THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 46, Issue of November 14, pp. 45539–45545, 2003
Printed in U.S.A.

Aggrecan Protects Cartilage Collagen from Proteolytic Cleavage*

Received for publication, April 9, 2003, and in revised form, July 28, 2003
Published, JBC Papers in Press, July 30, 2003, DOI 10.1074/jbc.M303737200

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The matrix components responsible for cartilage mechanical properties, type II collagen and aggrecan, are degraded in osteoarthritis through proteolytic cleavage by matrix metalloproteinases (MMPs) and aggrecanases, respectively. We now show that aggrecan may serve to protect cartilage collagen from degradation. Although collagen in freeze-thawed cartilage depleted of aggrecan was completely degraded following incubation with MMP-1, collagen in cartilage with intact aggrecan was not. Using interleukin-1-stimulated bovine nasal cartilage explants where aggrecan depletion occurs during the first week of culture, followed by collagen loss during the second week, we evaluated the effect of selective MMP and aggrecanase inhibitors on degradation. A selective MMP inhibitor did not block aggrecan degradation but caused complete inhibition of collagen breakdown. Similar inhibition was seen with inhibitor addition following aggrecan depletion on day 6–8, suggesting that MMPs are not causing significant collagen degradation prior to the second week of culture. Inclusion of a selective aggrecanase inhibitor blocked aggrecan degradation, and, in addition, inhibited collagen degradation. When the inhibitor was introduced following aggrecan depletion, it had no effect on collagen breakdown, ruling out a direct effect through inhibition of collagenase. These data suggest that aggrecan plays a protective role in preventing degradation of collagen fibrils, and that an aggrecanase inhibitor may impart overall cartilage protection.

Cartilage plays a critical role in joint function, where it acts as a shock absorber during joint loading and motion. The extracellular matrix molecules, type II collagen and aggrecan, provide the properties that enable the cartilage to serve this shock-absorbing function. Type II collagen is present as triple helical fibrils that provide cartilage with its structure and tensile strength (3). Aggrecan monomers consist of a core protein with three globular domains. The amino-terminal region contains two of these globular domains, G1 and G2, separated by an interglobular domain. The G2 is followed by a long region along which are attached the sulfated glycosaminoglycan side chains, keratan sulfate and chondroitin sulfate and by the C-terminal globular domain, G3 (4). Multiple aggrecan monomers bind to hyaluronan through their G1 domains to form huge aggregates that are trapped within the collagen network. Because of their high negative charge and water-binding capacity, the aggrecan molecules provide the mechanical properties of compressibility and elasticity to the cartilage. The mechanical properties of the matrix as a whole depend on both major components and their stabilization by intermolecular interactions. The ability of the cartilage to withstand mechanical stress, therefore, is dependent upon its structural integrity and upon the various interactions between the matrix components.

Cartilage degradation leading to a loss of joint function in osteoarthritis involves proteolytic cleavage of both aggrecan and collagen. Temporally, aggrecan is one of the first extracellular matrix components to undergo measurable loss in arthritic diseases (5). Indeed, there are several lines of indirect evidence suggesting that the presence of aggrecan may serve to protect type II collagen molecules from proteolytic degradation. In cartilage explant cultures stimulated to undergo matrix degradation with cytokines such as interleukin-1 (IL-1),[1] the aggrecan is degraded early, during the first week of culture, whereas the collagen is not rapidly degraded until later in the culture period when essentially all of the aggrecan has been lost (7, 8). Histological staining of cartilage from osteoarthritic joints indicates aggrecan loss occurs prior to loss of collagen, and in all areas where collagen loss or damage is seen, the staining for aggrecan staining is depleted (6). The loss of aggrecan, which in normal cartilage prevents the diffusion of large molecular weight molecules into the cartilage matrix, may allow an increased diffusion of cytokines and proteases from the synovial fluid into the cartilage tissue leading to further cartilage erosion. With the loss of aggrecan, the collagen fibrils are no longer well supported and can undergo mechanical damage. Finally, studies have shown an interaction between type II collagen and aggrecan molecules (9), and scanning electron microscopy shows aggrecan coating the collagen fibers. Thus, it could be envisioned that aggrecan physically prevents protease access to the collagen molecules raising the question of whether aggrecan serves to protect collagen from degradation, and conversely whether collagen degradation could be prevented by retention of cartilage aggrecan.

Although type II collagen and aggrecan are both degraded in osteoarthritis by proteolytic cleavage, different proteases are responsible. The matrix metalloproteinases (MMP) are thought to be responsible for type II collagen cleavage (10–13), whereas

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[1] The abbreviations used are: IL-1, interleukin-1; MMP, matrix metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs.

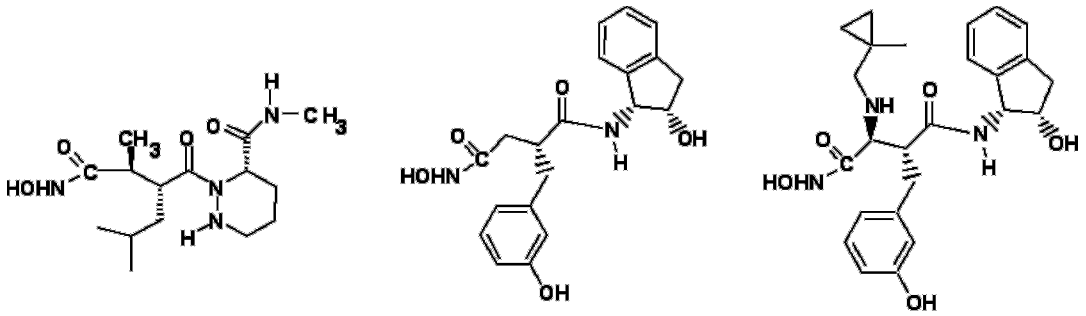

FIG. 1. **Structures of inhibitors used in this study.** XS309 is a potent nanomolar inhibitor of a number of MMPs, including MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13, but is a poor inhibitor of aggrecanase (Table I). In contrast ST109 and ST154 are potent, selective inhibitors of aggrecanase.

the aggrecanase enzymes, ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs) and ADAMTS-5 (1, 2), have been implicated in the cleavage of aggrecan (14–16). Selective MMP inhibitors that do not inhibit the aggrecanases do not prevent aggrecan degradation (1, 16, 17), suggesting that blockade of collagen degradation does not prevent aggrecan loss. The recent design and synthesis of selective aggrecanase inhibitors that do not inhibit the matrix metalloproteinases (18) now provide tools to examine the effect of cartilage aggrecan protection on type II collagen degradation.

In the studies reported herein, we have examined the ability of aggrecan preservation to prevent cartilage type II collagen degradation. This was evaluated by 1) comparing the ability of collagenase to degrade collagen in cartilage with normal aggrecan content or in aggrecan-depleted cartilage, and 2) by determining the ability of selective aggrecanase inhibitors devoid of collagenase inhibitory activity to block both aggrecan and type II collagen breakdown and release in cartilage explants.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, fetal calf serum, trypsin, and penicillin/streptomycin were purchased from Invitrogen. XS309 ([3S-[3R*,2-[2R*,2-(R*,S*)]-hexahydro-2-[2-[2-(hydroxyamino)-1-methyl-2-oxo ethyl]-4-methyl-1-oxopentyl]-N-methyl-3-pyridazinecarboxamide), ST109, (N1-[2(R)-hydroxy-1(S)-indanyl]-N4-hydroxy-2(R)-[3-(hydroxyphenyl)methyl]-butanediamide), and ST154 [N1-[2(R)-hydroxy-1(S)-indanyl]-N4-hydroxy-2(R)-[3-(hydroxyphenyl)methyl]-3(S)-(2,2-dimethylpropionamido)-butanediamide) were synthesized as previously described (16, 18, 19). XS309 is a potent nanomolar inhibitor of a number of MMPs, including MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13, but is a poor inhibitor of aggrecanase (Table I). In contrast, ST109 and ST154 are potent, selective inhibitors of aggrecanase (Fig. 1).

Aggrecanase Assay—Compounds were dissolved in 100% Me_2SO at 10 mM and then diluted in buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$) to the required concentration. Me_2SO alone at the highest final concentration used had no effect on the parameters tested. The compounds were evaluated against a semi-purified preparation of aggrecanase from IL-1-stimulated bovine nasal cartilage conditioned media (15, 16). IC_{50} values were determined against a bovine aggrecan monomer substrate and were based on the inhibition of aggrecan fragment generation that possessed an N terminus of "ARGS."

Preparation of Bovine Nasal Cartilage Explants—Cartilage was prepared from the nasal septum of mature steer (> 9 months of age) at the time of slaughter. Uniform cartilage disks were prepared from the nasal septum by Covance Research Products (Denver, PA) as previously described (16).

Digestion of Collagen in Freeze-thawed Cartilage Explants—Cartilage disks were frozen and thawed 3 times to render the chondrocytes non-viable and used as a substrate for enzymatic digestion. Each disk was cut into quarters and paired pieces from the same disk used for various treatments. Cartilage pieces were incubated 16 h with trypsin (5 μg/ml) in phosphate-buffered saline to deplete the matrix aggrecan or with buffer alone as a control (data not shown). At the end of the incubation 5% fetal calf serum was added for 60 min to inactivate the trypsin, and the disks were washed 4× with phosphate-buffered saline. Aggrecan-depleted cartilage and control cartilage pieces that contained a normal aggrecan matrix were incubated with 500 nM full-length MMP-1 or in buffer alone and the matrix integrity assessed visually after 24 h.

Cartilage Cultures—Bovine nasal cartilage disks were sliced into eighths and cultured as previously described (20). Briefly, each slice was weighed and placed into a well of a 96-well plate in a total volume of 180 μl of Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 1× antibiotic/antimycotic (Invitrogen) and equilibrated for at least 3 days prior to treatment. Cartilage was stimulated with IL-1 in serum-free Dulbecco's modified Eagle's medium containing 1× antibiotic/antimycotic in the absence or presence of inhibitors. Culture media were removed for analysis and replaced with fresh treatments every two to 3 days.

Aggrecan Degradation—Aggrecan degradation was monitored by assessing sulfated glycosaminoglycan released into the media using the dimethylmethylene blue (DMMB) dye assay with shark chondroitin sulfate as a standard (21). Aggrecan content of the cartilage was determined following digestion of the tissue with papain for 24 h at 65 °C as previously described (20).

Collagen Degradation—Collagen degradation products were assayed in the media or in the papain digest of the cartilage using a hydroxyproline assay (22). Fifty microliters of cell culture media or 10 μl of cartilage digest were hydrolyzed with 50 μl 12N HCl for 18 h at 100 °C. The hydrolysate was then dried in a vacuum desicator over NaOH pellets. The residue was dissolved in water (150 μl), transferred to a 96-well plate, and dried in a chemical hood. Sixty microliters of water was then added to each well followed by 20 μl of assay buffer (1-propanol/water/pH 6 buffer; 3:2:10 ratio). The pH 6 buffer consisted of 0.24 M citric acid, 0.88 M anhydrous sodium acetate trihydrate, 0.88 M anhydrous sodium acetate, 0.21 M acetic acid, 0.85 M sodium hydroxide, pH 6. The solution was allowed to shake until the residue was fully solubilized. Forty microliters of chloramine T reagent (0.282 g of chloramine T, 1 ml of water, 8 ml of pH 6 buffer, 1 ml of 1-propanol) was then added, and the samples were allowed to shake for 15 min at room temperature. Eighty microliters of DMBA reagent (2 g of dimethylaminobenzaldehyde, 1.25 ml of 1-propanol, 2.75 ml of perchloric acid) was added, and the plate was incubated on a shaker for 20 min at 70 °C. After the plate was allowed to cool, absorbances were determined at 570 nm on a Molecular Devices Thermomax microplate reader. Data were converted to nanograms of hydroxyproline based on a standard curve that consisted of hydroxyproline levels ranging from 20–2000 ng/well.

Evaluation of Denatured Collagen—Denatured type II collagen fragments retained within the tissue were removed as previously described by digesting the tissue with chymotrypsin (23) and then assayed using the hydroxyproline assay described above.

Statistical Analysis—Significant differences between groups were tested by Duncan's multiple range test when analysis of variance was significant ($p < 0.05$). Values shown are mean ± S.E. for glycosaminoglycan release ($n = 8$); mean ± S.D. for hydroxyproline levels ($n = 3$).

RESULTS

Collagenase Digestion of Cartilage—To determine whether the presence of aggrecan matrix affects the ability of collagenase to degrade cartilage collagen, freeze-thawed bovine nasal cartilage explants were treated with trypsin (5 μg/ml) for 16 h

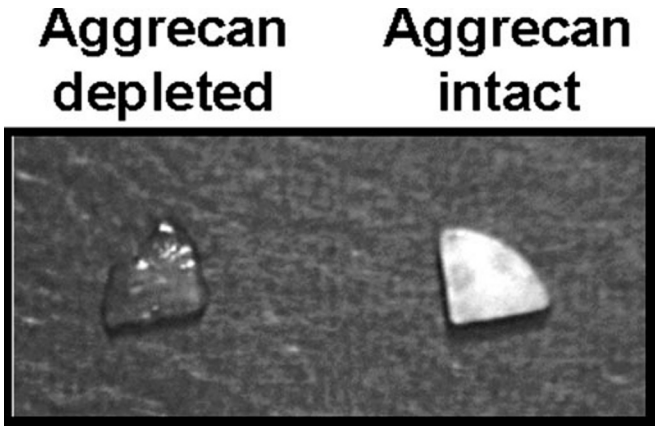

FIG. 2. **Presence of aggrecan protects collagen from collagenase digestion.** Paired pieces of freeze-thawed bovine nasal cartilage were incubated for 16 h with 5 μg/ml trypsin (*A*) to deplete aggrecan content of the tissue or with buffer (*B*) as an intact aggrecan control. Both slices were then incubated with 500 nM full-length human MMP-1 for 24 h.

to deplete the aggrecan content of the tissue. In a preliminary experiment, analysis of the aggrecan content in cartilage treated with trypsin under these conditions resulted in aggrecan depletion (data not shown). However, the cartilage was visually unchanged and appeared hard and white like the native cartilage not treated with trypsin. Incubation of aggrecan-depleted cartilage with MMP-1 (500 nM) for 24 h resulted in complete breakdown of the cartilage matrix to a gelatinous mass (Fig. 2). Under these same conditions, MMP-1 treatment of cartilage with an intact aggrecan matrix resulted in minimal changes of the cartilage matrix. These data suggest that the aggrecan matrix provides protection to the collagen fibrils from enzymatic cleavage.

Time Course of IL-1-stimulated Aggrecan and Collagen Degradation—Bovine nasal cartilage was incubated with IL-1 for 20 days, and media were removed and replaced with fresh treatments every 2–3 days. Media were assessed for aggrecan and collagen degradation products (Fig. 3). Aggrecan degradation products were released into the media by day 2 and 4 following IL-1 stimulation, with media glycosaminoglycan levels decreasing by day 6 and 8, suggesting depletion of aggrecan from the cartilage. In separate studies, assessment of cartilage digests indicated that aggrecan content of the tissue was depleted following 6–8 days of IL-1 exposure (24). As reported previously (8), collagen degradation occurred following the depletion of aggrecan. No detectable hydroxyproline levels were present in media at day 10, but increased levels were detected at day 12 and peaked by day 17.

Effect of an MMP Inhibitor on Aggrecan and Collagen Degradation—To evaluate the effect of a MMP inhibition on cartilage degradation, a broad-spectrum metalloproteinase inhibitor, XS309, was used. This compound has been shown to inhibit a variety of MMPs with K_i values in the nanomolar range, but is ineffective in inhibiting aggrecanase, with an IC_{50} of >10 μM (Table I) (24). XS309 was evaluated for its ability to block IL-1-stimulated aggrecan and collagen degradation monitored on day 4 and day 14, respectively, based on results of time course studies above. Inclusion of XS309 (10 μM) with IL-1 from time 0 had no effect on IL-1-stimulated aggrecan degradation (Fig. 4*A*), consistent with previous work suggesting that metalloproteinases are not involved in aggrecan degradation (17, 25, 26). However, consistent with the involvement of MMPs in type II collagen degradation, XS309 effectively blocked IL-1-induced collagen degradation in the same cultures at day 14 (Fig. 4*B*). A recent (27) report suggests that collagen degradation in this model is mediated by collagenase-3 (MMP-13) based on inhibition by a selective inhibitor of this enzyme. The fact that XS309 is an effective inhibitor of MMP-13 is consistent with the published observations using the selective MMP-13 inhibitor.

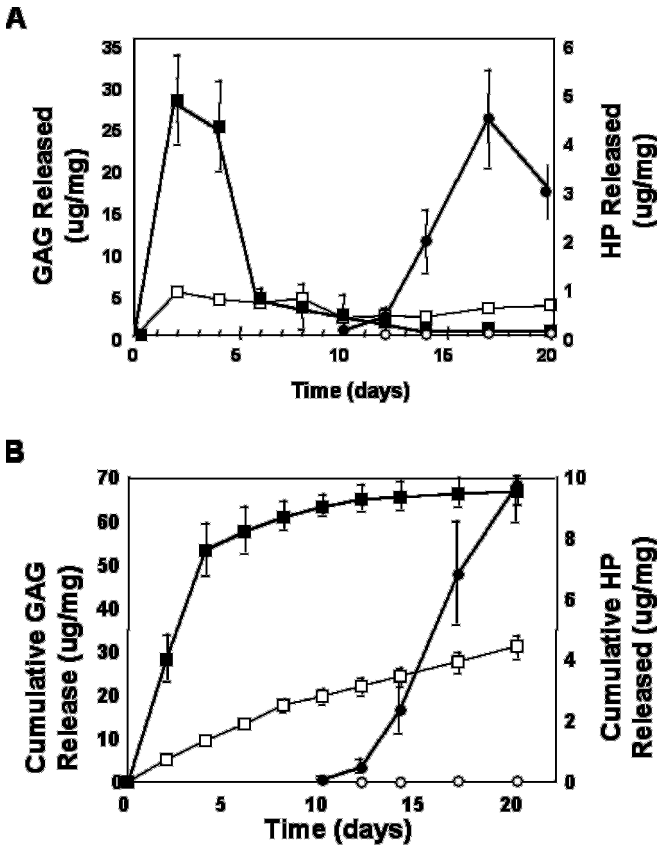

FIG. 3. **Time course of IL-1-induced aggrecan and collagen degradation in bovine nasal cartilage.** Bovine nasal cartilage was incubated in media without (*open symbols*) or with IL-1 (20 ng/ml) (*solid symbols*) for 20 days. Media were removed for analysis and replaced with fresh treatments every 2–3 days. Media were assessed for aggrecan and collagen degradation products as described in "Experimental Procedures." Aggrecan degradation (*squares*) is reported as μg glycosaminoglycan (*GAG*) per mg wet weight cartilage, and collagen degradation (*circles*) is reported as μg hydroxyproline (*HP*) per mg wet weight cartilage. Results are plotted as either the amount released during each time period (*A*) or as cumulative release over time (*B*). Values shown are mean ± S.E.

Based on the data shown in Fig. 3 and from several published reports (7, 28) there is no evidence to suggest that significant collagen degradation occurs prior to aggrecan depletion in this system. The temporal relationship between aggrecan and collagen degradation, therefore, suggests that it should be unnecessary for an MMP inhibitor to be present prior to aggrecan depletion to be effective in blocking collagen degradation. To test this hypothesis, we incubated cartilage for 6 days with IL-1 in the absence of XS309, and then added the inhibitor with IL-1 from day 6 through day 14. XS309 was equally effective in blocking collagen degradation when added 6 days after IL-1 treatment *versus* addition at time 0 (Fig. 4*B*). A similar observation was made when the compound was added 8 days after IL-1 treatment commenced (data not shown). These data suggest that there is no significant level of collagen degradation by MMPs occurring in response to IL-1 treatment prior to degradation and depletion of the aggrecan matrix.

Effect of Aggrecanase Inhibition on Aggrecan and Collagen Degradation—The contribution of aggrecanase to the degrada-

TABLE I
Inhibitor selectivity for aggrecanase and MMPs

The K_i values of these compounds against the MMPs were calculated using the fluorogenic peptide substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 at a concentration of 1 μM as previously described (39) and the IC_{50} values against aggrecanase were determined as described in "Experimental Procedures." NT; not tested.

	IC_{50}, aggrecanase	K_i					
		MMP-1	MMP-2	MMP-3	MMP-8	MMP-9	MMP-13
				nM			
XS309	>10000	<10	<10	29	<10	<10	10
ST109	251	30953	>3000	9519	596	3324	>5000
ST154	8	5248	>3000	>4500	NT	<1	>5000

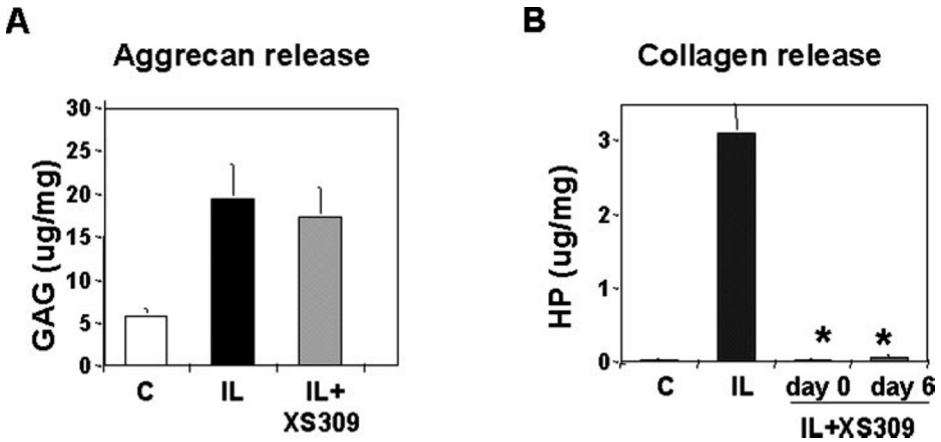

FIG. 4. **Effect of an MMP inhibitor on matrix degradation.** Bovine nasal cartilage explants were incubated without or with IL-1 (20 ng/ml) for 14 days with or without the MMP inhibitor, XS309 (10 μM), added either on day 0 at the time of IL-1 addition or at day 6 following depletion of aggrecan by IL-1. Media were evaluated at day 4 for aggrecan degradation products (*A*) and at day 14 for collagen degradation products (*B*). *HP*, hydroxyproline; *GAG*, glycosaminoglycan. *, significantly different from IL-1 alone at $p < 0.05$.

tion of the cartilage matrix was investigated using a selective aggrecanase inhibitor, ST109 (18). This compound inhibits aggrecanase with submicromolar potency but is inactive against a panel of MMPs including MMP-13 at concentrations up to 5 μM (Table I). The compound was added with IL-1 at time 0, and the effect on aggrecan and collagen degradation was monitored on day 4 and day 14, respectively (Fig. 5). IL-1 caused an increase in aggrecan degradation that was effectively blocked by the aggrecanase inhibitor, suggesting that aggrecan degradation induced by IL-1 is mediated by aggrecanase (Fig. 5*A*). In addition, the aggrecanase inhibitor caused a significant inhibition in IL-1-induced collagen degradation (Fig. 5*B*). These data suggest that retention of aggrecan by an aggrecanase inhibitor may be effective in protecting type II collagen as well.

Because the aggrecanase inhibitor was present at a relatively high concentration of 10 μM, we were concerned that the compound may have broken selectivity for aggrecanase and had some direct effect on collagenase activity. To test this hypothesis, the compound was added on day 6, following aggrecan depletion. When added on day 6, ST109 had no effect on collagen degradation measured on day 14 (Fig. 5*B*). These data suggest that both aggrecan and collagen protection by the aggrecanase inhibitor are through the inhibition of aggrecanase, and not through inhibition of the MMPs involved in collagen degradation.

Although these data suggest that inhibition of aggrecanase leads to overall cartilage protection, degradation appears to be only delayed. Inhibition of aggrecan breakdown was lost by day 6 of culture leading to eventual, although delayed, depletion of aggrecan (data not shown). This loss of aggrecan protection was associated with a loss of collagen protection in the same cultures beginning at day 17 (Fig. 6). These data suggest that ST109 may lack the potency required for long-term cartilage protection in this aggressive model of cartilage degradation, and/or that additional mechanisms may be involved in aggrecan depletion. To evaluate these possibilities we employed a second, selective, more-potent aggrecanase inhibitor.

ST154 is a selective aggrecanase inhibitor with improved potency against aggrecanase (ST109 IC_{50} = 250 nM; ST154 IC_{50} = 8 nM). When we evaluated ST154 in the cartilage explant assay, aggrecan degradation was inhibited for a longer period, which extended aggrecan protection (Fig. 7*A*). In fact, when lower levels of IL-1 were used to stimulate cartilage breakdown, ST154 prevented aggrecan loss through the entire culture period of 21 days (Fig. 7*B*). The extended aggrecan protection seen with ST154 resulted in improved collagen protection in the same cultures, with a decrease in collagen release extended to 21 days (Fig. 8*A*). This result was substantiated by a maintenance of cartilage collagen content with ST154 as determined by monitoring hydroxyproline levels in papain-digested cartilage following 23 days in culture (Fig. 8*B*).

Although total collagen content in cartilage treated with the aggrecanase inhibitor was similar to the control, the possibility exists that denatured collagen fibrils are retained within the matrix. To address this question, the cartilage was treated with chymotrypsin to release any cleaved/denatured collagen fibrils that were retained within the cartilage matrix (23), and the cartilage was analyzed for hydroxyproline content. Evaluation of native collagen content of the tissue following chymotrypsin treatment (Fig. 8*C*) indicates that the collagen fibrils remaining in the cartilage have been protected from cleavage in the presence of the selective aggrecanase inhibitor.

DISCUSSION

The data presented herein is the first direct demonstration that cartilage aggrecan serves to protect type II collagen from enzymatic cleavage. We demonstrate that 1) digestion of type II collagen in freeze-thawed cartilage by incubation with collagenase is dramatically less using cartilage where the normal aggrecan is intact than in cartilage in which the aggrecan has

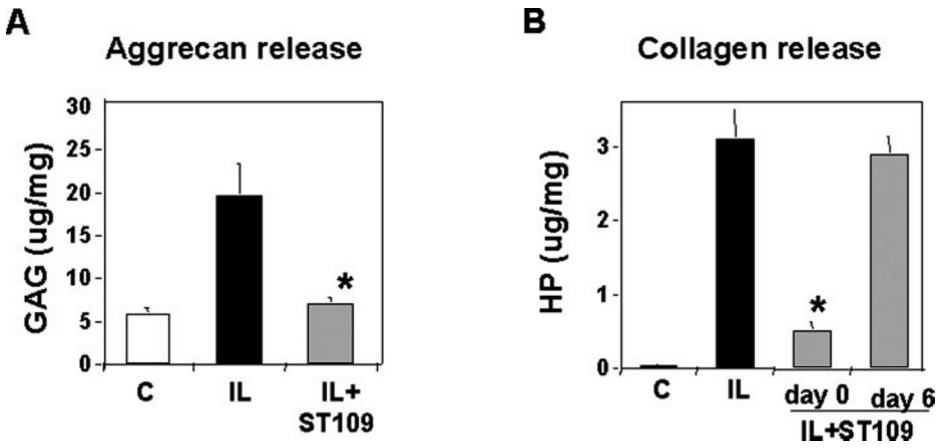

FIG. 5. **Effect of an aggrecanase inhibitor on matrix degradation.** Bovine nasal cartilage explants were incubated without or with IL-1 (20 ng/ml) for 14 days with or without the aggrecanase inhibitor, ST109 (10 μM), added either on day 0 at the time of IL-1 addition or at day 6 following depletion of aggrecan by IL-1. Media were evaluated at day 4 for aggrecan degradation products (*A*) and at day 14 for collagen degradation products (*B*). *HP*, hydroxyproline; *GAG*, glycosaminoglycan. *, significantly different from IL-1 alone at $p < 0.05$.

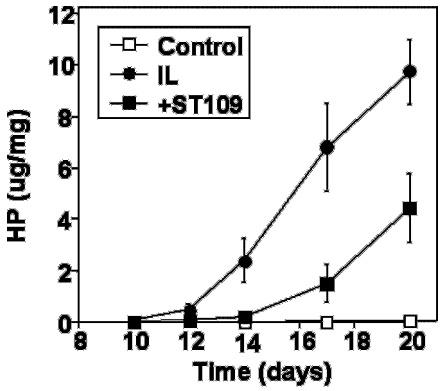

FIG. 6. **Loss of effect of an aggrecanase inhibitor on collagen degradation over time.** Bovine nasal cartilage explants were incubated with IL-1 (20 ng/ml) for 20 days with or without the aggrecanase inhibitor, ST109 (10 μM), added on day 0 at the time of IL-1 addition, and media were evaluated on day 10–20 for collagen degradation products. *HP*, hydroxyproline.

been depleted by treatment with trypsin; 2) a selective aggrecanase inhibitor is capable of blocking cleavage of collagen in IL-1-stimulated cartilage explants; 3) inhibition of collagen depletion corresponds with inhibition of aggrecan depletion; and 4) direct inhibition of collagenase activity is not responsible for the observed effect. Thus, these data support the notion that selective inhibitors of aggrecanase may lead to overall cartilage protection by blocking both the degradation of aggrecan and type II collagen.

One question raised by the inhibition of type II collagen degradation in response to selective aggrecanase inhibitors is whether aggrecanases may contribute directly to type II collagen breakdown through enzymatic digestion of the fibrils. Based on our data showing that type II collagen degradation can be completely blocked by addition of an MMP inhibitor 6–8 days after initiation of IL-1 stimulation, it is unlikely that aggrecanases contribute to the initial cleavage of the collagen fibril. During this early period, aggrecanases are clearly active as evidenced by the digestion of aggrecan, but little or no degradation of collagen has been detected in the early stages of this model (8, 29, 30). In addition, studies have shown that incubation of purified aggrecanases with type II collagen did not result in cleavage (31).[2] These data suggest that aggrecanases are not directly involved with type II collagen breakdown and support the hypothesis that the protection of collagen by aggrecanase inhibitors is through protection of the aggrecan matrix.

Although the compounds tested in this system are nanomolar inhibitors of the bovine aggrecanase enzymes, concentrations in the micromolar range were required to see full protection of aggrecan and collagen. There are several factors that may potentially contribute to this discrepancy between the two systems in this *in vitro* model of accelerated cartilage matrix destruction, including local concentrations of enzyme, access of inhibitors to the cartilage matrix, etc. This does not necessarily imply that such high concentrations would be required *in vivo*, as this IL-1-stimulated bovine nasal cartilage explant system represents an example of aggressive cartilage degradation. The serum-free conditions used in this assay, combined with the high concentrations of IL-1 with repeated exposure to fresh cytokine results in a highly robust degradative model. The cartilage undergoes both aggrecan and collagen depletion in a matter of weeks. In contrast, the destruction of cartilage due to osteoarthritis occurs over many years. The fact that the degradative events leading to cartilage dissolution occur in a matter of weeks in this system suggests a dramatically accelerated destruction.

Our data, consistent with previous reports (8, 29), demonstrate that aggrecan degradation precedes collagen degradation during stimulated cartilage breakdown in explant cultures. The delay in collagen breakdown in these cultures appears to be inconsistent with the finding that many MMPs, including those capable of degrading type II collagen, are induced in response to IL-1 in cartilage explants. One explanation for this finding is that the proteases responsible for collagen degradation are not active in the early cultures. Reports that metalloproteinases, induced in response to IL-1 in explant cultures at the early timepoints, are present in the media as zymogens (16, 29, 32). However, in studies evaluating MMP-3 production in this system, we showed that, although only the zymogen was detected in the media, the active form could be detected within the cartilage at early time periods (33). Another potential explanation for the lack of MMP activity in the early phase of these cultures is that the active form of the enzymes are generated but that they are complexed to a matrix component or to an endogenous inhibitor such as one of the tissue inhibitor of metalloproteinases. Finally, these data are consistent with the hypothesis that aggrecan may be serving to protect collagen from proteolytic cleavage.

There has been much circumstantial evidence over the past

[2] M. D. Tortorella and E. C. Arner, unpublished observations.

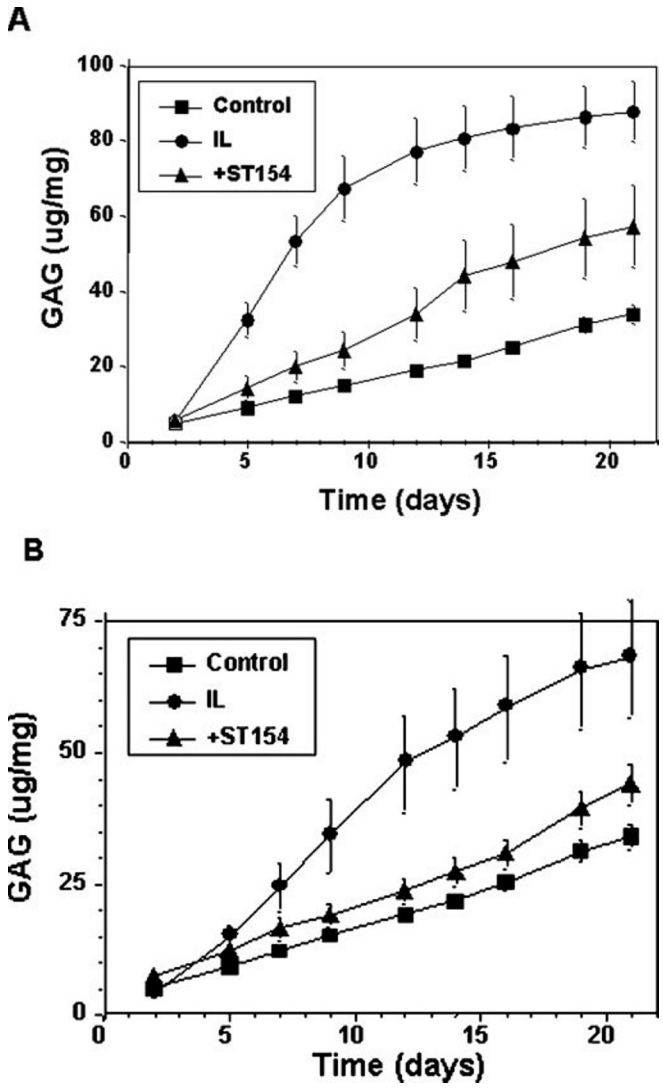

FIG. 7. **More potent selective aggrecanase inhibitor retains protection of aggrecan.** Bovine nasal cartilage was incubated in control media (*squares*), IL-1 (*circles*), or IL-1 plus the selective aggrecanase inhibitor, ST154 (*triangles*), and media were removed and replaced with fresh treatments every 2–3 days. Media were analyzed for aggrecan degradation products and cumulative glycosaminoglycan (*GAG*) released per mg wet weight cartilage are plotted *versus* time of incubation using an IL-1 stimulus of 5 ng/ml (*A*) or 2 ng/ml (*B*).

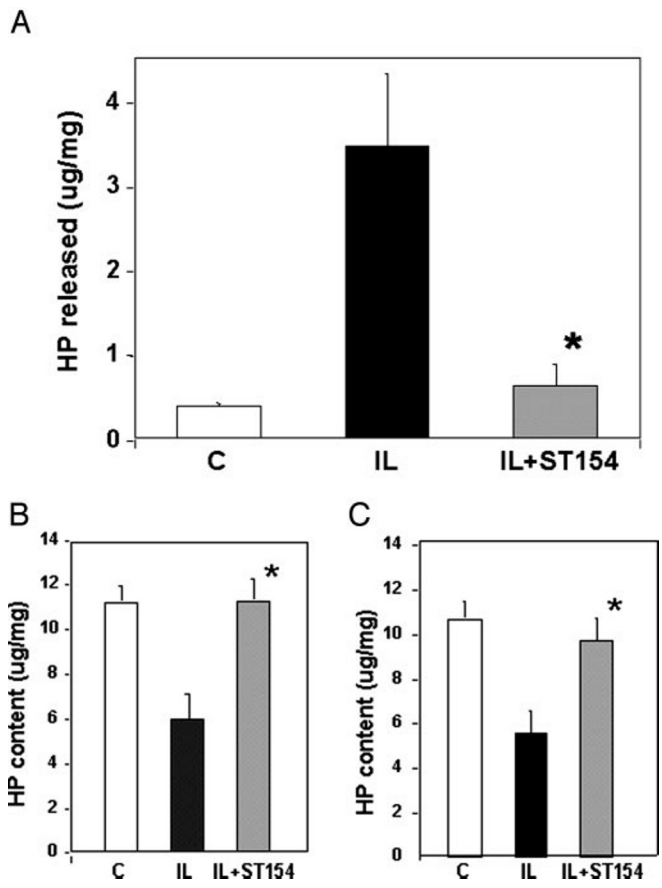

FIG. 8. **Prolonged aggrecan protection by potent, selective aggrecanase inhibitor extends time of inhibition of collagen degradation.** Media from the study shown in Fig. 7*A* were analyzed for collagen degradation products on day 21 (*A*). On day 23, papain digests of the cartilage explants were analyzed for hydroxyproline (*HP*) content as a measure of total collagen content (*B*) or native collagen content by first incubating with chymotrypsin to release any cleaved/denatured collagen prior to papain digestion (*C*). *, significantly different from IL-1 alone at $p < 0.05$.

10 years suggesting that aggrecan may play a protective role in preventing collagen loss from cartilage. Histopathological evaluations of osteoarthritic cartilage have consistently shown loss of aggrecan in regions of the cartilage where a decrease in collagen is detected (6, 34), and loss of aggrecan staining has been detected in regions of cartilage showing no detectable collagen loss, consistent with the hypothesis that aggrecan loss may precede collagen degradation. Several cartilage explant studies (8, 29, 30), including the present work, have illustrated that there is no dramatic loss of collagen until the majority of the aggrecan has been lost from the cartilage. Although there have been a number of hypotheses as to the reason for this sequence of events, no conclusive data have been generated.

We have now shown that the preservation of aggrecan prevents proteolytic cleavage of cartilage type II collagen. However, the mechanism by which aggrecan protects collagen from enzymatic degradation remains to be elucidated. One possibility is that aggrecan may protect collagen from degradation by providing an alternative substrate for proteases involved with collagen degradation. Although several of the MMPs have been

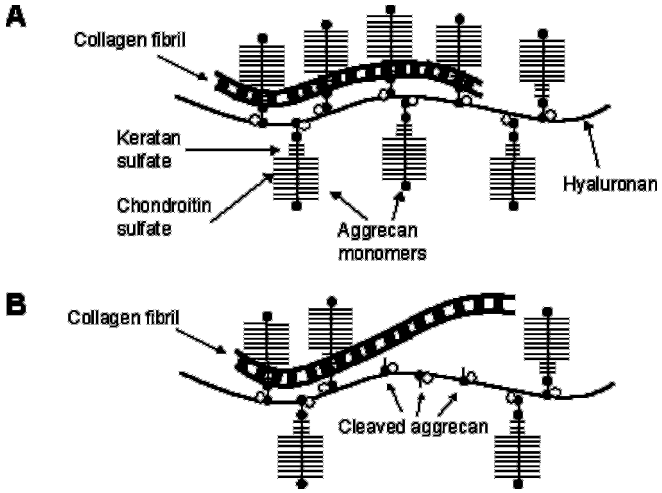

FIG. 9. **Proposed mechanism of collagen protection by preservation of aggrecan.** Interaction of collagen fibrils with the KS-rich region of multiple aggrecan molecules would position the collagen fibril in such a manner that it would be protected by the highly sulfated CS-rich regions of aggrecan preventing access to the fibril (*A*). Cleavage of the aggrecan within the interglobular domain between G1 and G2 releases the protective C-terminal portion of the aggrecan monomers, thus exposing the type II collagen fibril for cleavage by collagenases (*B*).

shown to be capable of cleaving aggrecan, they are much less efficient in cleaving this substrate than in cleaving collagen as well as several logs less potent than aggrecanases in cleaving aggrecan. These data, together with the lack of detection of MMP-generated aggrecan neoepitopes either in the media or cartilage in this or similar explant systems (15, 17, 25, 26, 32, 35), suggest that this is not a viable explanation. Alternatively, the aggrecan molecules may physically protect the type II collagen by preventing access of the proteases. In normal cartilage these matrix molecules are so tightly associated that they require denaturing solvents for extraction, and histochemical studies have indicated a close spatial relationship between proteoglycans and collagen in articular cartilage (36) as well as in other tissues (37, 38). More recent studies from Hedlund *et al.* (9) demonstrate that the KS-rich region of aggrecan binds type II collagen, and that the aggrecan KS-rich region was preferentially localized within the proximity of collagen fibrils of bovine articular cartilage. The data from these studies strongly suggest that located within the aggregate, the collagen fibrils interact with proteoglycan aggregates near the central hyaluronan core. This type of interaction via the KS-rich region of multiple aggrecan molecules would position the collagen fibril in such a manner that it may be protected by the highly sulfated CS-rich regions of the aggrecan preventing access to the fibril (Fig. 9*A*). If this is the case, then cleavage of the aggrecan within the interglobular domain between G1 and G2 by the aggrecanases would release the protective C-terminal portion of the aggrecan monomers, thus exposing the type II collagen fibril (Fig. 9*B*).

In conclusion, our studies indicate that cartilage aggrecan plays a protective role in preventing degradation of type II collagen fibrils, and suggests that this is due to interference with the ability of collagenases to cleave the collagen fibrils *in situ* within the cartilage matrix. In addition, the data provided herein suggest that a selective aggrecanase inhibitor will impart overall cartilage protection by inhibiting both aggrecan and collagen breakdown.

Acknowledgments—We thank Drs. Thomas Schmid, Ada Cole, and Klaus Kuettner for their help and guidance in setting up the hydroxyproline assay. We also thank Liana Bauerle, Diane Lombardo, Julian Austin, and John Giannaras for expert technical assistance.

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