# Pathways of aggrecan processing in joint tissues

## Implications for disease mechanism and monitoring

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Aggrecan is an essential matrix constituent of the soft tissues of the joint, which include the articular and meniscal cartilages and the supporting intraarticular ligaments. Our understanding of the pathways of aggrecan turnover in these tissues is limited. However, recent evidence obtained from analysis of human synovial fluids (Sandy et al. 1992, Lohmander et al. 1993), human articular cartilages (Bayne et al. 1995) cartilage explant cultures (Sandy et al. 1991) and chondrocyte cultures (Lark et al. 1995) has suggested a central role for a novel proteoglycan-degrading proteinase (aggrecanase) which cleaves the Glu 373- Ala374 bond within the interglobular domain of aggrecan.

Cleavage of this bond brings about complete separation of the CS-bearing regions from the N-terminal G1 domain. The identity of the proteinase responsible is presently unknown, however it does not appear to be one of the known matrix metalloproteinases, MMP -1, 2, 3, 7, 8, 9 or 13, all of which primarily cleave the Asn341-Phe342 bond of the aggrecan interglobular domain. On the other hand, MMP-8 also cleaves the Glu373-Ala374 bond slowly (Hardingham and Fosang 1995) suggesting that an MMP-8-like proteinase might be responsible for this cleavage in vivo, where it can occur independent of cleavage at the MMP-sensitive Asn341-Phe342 bond (Lark et al. 1995, and data herein). It is also presently assumed that aggrecanase-type activity is responsible for cleavage at the four other sites within the CS domain with similar sequences (Figure 2).

The discovery of aggrecanase products initiating at Ala 374 in the synovial fluids of patients with various joint conditions (Sandy et al. 1992, Lohmander et al. 1993) has provided a new insight into joint disease mechanisms. It suggests that aggrecanase is acting within joint tissues to promote excessive aggrecan catabolism with subsequent release of such fragments into the fluid thereby compromising tissue function. On the other hand the tissue source of these fragments is unknown, although it has been generally assumed that these products derive from articular cartilage. It is also unclear why there are undetectable levels of fragments with the N-terminii (Gly1546, Gly1715, Ala1820 and Leu1920) predicted from aggrecanase activity within the CS domain. In the present paper we describe results which implicate aggrecanase in aggrecan turnover in a range of joint tissues and which also begin to examine the precise mechanisms of C-terminal processing of aggrecan.

## Materials and methods

Aggrecan (aA1) was prepared from Swarm rat chondrosarcoma tumor by associative extraction and CsCl gradient centrifugation. Human OA cartilages were obtained at knee joint replacement surgery and OA knee fluids were obtained from individuals with late stage primary OA. Aggrecan isolation from bovine tissues was as described (Koob et al , 1995). Antisera anti-ATEGQV (G1-specific) and anti-TYKHRL (G3-specific) were produced at the Tampa Unit of Shriners Hospitals. They were raised in rabbits against the KLH-conjugated peptides (CATEGQVRVNSIYQDKVSLP) and (CTYKHRLQKRTMRPTRRSRPSMAH). The preparation and characterization of anti-CDAGWL, anti-NITEGE and anti-FVDIPEN, and detailed methods for Western analysis have been described (Lark et al, 1995). Anti-TFKEEE (which detects the new C-terminal generated by cleavage at Glu1459-Gly1460), anti-APTAQE (which detects the new C-terminal generated by cleavage at Glu1564-Ala1565) and anti-PTVSQE ( which detects the new C-terminal generated by cleavage at Glu1664-Leu 1665)were provided to us by Dr. Michael Lark of Merck and Co., Rahway, NJ.

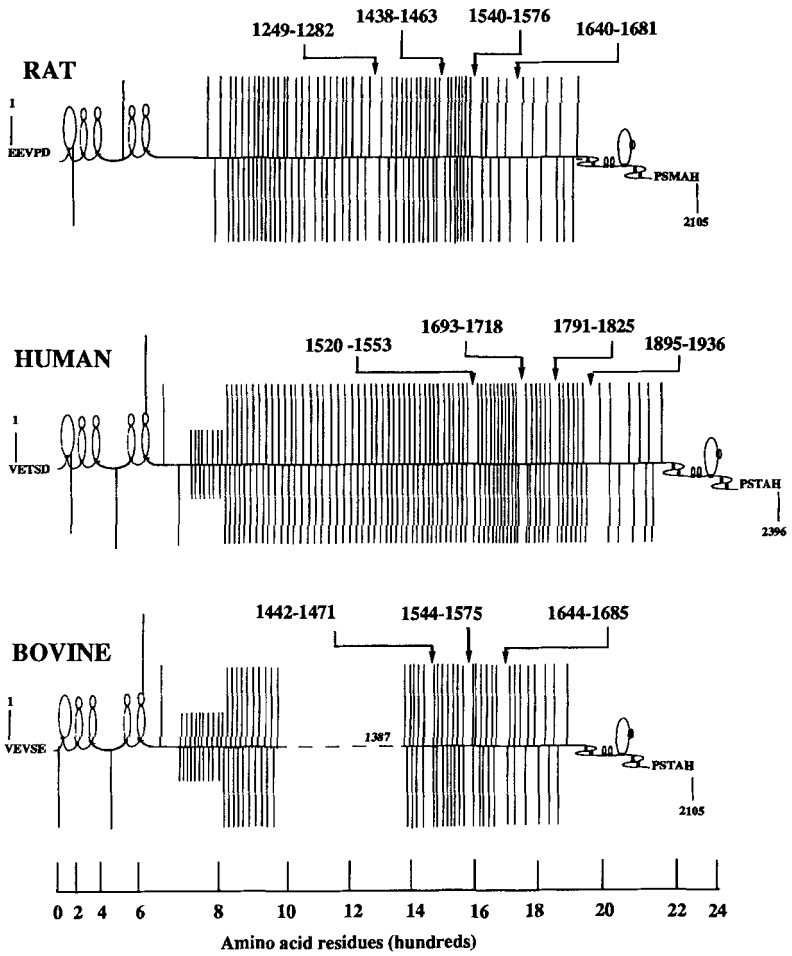


Figure 1. Schematic representation of rat, human and bovine aggrecan showing "gaps" in the chondroitin sulfate attachment region.The position of SG and GS sequences (putative attachment sites for CS) are shown by long lines and the position of serine residues in the KS domain are shown by short lines. The numbers above each model refer to residue numbers of the serine residues which form the boundary of each "gap" region. There is a portion of the bovine core model missing (dotted line) since full-length sequence data is not yet available. Therefore the bovine residue numbers have not been determined but are based on the rat model.

## Results

### *Aggrecan catabolism within the CS attachment region*

It is now generally accepted that turnover of aggrecan in vivo involves not only cleavage within the interglobular domain but also proteolysis at site(s) within the CS attachment region. Such cleavages remove the G3 domain of aggrecan and reduce the size of the CS-attachment region to varying degrees. Western analysis of normal human articular cartilage aggrecan with antibodies to the G1 and G2 domains (Vilim and Fosang 1994) has shown that a range of sizes of C-terminally truncated molecules are present and analysis of bovine articular cartilage aggrecan by peptide quantitation and rotary shadowing (Flannery et al. 1992b) has shown that only 30–50% of G1-G2-CS-bearing molecules carry the G3 domain. This clearly shows that truncation from the C-terminal, without concomitant interglobular domain cleavage, is a quantitatively significant pathway of aggrecan catabolism in articular cartilage.

There is however no definitive data on the nature of the protease(s) responsible for C-terminal truncation and the possible physiological importance of this process is unclear. Indirect evidence, based on core fragment sizes in bovine (Ilic et al. 1992) and human synovial fluid (Lohmander et al. 1993) suggests that the major CS-bearing aggrecan fragments generated in vivo are also the products of the aggrecanase type

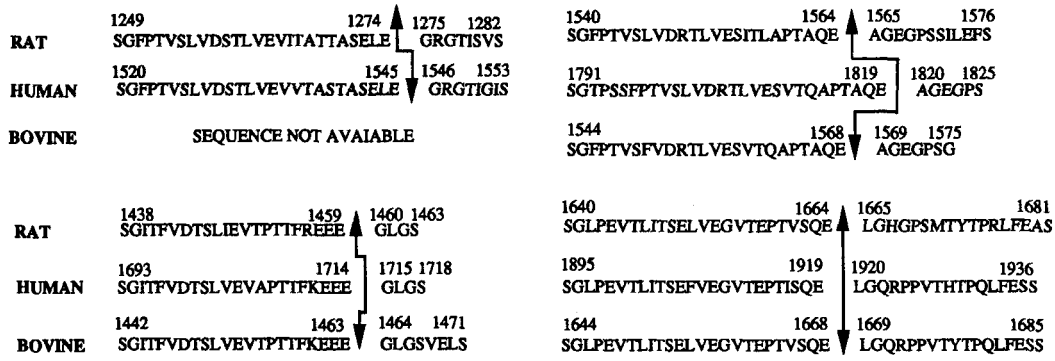


Figure 2. Comparison of the sequence of "gap" regions in rat, human and bovine aggrecan illustrating high degree of conservation in these regions and the putative aggrecanase sites. See Figure 1 for explanation of numbering.

of activity. Such fragment identification based on electrophoretic migration alone may however be misleading for two reasons. Firstly, variable O-linked glycosylation of aggrecan core fragments, which was not examined in these studies, could alter electrophoretic properties. Secondly, since minor variation in the peptide length would not be detected on the electrophoretic systems used, it is possible that cleavages adjacent to the aggrecanase sites could be misinterpreted as aggrecanase-dependent cleavages. Nonetheless, these studies make it clear that the CS-attachment region of aggrecan has a number of proteolytically sensitive stretches which correspond to regions where there is an absence of SG or GS sequences for CS substitution (Flannery and Sandy 1993).

These "gap" regions are readily seen when a map of SG and GS sites of rat, human and bovine aggrecan are compared (Figure 1). Interestingly, the sequences across these "gap" regions are highly conserved between species and each contain sites (shown by arrows on Figure 2) which exhibit strong sequence similarity to the Glu 373-Ala 374 aggrecanase site. While there is direct evidence for cleavage at one or more of these sites in bovine explant systems in the presence of retinoic acid (Ilic et al, 1992; Plaas and Sandy, 1993), or IL-1 (Loulakis et al. 1992) more definitive studies are required to determine whether C-terminal proteolysis of aggrecan occurs at these sites in vivo.

### *Identification of cleavage sites in the CS attachment domain*

In order to further define the proteolytic events involved in C-terminal truncation we have analyzed the aggrecan species present in the Swarm rat chondrosarcoma tumor. The ratinale for this approach was that aggrecan from this source has been analyzed in detail at the protein, GAG, and pligosaccharide levels. Particularly advantageous to a catabolic study is the absence of KS substitution which generally complicates interpretation of core analyses on Western blots. We examined CsCl purified aggrecan (aA1 fraction) by SDS-PAGE and Western analysis with antisera to the aggrecan G1 domain and G3 domains (Figure 3). The immunoblot revealed the presence of six major G-1 containing species. Their C-terminii were established by the use of the G3 antiserum and a range of antisera specific to aggrecanase and metalloproteinase cleavage sites (see Methods and Figure 2) and suggested structures for eight of the core protein species are also shown on Figure 3.

As expected, both full length core (peptide 1) and the extensively characterized catabolic G1 domain species (peptides 9 and 10), were readily detectable in the tumor preparations. The two small G1 domain species have previously been described in normal and arthritic human cartilages (Flannery et al. 1992a, Bayne et al. 1995). Western analysis with anti-FVDIPEN and anti-NITEGE of six human OA cartilages and ten human OA synovial fluids showed that both G1 species are present in all samples and a typical data set is shown in Figure 4. The anti-FVDIPEN antiserum detects a 52kDa species and the anti-NITEGE a 62kDa species in both cartilage and fluid.

In addition to the small catabolic G1 products, the chondrosarcoma extracts were probed with C-terminal-specific antisera and found to contain species apparently terminating at Glu 1459 (peptide 3), Glu 1564 (peptide 5) and Glu 1664 (peptide 2) (data not shown). These products would result from aggrecanase-type activity within the CS-domain. When probed with the G3 antiserum the same sample confirmed that peptide 1 represents a full length core species, but also revealed the presence of four other G3-

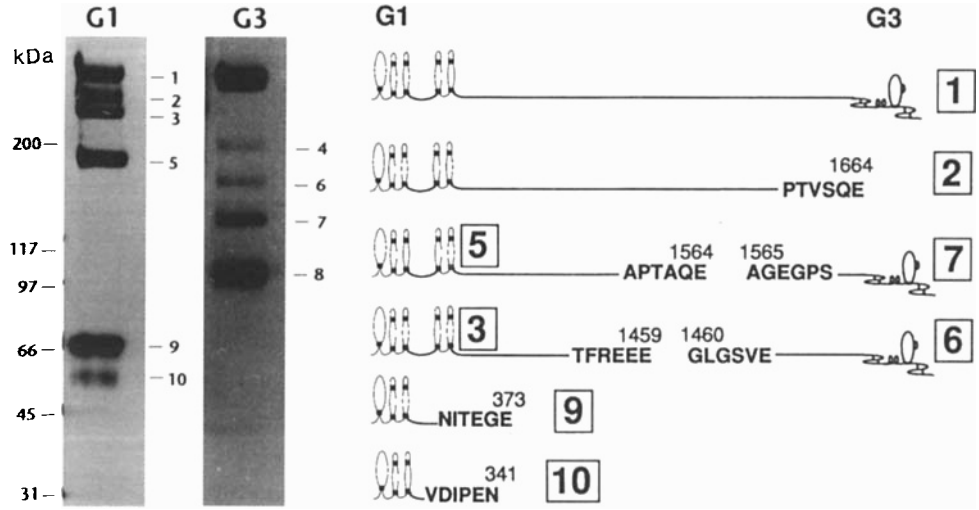


Figure 3. Western analysis of rat chondrosarcoma aggrecan (a A1) and suggested structures for the G1-bearing and G3-bearing species. The Western blot with G1-specific and G3-specific antisera is shown on the left with suggested structures for eight of the peptides shown to the right.

containing species. Two of these (peptides 6 and 7) were shown by N-terminal analysis to initiate at Ala 1565 and Gly 1459 respectively and thus represent the complimentary fragments to the G1 containing species 3 and 5 (Figure 3). Further analysis is required to establish the N-terminii of the G3-containing peptides 4 and 8 and to explain the discrepancy between the immunoreactivity and apparent size on SDS-PAGE of peptides 3 and 5.

Nonetheless, all of the fragments identified in the tumor as being generated by cleavage within the CS domain (peptides 2, 3, 5, 6 and 7) are products of aggrecanase-type activity suggesting that this proteinase type is largely responsible for C-terminal processing in this tissue. Further, since peptides 2 , 3 and 5 accumulate, it is clear that C-terminal processing of some molecules occurs before interglobular domain cleavage. Indeed, it is possible that C-terminal processing is a prerequisite of interglobular domain cleavage. Moreover, since products from both sides of the Glu1564-Ala1565 and Glu1459-Gly1460 sites have been identified, aggrecanase would appear to function here as an endopeptidase which can cleave these two sites independently.

In relation to aggrecanase action within the CS domain of human aggrecan it is interesting that the major high-density G1-bearing species in human cartilages (Vilim and Fosang, 1994) were shown to have apparent sizes of 380 kDa, 320kDa, 240kDa and 180kDa. One or more of these may be represented by peptides 1, 2, 3 or 5 (Figure 3), however definitive identification of these species will require analysis with cleavage site-specific antisera, such as those described in the present work. In the same way it will be interesting to investigate the presence of aggrecanase-generated G3-bearing fragments in cartilage and synovial fluids, since molecules of this kind (equivalent to peptides 6 and 7; Figure 4) may represent the non-aggregating species commonly found in cartilages extracts.

### *Aggrecan catabolism in intra-articular tissues*

Figure 3 illustrates the likely heterogeneity of aggrecan core species in mature articular cartilages. Even though articular cartilage is clearly the major reservoir of aggrecan in the mature joint, it does not necessarily follow that it is the only source of fragments in the fluid. Other intraarticular tissues, such as meniscus and ligaments contain aggrecan (Koob et al. 1995) and may therefore release it under any condition which alters the metabolism of the joint. To begin to examine this we have performed comparative assays for aggrecan species in cartilage, menisci and meniscal ligaments of fetal, calf and steer stifle joints (Figure 5). The antiserum (anti-CDAGWL) used in this study recognizes a sequence in the PTR loops of both aggrecan G1 domain and link protein (Lark et al. 1995). The products detectable in the fetal and calf samples were similar in that they all contained high molecular weight aggrecan core (equivalent to peptide 1; Figure 3). Cartilage and meniscus from steer also accumulate a considerable amount of core species migrating as a doublet between 100–130kDa and this represents a C-termi-

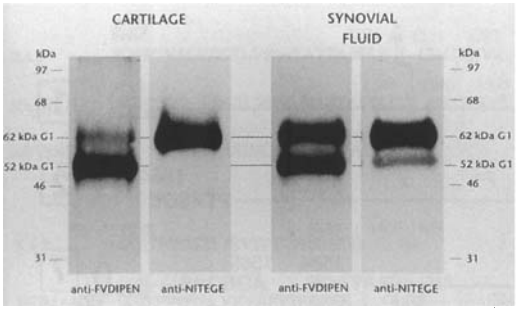


Figure 4. Western analysis of human osteoarthritic cartilage extracts and synovial fluids. Shown is a typical Western blot of human OA cartilage extract (Cs Cl, A1D4 fraction) and human OA synovial fluid (CsCl, A1 fraction) probed with the anti-FVDIPEN and the anti-NITEGE antiserum. Fragments derived from about 10µg of GAG were loaded on each lane. The anti-FVDIPEN reacts with a 52kDa species and the anti-NITEGE with a 62kDa species. The apparent reactivity of the anti-FVDIPEN antiserum with a 62kDa species, particularly in synovial fluid samples has not been further investigated. The apparent reactivity of the anti-NITEGE antiserum with a 54kDa species in synovial fluid was non-specific in that it was obtained with secondary antibody alone. There was no significant immunoreactivity elsewhere on these blots.

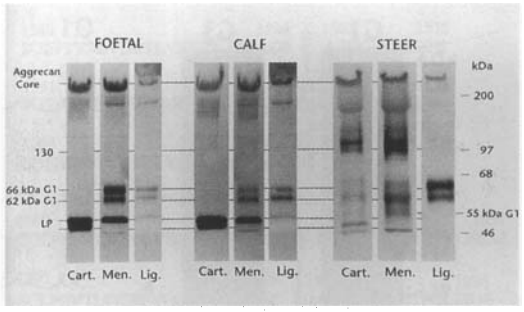


Figure 5. Western analysis with anti-CDAGWL of developing bovine joint tissues.Shown are the immunoreactive species in A1 preparations (CsCl gradient) of bovine cartilage, meniscus and ligaments. Fragments derived from about 30µg of GAG were loaded on each lane. Note the dominant 62kDa /66kDa doublet in meniscus and ligament samples.

nally truncated aggrecan core species not seen in the chondrosarcoma extracts. Somewhat unexpected, however, was the much greater abundance of aggrecan G1 domains (62/66kDa double-band) in all meniscus and ligament samples, compared with cartilage. Both of these fragments have been identified as aggrecanase-generated G1 based on their strong reactivity with the anti-NITEGE antiserum and elimination of this reactivity by pre-treatment of samples with MMP-3 (data not shown). The presence of two forms appears to be due to variable O-glycosylation of the interglobular domain between Asn341 and Glu373 since the MMP-3 treatment generated a single 55kDa form of G1 in these samples (Koob and Hernandez, unpublished). A native 55kDa G1 domain species which is probably the MMP-generated product (see peptide 10 in Figure 3) was only observed in steer meniscus. Taken together these data show that aggrecanase-mediated cleavage of the Glu 373-Ala 374 bond is a normal process in matrix turnover of bovine meniscal fibrocartilage and ligament.

The abundance in the adult meniscus and ligamentous attachments of aggrecanase-generated G1 species opens the possibility that the G1 catabolic fragment in adult human synovial fluids (Figure 4) may arise from these tissues. Evidence for such a possibility is supported by experiments in which fetal articular cartilage and meniscus were maintained in explant culture and the media analyzed for the presence of aggrecan catabolic products. Both G1-species were continuously released from the meniscus over 11 days of culture, whereas the same product was barely detectable in the media from articular cartilage maintained under identical conditions. Since the G1 was not extractable from meniscus with isotonic salt but required 4M guanidinium HCl for solubilization, it is most likely that release from meniscus and ligaments from the joint would also be a cell-driven process.

Indeed, the involvement of a cell-driven process in the release of such G1 fragments has been observed in experiments with bovine cartilage explants treated with IL-1 or retinoic acid to induce aggrecan catabolism (Plaas, Sandy, Grodzinsky & Bonassar, unpublished observations). Interestingly, in these studies aggrecanase-generated G1 was released into the culture medium much later than the aggrecanase-generated CS-bearing fragments, and release of hyaluronan has also a late event.

Establishing the tissue source of CS-bearing fragments in synovial fluids is also important since this should further delineate the metabolic response within different joint tissues and at the same time may reveal new degradative mechanisms for joint tissue matrices. In addition, knowledge of tissue origin is critical if the analysis of synovial fluids is to be useful in the broad assessment of joint diseases. In this regard, we examined the possibility that analysis of the sulfation pattern of chondroitin sulfates might reveal tissue-specific patterns. CS mapping was done by chondroitinase digestion, 2-aminopyridine derivitization and separation of fluorescent products by anion exchange HPLC (Plaas et al. 1995). A typical example of such HPLC chromatograms is shown in Figure 6. The major components shown are the inter-

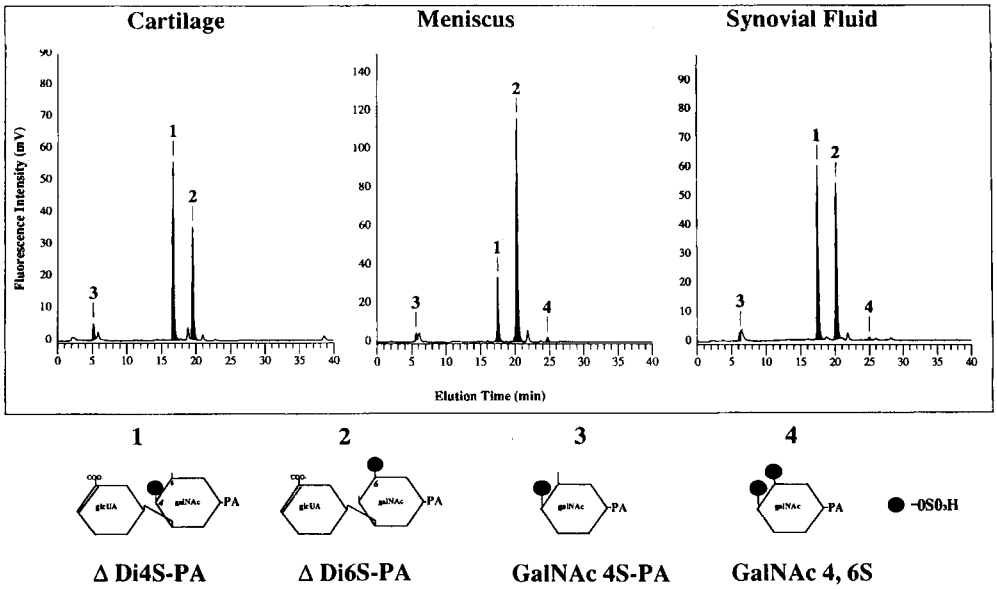


Figure 6. Anion exchange HPLC chromatogram of fluorotagged chondroitinase ABC digests. Typical profiles for quantitation of internal structures are shown. Since the terminal structures are present at about 1/20th the abundance of the internal disaccharides, quantitative analysis of the terminii are obtained at up to 20-fold higher loading than for the analyses shown here.

nal disaccharides, ΔDi4S and ΔDi6S, and the non-reducing terminal monosaccharides GalNAc-4S and GalNAc-4, 6S. Calculation of the molar ratios (ΔDi4S/ΔDi6S and GalNAc-4S/GalNAc-4, 6S) obtained for cartilage and meniscus of fetal, calf and steer stifle joints (Table 1) shows that the CS structure for aggrecan from these two tissues is markedly different at all stages of development. In addition, analysis of synovial fluid CS from the fetal stifle joint (Table 1) shows that its sulfation pattern is markedly different from that in either the meniscus or the cartilage of that joint but very similar to that in the carpal joint fluid of the same animal. These results indicate that CS accumulating in normal synovial fluid is not representative of the CS population present in the articular cartilage alone. A capacity to determine the tissue source of synovial fluid CS and G1 fragments in disease states should clarify tissue-specific processes and therefore may further our understanding of the pathological changes within the entire joint.

**Table 1. Chondroitin sulfate analysis of bovine cartilages, meniscus and synovial fluids**

| | ΔDi4S/ΔDi6S[1] | GalNAc-4S/GalNAc-4,6S[2] |
|---|---|---|
| *Meniscus* | | |
| Fetal calf | 0.25 | 1.33 |
| Calf | 0.22 | 0.66 |
| Steer | 0.21 | 1.75 |
| *Cartilage* | | |
| Fetal calf | 1.38 | 3.3 |
| Calf | 1.42 | 2.1 |
| Steer | 1.65 | 2.7 |
| *Synovial fluid* | | |
| Stifle joint | 1.42 | 2.1 |
| Carpal joint | 1.25 | 2.0 |

[1] Determined by capillary zone electrophoresis.
[2] Determined by Anion Exchange HPLC.

## Discussion

Catabolism of aggrecan in the joint would appear to be a process which involves aggrecanase cleavage of the core protein at multiple sites to generate a heterogenous mixture of tissue-associated and released products. This process is not confined to cartilage but occurs in all aggrecan-containing tissues. An understanding of the structures of these products and the development of assay procedures for individual components is now emerging. The increasing use of cleavage-site specific antisera should soon reveal the extent to which aggrecanase or other proteinases are primarily responsible for all steps of aggrecan catabolism in different joint tissues.

These approaches will also form an important basis of knowledge for the ongoing development of synovial fluid analyses in monitoring disease progres-

sion. For example, an ability to identify the tissue source may be particularly important following meniscal or ligament injury when the concentration of aggrecan fragments in the fluid is known to be markedly elevated (Lohmander et al. 1989). Moreover since it is clear that even partial meniscectomy in experimental models and following human joint injury predisposes to cartilage failure, degradative changes in aggrecan metabolism in the meniscus may be a critical early event in this process. The availability of specific markers of meniscus catabolism may therefore provide a diagnostic tool to detect these early events and to predict the likelihood of later articular degeneration. In addition, specific assays will be required to determine whether aggrecan fragment release is due to both C-terminal and N-terminal proteolysis and whether it is represented primarily by newly synthesized or resident matrix molecules. In this regard, it now seems likely that a combination of immunoreagents to identify specific aggrecan core fragments and quantitative assays for specific carbohydrate structures will contribute significantly to our understanding in this area.

## Acknowledgements

We acknowledge the Shriners of North America (grants to JDS, AHKP and TJK) for continued financial support of this work. In addition we thank Dr. Michael Lark, Dr Steve Carlson and Dr. Mineo Iwata for the provision of antisera and Dr. James Kimura for the supply of rat chondrosarcoma aggrecan. We also acknowledge the expert assistance of our collaborators, John Gordy, Kelli Herrlinger-Garcia, Dan Hernandez, and Dr. Peter Neame at the Tampa Unit.

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