was normalised to total aggrecan content. The blots were exposed to X-ray film until the most strongly-detected band in the series approached saturation (a-h), with the exception of panel i which shows the same FREEE1467 blots as panel h, but over-exposed to reveal low levels of FREEE1467 epitope released from ADAMTS-5-Δcat cartilage. The blots from one experiment are shown and are representative of two separate experiments.

Figure 5. Characterisation of SELE1279 and FREEE1467 fragments a) Conditioned media from wildtype femoral head cartilage treated with retinoic acid was analysed by Western blotting with antibodies recognising the G1 domain (lane 1), FREEE1467 (lanes 2, 5), SELE1279 (lanes 3, 6) and 374ALG (lane 4) fragments. b) Schematic of the fragments detected by Western blotting. The boxed characters in a) and b) correspond to fragments identified previously in human synovial fluids (22) and rat chondrosarcoma (21). The band with the unidentified N-terminus is labeled X.
Table 1. ADAMTS-5 mRNA levels in cartilage stimulated with retinoic acid or IL-1α.

ADAMTS-5 mRNA was measured in cartilage treated with $10^{-5}$M retinoic acid or 10ng/ml IL-1α, in wildtype, ADAMTS-4-Δcat and ADAMTS-5-Δcat mice. ADAMTS-5 mRNA expression was quantitated relative to an 18s rRNA internal reference ($\Delta C_T$ value).

|                | Genotype    | Days in culture | Fold difference in ADAMTS-5 expression | Genotype    | Days in culture | Fold difference in ADAMTS-5 expression |
|----------------|-------------|-----------------|----------------------------------------|-------------|-----------------|----------------------------------------|
| retinoic acid  | Control     | 0               | 1                                      | IL-1α       | Control         | 1                                      |
|                |             | 1               | 2.7                                    |             | 1               | 6.7                                    |
|                |             | 2               | 5.0                                    |             | 2               | 8.6                                    |
|                |             | 3               | 8.6                                    |             | 3               | 17.8                                   |
| retinoic acid  | TS-4-Δcat   | 0               | 1                                      | IL-1α       | TS-4-Δcat       | 0                                      |
|                |             | 1               | 2.5                                    |             | 1               | 7.2                                    |
|                |             | 2               | 4.6                                    |             | 2               | 10.7                                   |
|                |             | 3               | 8.1                                    |             | 3               | 18.8                                   |
| retinoic acid  | TS-5-Δcat   | 0               | 1                                      | IL-1α       | TS-5-Δcat       | 0                                      |
|                |             | 1               | 3.5                                    |             | 1               | 7.1                                    |
|                |             | 2               | 6.5                                    |             | 2               | 9.8                                    |
|                |             | 3               | 8.2                                    |             | 3               | 17.7                                   |
Figure 1

(a) Aggrecan release (%)

- wildtype
- ADAMTS-4-Δcat
- ADAMTS-5-Δcat

Days in culture with retinoic acid

(b) Aggrecan release (%)

- wildtype
- ADAMTS-4-Δcat
- ADAMTS-5-Δcat

Days in culture with IL-1α
Figure 2

a) Diagram showing the interactions between NVTEGE<sub>373</sub> ALGSV and SSELE<sub>1279</sub> GRGTT with G1, G2, and G3.

b) Table listing peptides for human, bovine, rat, and mouse proteins.

c) Table listing peptides for human, bovine, rat, and mouse proteins.

d) Western blot analysis showing α-ALGS.

e) Western blot analysis showing α-SELE.

f) Western blot analysis showing α-FREEE.
### Retinoic acid

| Days | Wildtype | TS-4 Δ-cat | TS-5 Δ-cat |
|------|----------|------------|------------|
| 1    | ![Image](https://via.placeholder.com/150) | ![Image](https://via.placeholder.com/150) | ![Image](https://via.placeholder.com/150) |
| 2    | ![Image](https://via.placeholder.com/150) | ![Image](https://via.placeholder.com/150) | ![Image](https://via.placeholder.com/150) |
| 3    | ![Image](https://via.placeholder.com/150) | ![Image](https://via.placeholder.com/150) | ![Image](https://via.placeholder.com/150) |

### α-NITEGE extracts

- a)
- b)

### α-ALGS medium

- c)
- d)

---

**Figure 3**
### Retinoic acid

| Days | Wildtype | TS-4 Δ-cat | TS-5 Δ-cat |
|------|----------|------------|------------|
| 1    | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| 2    | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| 3    | ![Image](image7) | ![Image](image8) | ![Image](image9) |

**Figure 4**

### IL-1α

| Days | Wildtype | TS-4 Δ-cat | TS-5 Δ-cat |
|------|----------|------------|------------|
| 1    | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| 2    | ![Image](image13) | ![Image](image14) | ![Image](image15) |
| 3    | ![Image](image16) | ![Image](image17) | ![Image](image18) |

**Figure 4**
Figure 5
ADAMTS-5 deficiency does not block aggrecanolyis at preferred cleavage sites in the chondroitin sulphate-rich region of aggrecan
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