

Aggrecan degradation in osteoarthritis and rheumatoid arthritis

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Aggrecan turnover is critically important to maintain extracellular matrix homeostasis in articular cartilage. Cartilage aggrecan metabolism has been studied in chondrocyte cell cultures, cartilage explant cultures, and in whole animal models. In many of these studies, matrix metalloproteinases (MMPs) are proposed to degrade cartilage aggrecan. MMP expression appears elevated in joint tissues from patients with osteoarthritis (OA) and rheumatoid arthritis (RA) (Dean et al. 1989, Wolfe et al. 1993) and inhibitors of both MMPs and thiol proteinases have been shown to block aggrecan (Buttle et al. 1992) and type II collagen (Mort et al. 1993) degradation in cartilage explant cultures. Using antibodies and cDNA probes, elevations in expression and concentration of many of these enzymes in animal models and in OA and RA have been described. Human cartilage aggrecan has now been cloned and sequenced (Doege et al. 1991). This information, in combination with the ability to sequence aggrecan and aggrecan fragments at the protein level, has resulted in the identification of several aggrecan fragments which appear to result from proteinase degradation. In this report, we describe data which suggest that MMPs may in part be responsible for aggrecan catabolism in normal articular cartilage, as well as in the elevated catabolism seen in OA and RA.

Matrix metalloproteinases in human OA, RA and in animal models

Since MMP expression appears elevated in OA and RA (Dean et al. 1989, Wolfe et al. 1993) and since inflammatory cytokines upregulate the expression of these enzymes in both synoviocytes (MacNaul et al. 1990) and chondrocytes (Goldring et al. 1994), we chose to quantify the levels of two of these MMPs, stromelysin-1 (EC 3.4.24.17, MMP-3) and collagenase (EC 3.4.24.7, MMP-1). Stromelysin is thought to be important in cartilage matrix turnover since it

degrades a number of the matrix components including aggrecan (Fosang et al. 1991, Flannery et al. 1992), link protein (Nguyen et al. 1989), type IX collagen and type II collagen telopeptide (Wu et al. 1991). Stromelysin also can activate other members of the MMP family (Murphy et al. 1987). Collagenase was also evaluated since it degrades type II collagen, the major collagen type in cartilage. Using ELISAs, we showed that there were extremely high levels of stromelysin (micromolar in some cases) in joint fluids of patients with OA and RA (Walakovits et al. 1992, Lohmander et al. 1993a, Lohmander, et al. 1993b). Interestingly, the majority of the enzyme in the joint fluid is either free, inactive proenzyme or stromelysin complexed to its natural inhibitors tissue inhibitor of metalloproteinase-1 (TIMP-1) or alpha-2-macroglobulin. In contrast, the joint fluid collagenase concentrations were only about one tenth of those seen for stromelysin (Walakovits et al. 1992, Lohmander et al. 1995). When the levels of stromelysin in these patients were compared to the TIMP-1 levels, there was excess TIMP in the control population but the balance shifted to one of excess stromelysin in the patients with joint disease. These data are consistent with data published from extracts of human OA cartilage which suggested that there was an excess of a metallo-proteoglycanase, thought to be stromelysin, over TIMP (Dean et al. 1989).

MMP expression has also been evaluated in animal models. In a simple inflammatory model in rabbits injected with a IL-1, we have shown that there is at least a 100-fold increase in stromelysin levels (McDonnell et al. 1992). In the rat streptococcal cell wall (Case et al. 1989), and CIA models (Hasty et al. 1990), there are detectable levels of MMPs at the cartilage-synovial interface. In the rabbit meniscectomy model (Mehraban et al. 1994), elevations in MMP mRNA in cartilage and synovium have also been

reported. Together these data indicate that there are significant elevations of MMPs in human OA and RA as well as in animal models of these diseases.

Identification of aggrecan fragments in OA and RA

When aggrecan fragments from the medium of catabolic cartilage explant cultures were sequenced it appeared that there were specific, reproducible cleavages taking place C-terminal to glutamic acid (Sandy et al. 1991). One of the major sites for this activity appeared between Glu³⁷³-Ala³⁷⁴ in the aggrecan interglobular domain. Fragments consistent with this cleavage with the N-terminal sequence A³⁷⁴RGSV, were detected in joint fluids of patients with both OA (Sandy et al. 1992, Lohmander et al. 1993) and inflammatory joint diseases (Lohmander et al. 1993). Stromelysin does not appear to be the enzyme responsible for this cleavage since it cleaves aggrecan between Asn³⁴¹-Phe³⁴² (Flannery et al. 1992). However, sequencing of aggrecan fragments from OA cartilage showed that this tissue did contain fragments resulting from stromelysin-like MMP cleavage (Flannery et al. 1992). These initial sequencing results suggested that there are two activities within cartilage that were responsible for aggrecan turnover, one which cleaves between Asn³⁴¹-Phe³⁴² and a second that cleaves between Glu³⁷³-Ala³⁷⁴.

A number of the MMPs have the ability to cleave aggrecan between Asn³⁴¹-Phe³⁴² (Fosang et al. 1991, Flannery et al. 1992). In contrast, the only enzyme which appears to have any activity against aggrecan at the Glu³⁷³-Ala³⁷⁴ bond is neutrophil collagenase (Fosang et al. 1994). However, neutrophil collagenase seems to have a predominant *in vitro* activity at the Asn³⁴¹-Phe³⁴² MMP site.

Since cleavage at the Glu³⁷³-Ala³⁷⁴ bond appears so reproducible and since no enzyme with this substrate specificity has been identified, the activity responsible for this cleavage was named "aggrecanase". It is possible that "aggrecanase" is a member of the MMP family since MMP inhibitors can completely block the release of "aggrecanase"-generated aggrecan fragments from cartilage explant cultures (Buttle et al. 1992). Since the sequencing data (Flannery et al. 1992, Sandy et al. 1992, Lohmander et al. 1993) suggested that both stromelysin-like MMP and "aggrecanase" activities were responsible for cartilage aggrecan catabolism, we developed antibodies which detect the C-terminal neopeptides on the G1 fragments generated by these catabolic activities to characterize these fragments in cartilage.

Monospecific antibodies which detect aggrecan catabolic products

Polyclonal anti-peptide antisera were generated against NITEGE (for the "aggrecanase"-generated G1 fragment) and FVDIPEN (for the stromelysin-like MMP-generated G1 fragment) (Lark et al. 1995 a and b). In both cases, the immunodominant epitopes reside in the most C-terminal amino acids; however, both antisera require the entire antigen sequence for optimal recognition. Neither antiserum recognizes intact aggrecan. When aggrecan is digested with stromelysin or gelatinases A or B, a FVDIPEN-positive band migrating with a Mr ~55 kD is generated. Using the anti-NITEGE antiserum, we have further shown that neither intact aggrecan, nor G1 fragments generated by enzymes including stromelysin, collagenase, gelatinases A or B, cathepsins B or G or human leukocyte elastase are recognized by this antiserum. However, an activity which cleaves aggrecan at this site (independent of cleavage at the Asn³⁴¹-Phe³⁴² site) is expressed in retinoic acid-stimulated rat chondrosarcoma cells (Lark et al. 1995). The enzyme, "aggrecanase", responsible for this cleavage has not yet been purified.

Characterization of MMP- and "aggrecanase"-generated G1 fragments generated *in vivo*

In extracts of human OA cartilage, fragments recognized by both the anti-NITEGE and anti-FVDIPEN antisera were detected (Figure 1) (Bayne et al. 1995). The NITEGE-positive band comigrated with the G1 fragment generated in the retinoic acid stimulated rat chondrosarcoma cell system and the FVDIPEN-positive band comigrated with stromelysin-generated aggrecan G1. At the immunohistochemical level, both epitopes appeared to be most abundant toward the articular surface of the cartilage where they were found in all matrix zones (Figure 2 A, C and E). The staining was extremely intense in areas of extensive damage. A similar pattern of staining was detected in the specimens from patients with RA (Figure 2 B, D and F). When sequential sections were evaluated using both antibodies, there was significant codistribution of both neopeptides; however, in some cases there was very intense staining for the NITEGE neopeptide with little FVDIPEN staining whereas in other areas there was staining for the FVDIPEN neopeptide with no NITEGE staining.

To determine if the G1 fragments detected in the cartilage extracts represented a significant proportion of the total available aggrecan, the FVDIPEN-positive G1 fragment was quantified in the cartilage extracts before and after exhaustive stromelysin

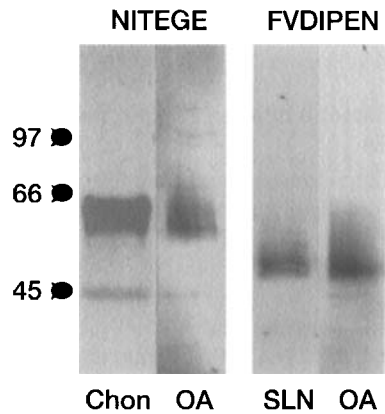


Figure 1. Western blotting of aggrecan G1 fragments.
G1 fragments generated in retinoic acid-stimulated rat chondrosarcoma cultures (Chon.) and an A1D4 fraction from an OA cartilage extract (OA) were evaluated by western blotting stained with anti-NITEGE antiserum and compared to a G1 fragment generated by stromelysin cleavage of aggrecan (SLN) and an A1D4 fraction from an OA cartilage extract (OA) stained with anti-FVDIPEN antiserum.

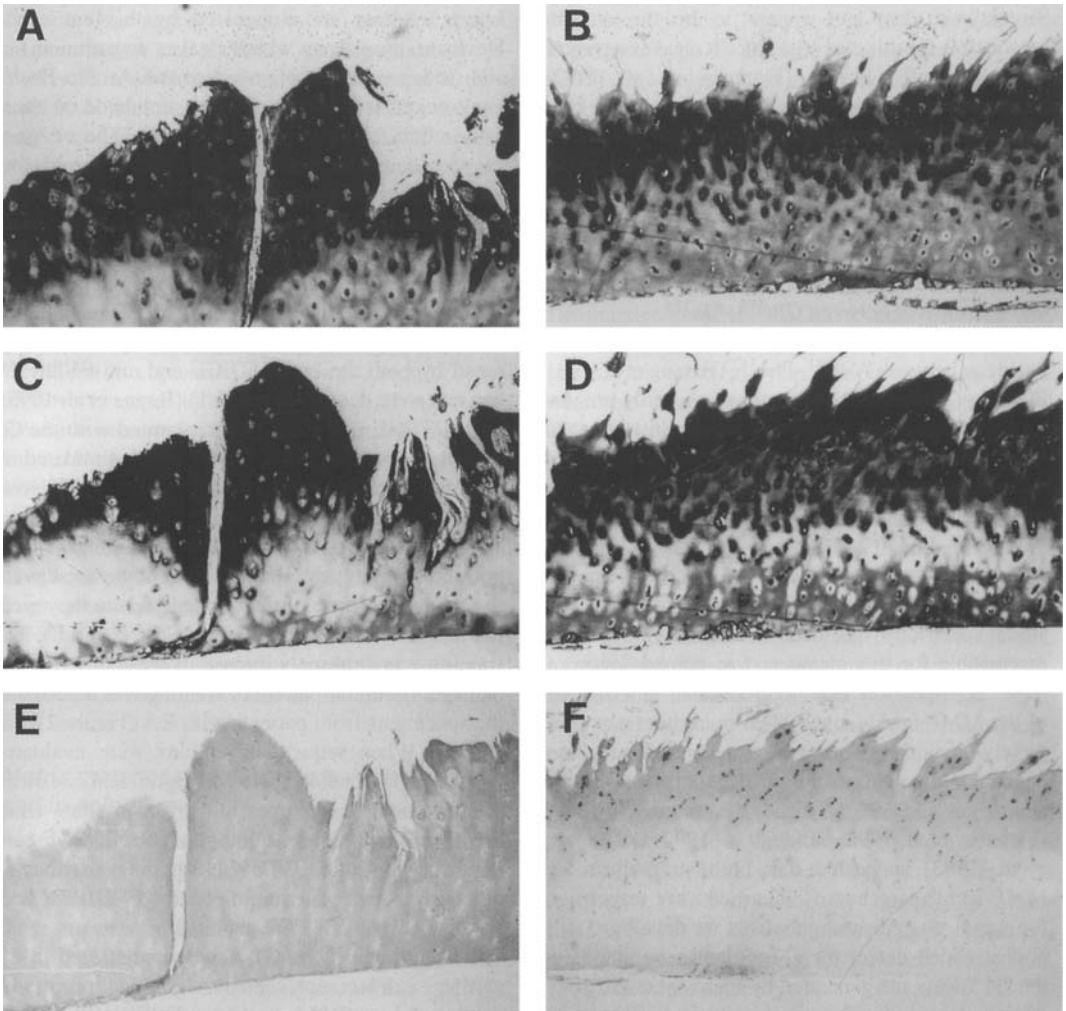


Figure 2. Immunoperoxidase staining of cartilage aggrecan G1 fragments.
Sections of cartilage from a patient with OA (A, C and E) or RA (B, D and F) were stained with anti-FVDIPEN (A and B) and anti-NITEGE (C and D) antisera and compared to sections stained with preimmune serum.

digestion. In the case of the RA samples, there was a 5-fold increase in FVDIPEN signal following stromelysin digestion and in the case of the OA samples, there was a 10-fold increase following digestion. These data indicate that at least 10% of the aggrecan in the OA cartilage (20% in the case of RA) had been catabolized and remained resident within the tissues as an FVDIPEN-positive G1 fragment. By comparison, in an age-matched normal population, the levels of FVDIPEN neopeptide relative to available substrate was similar to that seen in the samples from patients with OA. In contrast, there was little FVDIPEN-positive detectable signal in fetal tissues. However, after stromelysin treatment, there was greater than a 1,000-fold increase in signal. Together these data indicate that there is little FVDIPEN-positive G1 in fetal tissues but with age this epitope increases. Furthermore, the levels of epitope in samples from patients with OA are similar to those from patients with no diagnosed joint disease; however, the levels of the FVDIPEN-positive G1 fragment appear higher in the samples from patients with RA.

A similar analysis has been undertaken in the mouse CIA model (Singer et al. 1995 a and b). In the 8–12 week old mouse, there is no detectable immunostaining for either the FVDIPEN or NITEGE neopeptides in normal articular cartilage. At a very early stage in the mouse CIA model, before overt clinical signs are evident, both neopeptides are detected within the articular cartilage in a pattern similar to that seen in human cartilage. These data suggest that in this model both “aggrecanase” and stromelysin-like MMP aggrecan cleavage takes place in articular cartilage, similar to that seen in human RA and OA.

Models for aggrecan catabolism in OA and RA

The predominant aggrecan fragments in joint fluids have the N-terminus A³⁷⁴RGSVIL (Sandy et al. 1992, Lohmander et al. 1993). Presumably these fragments enter the joint fluid following “aggrecanase” cleavage in the articular cartilage. The data outlined in this report indicate that there are G1 fragments resident in articular cartilage that have been generated by stromelysin-like MMPs as well as by “aggrecanase”. In many regions of cartilage, both of these neopeptides are present but in some regions only one or the other of these neopeptides is found. We have shown that MMPs like stromelysin (Flannery et al. 1992) and “aggrecanase” (Lark et al. 1995) both have the ability to cleave aggrecan, independent of the other enzyme.

Taking these data together, three models of aggrecan turnover can be proposed:

A) Both stromelysin-like MMPs and “aggrecanase” are active at different sites and possibly at different times within the cartilage (Figure 3A). NITEGE- and FVDIPEN-positive G1 fragment don’t codistribute in some cases, suggesting that these enzymes may act independently. As the MMP-cleaved glycosaminoglycan-rich fragment moves from the cartilage into the joint fluid it may get further processed by “aggrecanase”. This would result in release of both a large aggrecan fragment with the N-terminus A³⁷⁴RGSV and a small peptide fragment with the N-terminus F³⁴²FGVG. It has recently been reported that such a small fragment may be found in human joint fluids (Fosang et al. 1995).

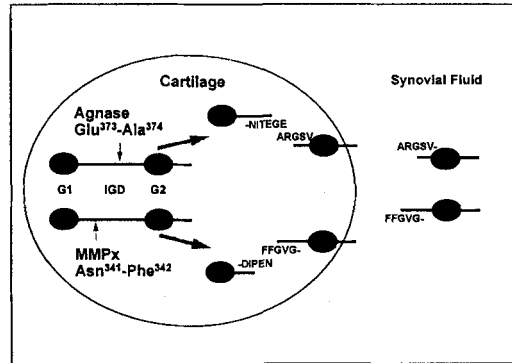
B) It is possible that “aggrecanase” may initially cleave aggrecan resulting in release of large aggrecan fragments into the joint fluid with the N-terminus A³⁷⁴RGSV and accumulation of HA-bound G1 fragments with the C-terminus NITEGE³⁷³. Stromelysin could then cleave the resident NITEGE-positive G1 fragments generating G1 fragments with the C-terminus FVDIPEN³⁴¹ and releasing a small peptide fragment into the joint fluid with an N-terminus of F³⁴²FGVG (Figure 3B). If this mechanism were functioning, little NITEGE-positive G1 fragment would be found in cartilage from patients with advanced disease. The seemingly large levels of NITEGE-positive G1 in cartilage from patients undergoing joint replacement surgery and the resistance of this fragment in tissue sections to cleavage by stromelysin (Bayne et al. 1995), raises questions about this second model.

C) A third possibility is that stromelysin-like MMPs could cleave aggrecan first and that “aggrecanase” would then cleave the large aggrecan fragment as it moves through the cartilage into the joint fluid (Figure 3C). The joint fluid data are consistent with this possibility. However, since “aggrecanase” cleaves C-terminal to the MMP site, no “aggrecanase”-generated G1 fragment terminating in NITEGE³⁷³ would be detected in the tissue, if this mechanism were in place. Therefore, it is unlikely that this mechanism is functioning in cartilage.

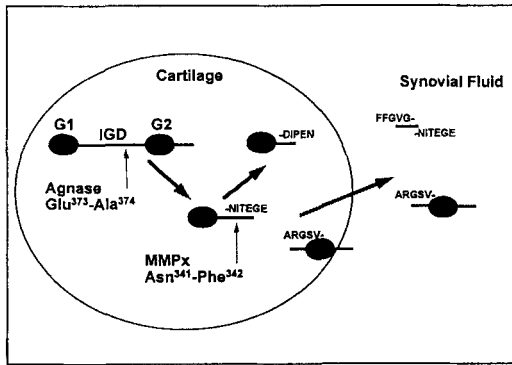
Using the antibodies described in this report, in combination with cell culture and explant culture model systems, we may be able to begin to unravel the mechanisms that are controlling aggrecan turnover in normal articular cartilage and compare those to mechanisms controlling catabolism in joint diseases such as OA and RA.

Figure 3. Hypothetical models for proteolytic breakdown of aggrecan in cartilage.

(A) Aggrecanase (Agnase) and a classic matrix metalloproteinase activity (MMPx) independently cleave aggrecan interglobular domain (IGD). The G1-domain may remain bound to hyaluronan in matrix, while the remainder of the aggrecan molecule (including the G2-domain) are free to diffuse into joint fluid. Cleavage epitopes NITEGE and -DIPEN may be detected in tissue, while ARGSV- and FFGVG- may be detected in synovial fluid.

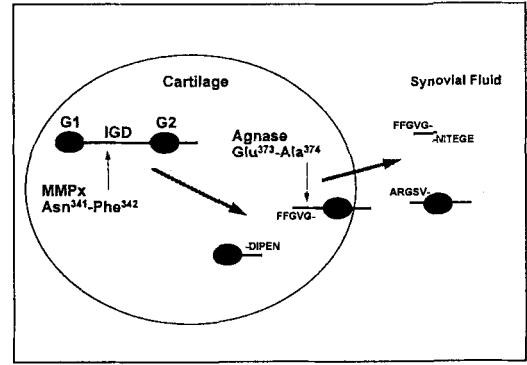


A



B

(B) Primary cleavage of IGD by aggrecanase, followed by secondary cleavage by MMPx activity. Depending on efficiency of second cleavage step, both NITEGE and -DIPEN may be detected in cartilage matrix, while ARGSV- and a small 30 amino acid peptide with FFGVG- and -NITEGE termini will be detected in joint fluid.



C

(C) Primary cleavage of IGD by MMPx, followed by aggrecanase cleavage of a 30 amino acid peptide from liberated G2-containing fragment. Only -DIPEN would be detected in matrix, while fragments with ARGSV- or FFGVG-termini would be present in joint fluid.

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