Differential Expression of Aggrecanase and Matrix Metalloproteinase Activity in Chondrocytes Isolated from Bovine and Porcine Articular Cartilage*

(Received for publication, January 26, 1998, and in revised form, August 21, 1998)

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The release of aggrecan catabolites from cartilage is an early event in the pathogenesis of degenerative joint diseases. The enzymes involved in this process are unknown, controversial, and the subject of intense investigation. In this paper we have utilized a recombinant substrate containing the interglobular domain (IGD) of aggrecan to study specifically aggrecanase versus matrix metalloproteinase (MMP) catabolism in this domain of aggrecan. Our studies have shown that (i) there are species differences in the expression of latent versus active MMP activity on the aggrecan IGD; (ii) interleukin-1α exposure induces both aggrecanase and MMP activities, whereas retinoic acid induces only aggrecanase activity and inhibits the MMP activity on the aggrecan IGD; (iii) activators of latent MMP activity (p-aminophenylmercuric acetate and trypsin) significantly reduce aggrecanase activity; (iv) the time course of the appearance of aggrecanase versus the MMP catabolism of aggrecan IGD differs; (v) aggrecanase is a proenzyme with metalloprotease characteristics; however (vi) the physiological (tissue) inhibitors of MMPs show weak inhibition (TIMP-1) or no inhibition (TIMP-2) of aggrecanase activity. Collectively, these studies show that aggrecanase and MMP catabolism of the aggrecan IGD are independent and uncoupled.

The loss of aggrecan from the articular cartilage matrix is one of the earliest pathophysiological hallmarks of diseases such as osteoarthritis and rheumatoid arthritis. The depletion of this macromolecule from articular cartilage is thought to involve the potential cleavage of the core protein at two major sites within the interglobular domain (IGD) of the molecule (1–6). Additional cleavages also take place within the COOH-terminal domains of the molecule (1–3). The two predominant catabolic sites within the IGD of the molecule have been well documented as occurring between amino acid residues Asn1341 and Phe1342, and Glu373 and Ala374, respectively (1, 4, 7). The former cleavage site is thought to be generated by one of the known matrix metalloproteinases (MMPs 1, 2, 3, 7, 8, 9, 10, and 13), whereas the latter cleavage site is generated by an unknown enzyme activity, termed “aggrecanase” (8). Two recent studies have reported that MMP-8 (9) and membrane type 1 MMP (10) can cleave the aggrecan IGD at both of these cleavage sites, suggesting that these enzymes may account for some of the aggrecanase activity observed in vivo.

In several studies, using in vitro culture of articular cartilage explants or chondrocytes in the presence or absence of known catabolic agents such as retinoic acid (RA) or interleukin-1 (IL-1), the major proteoglycan degradation products released from the tissue or cell cultures have been shown to contain the new NH2 terminus 374ARGSVI... generated by the enzyme aggrecanase (2, 11, 12). However, in a single experimental system the NH2 terminus 342FFGVG has also been detected (13). In addition, analysis of synovial fluid from patients diagnosed with a variety of arthritic conditions has also indicated that the major product detectable by amino acid sequence analysis has contained the amino terminus 374ARGSVI... suggesting aggrecanase as the predominant activity accounting for aggrecan release from cartilage (7, 14). However, more recent analysis of synovial fluids using enzyme-linked immunosorbent assay techniques has also shown the presence of proteoglycan fragments that have the NH2 terminus 342FFGVG..., which suggests that MMPs are also involved in aggrecan catabolism (5, 15). Recent studies using polyclonal neoepitope antibodies to the COOH-terminal epitopes generated after cleavage of aggrecan by either the MMPs or aggrecanase have shown that in tissue sections from human cartilage both products are present (6). These studies have implied that the two catabolic pathways (aggrecanase or MMPs) are independent of each other. However, as this analysis represents fragments that could have accumulated in the tissue over many years, the results do not give an indication of the initial or current enzyme activity responsible for aggrecan catabolism.

In recent work (16) we have produced and characterized an artificial substrate (rAgg1) containing the IGD of human aggrecan, which can be cleaved at either the MMP site or the aggrecanase site (16). In this previous work we demonstrated the ability of this artificial substrate to be cleaved at the aggrecanase site by conditioned medium from RA-treated rat and bovine chondrocyte agarose culture medium. The cleavage of the artificial substrate was clearly distinguishable from endogenous aggrecanase-generated aggrecan catabolites (16). Thus at the time of analysis, cleavage of this artificial substrate at a specific site detected by a neoepitope monoclonal antibody (BC-3) indicates aggrecanase activity in conditioned medium.
In this present study we have used a modification of this artificial substrate (rAgg1mut) in which the alternate splice variant reported previously (16) was removed to produce a homogeneous substrate still containing the complete human IGD sequence (10). This modified substrate, in conjunction with neoepitope antibodies to the two major cleavage sites in the IGD of aggrecan, has been used to investigate the differential expression of MMP activity and aggrecanase activity in two different animal species (porcine and bovine). In addition, we have used this experimental system to characterize and compare the inhibitory profile of aggrecanase with that of the MMP(s) that are present or active in these culture systems. These studies for the first time clearly demonstrate the uncoupled activity of aggrecanase and MMPs in aggrecan catabolism.

**EXPERIMENTAL PROCEDURES**

**Materials—**Alkaline phosphatase-conjugated second antibody and substrate used in Western blot analysis were obtained from Promega as the Protoblot Western blot AP system (catalog no. W3920). Nitrocellulose (0.2-μm pore size) was obtained from Schleicher and Schuell. Recombinant human IL-1α was obtained from Sigma. Monoclonal antibodies BC-3 and BC-14 were prepared as ascitic fluid (11). TIMP-1 and TIMP-2 were kindly provided by Dr. T. Cawston, University of Newcastle. rAgg1mut was prepared at Hoechst Marion Roussel (10). Collagenase type II prepared from *Clostridium histolyticum* and Dulbecco’s modified Eagle’s medium (DMEM) with 4,500 mg/ml glucose were obtained from Life Technologies, Inc. All-trans-RA, trypsin (tosyl-phenylalanil chloromethyl ketone-treated type XIII from bovine pancreas), and actinonin were obtained from Sigma.

**Culture of Bovine or Porcine Chondrocytes in Agarose in the Presence or Absence of RA or IL-1α—**Bovine or porcine articular chondrocytes were isolated from the metacarpal- and metatarsophalangeal joints of 10-day-old calves, 1–3-year-old cows, or 3–6-month-old pigs using established procedures (17, 18). Briefly, cartilage was removed from the joint line using sterile conditions. The tissue was then digested in 0.1% Pronase in DMEM containing 5% fetal bovine serum for 1 h at 37 °C with agitation, washed, and further digested with 0.04% collagenase in DMEM containing 5% fetal bovine serum overnight at 37 °C with agitation. Cells were filtered through a 40-μm Nitex filter and washed before cell numbers were established. Cells were resuspended at 12 × 10^6 cells/ml in DMEM, mixed 1:1 with a 2% (w/v) solution of Seaplaque agarose in DMEM, and plated into 60-mm dishes precoated with neoepitope antibodies to the two major cleavage sites in the IGD of aggrecan for 24 h at 37 °C. To assess better the unbalanced expression of MMP activity and aggrecanase activity in these culture systems, conditioned medium from porcine chondrocyte cultures treated with IL-1α was incubated with a range of specific metalloproteinase inhibitors over the dose ranges indicated: 1,10-phenanthroline dissolved in Me2SO (dose range 2–10 mM), EDTA dissolved in DMEM (10–0.1 mM), actinonin dissolved in distilled water (500–1 μM), TIMP-1 dissolved in DMEM (255 μM), and TIMP-2 dissolved in DMEM (285 μM). As indicated earlier the highest final concentration of Me2SO in any of the digest was 5% v/v. Chemical inhibitors were preincubated as described above, and TIMPs were preincubated with the medium for 2 h before the addition of rAgg1mut to facilitate maximal binding of the physiological inhibitors. Samples were then incubated for a further 24 h with the substrate at 37 °C before processing as described previously. Western blots were analyzed desitometrically and the percentage inhibition calculated from the standard curves described above. Quantitative data from two separate experiments were analyzed for differences in aggrecanase activity (BC-3 immunoreactivity) and MMP (BC-14 immunoreactivity) inhibition by specific inhibitors using a two-factor analysis of variance.

**RESULTS**

**Immunohistochemical Analysis of rAgg1mut Digestion Products Generated from Bovine or Porcine Chondrocyte Agarose Culture Medium Treated with and without RA or IL-1α—**Western blot analysis (Fig. 1) with monoclonal antibody BC-3 (recognizing the new NH2-terminal ARGSV1 . . . generated by aggrecanase) showed positive staining in the RA- and IL-1α-treated bovine and porcine cultures (Fig. 1A, lanes 2 and 5 (RA) and lanes 3 and 6 (IL-1α)). No staining was seen in the control cultures from either animal species (Fig. 1A, lanes 1 and 4). Western blot analysis of conditioned medium obtained from bovine chondrocytes with monoclonal antibody BC-3 showed no evidence of MMP activity (Fig. 1B, lanes 1–3). In contrast, similar analysis on porcine cultures showed BC-14 positive staining indicating the presence of MMP activity (Fig. 1B, lanes 4 and 6).

To ascertain whether or not there was any latent MMP activity, conditioned medium from porcine chondrocyte cultures treated with IL-1α was incubated with a range of specific metalloproteinase inhibitors over the dose ranges indicated: 1,10-phenanthroline dissolved in Me2SO (dose range 2–10 mM), EDTA dissolved in DMEM (10–0.1 mM), actinonin dissolved in distilled water (500–1 μM), TIMP-1 dissolved in DMEM (255 μM), and TIMP-2 dissolved in DMEM (285 μM). As indicated earlier the highest final concentration of Me2SO in any of the digest was 5% v/v. Chemical inhibitors were preincubated as described above, and TIMPs were preincubated with the medium for 2 h before the addition of rAgg1mut to facilitate maximal binding of the physiological inhibitors. Samples were then incubated for a further 24 h with the substrate at 37 °C before processing as described previously. Western blots were analyzed desitometrically and the percentage inhibition calculated from the standard curves described above. Quantitative data from two separate experiments were analyzed for differences in aggrecanase activity (BC-3 immunoreactivity) and MMP (BC-14 immunoreactivity) inhibition by specific inhibitors using a two-factor analysis of variance.
activity present in the bovine control or RA-conditioned medium, the medium from these cultures was preincubated with APMA before the addition of rAgg1mut substrate. Subsequent immunoblotting of rAgg1mut digestion products with monoclonal antibody BC-14 is shown in Fig. 2A. This analysis showed that the addition of APMA to control medium resulted in the activation of latent MMPs in the bovine control medium (lane 2) but not in the RA-conditioned medium (lane 4). Similar analyses performed on control and RA medium from porcine chondrocyte cultures (lanes 5–8) showed that control medium from porcine chondrocyte cultures contained additional latent MMP activity that was activated by APMA (lanes 5 and 6). In contrast, the RA-treated cultures showed no evidence of latent MMP activity (Fig. 2, lanes 7 and 8) similar to that observed in bovine chondrocyte cultures.

The effect of APMA on aggrecanase activity was also assessed by BC-3 Western blot analysis of rAgg1mut fragments in conditioned medium from control porcine chondrocyte cultures (Fig. 2B). In this conditioned medium aggrecanase activity was not activated in the presence of APMA (lanes 1 and 2). However, as there was increased MMP activity in APMA-activated control conditioned medium (Fig. 2A), the lack of aggrecanase activity could have been caused by limiting substrate conditions with the active MMP present. Therefore, we also examined the effect of APMA on aggrecanase activity in the RA-conditioned medium from porcine cultures that had no MMP activity. In this RA-conditioned medium (Fig. 2B, lane 3) aggrecanase activity was decreased in the presence of APMA (Fig. 2B, lane 4). In a dose-response experiment, aggrecanase activity in RA-conditioned medium was not inhibited by 0.1 mM APMA but was inhibited by 0.5 mM APMA (results not shown). In addition, preincubation of conditioned medium with trypsin (80 nM for 1 h) resulted in a loss of aggrecanase activity but an increase in MMP activity in IL-1α-conditioned medium (results not shown).

**Time Course of the Appearance of BC-3 and BC-14 Epitope Generated by the Digestion of rAgg1mut with IL-1α-conditioned Medium From Porcine Chondrocyte Cultures**—The results for the time course of the appearance of both aggrecanase and MMP activity were reproducible in three separate experimental cultures. A representative Western blot of these experiments is shown in Fig. 3. At all time points assayed there were no detectable BC-3-positive rAgg1mut digestion products in the control cultures (Fig. 3A). However, BC-3-positive rAgg1mut fragments were detectable in IL-1α-treated cultures as early as 24 h after exposure to this cytokine (Fig. 3A). From 24 to 96 h in culture there was an apparent increase in the amount of BC-3-positive rAgg1mut fragments generated by IL-1α-conditioned medium. These results demonstrate that aggrecanase activity is present only in IL-1α-conditioned medium and that this activity increases with time in culture, being maximal at 96 h.

In contrast, BC-14-positive rAgg1mut fragments were generated by control conditioned medium by 24 h of culture (Fig. 3B). This MMP activity in control conditioned medium decreased significantly between 24 and 72 h but then appeared to increase again at 120 h. The MMP activity in IL-1α-conditioned medium from 24- and 48-h cultures was similar to the control medium. However, after 72 h there was a significant increase in MMP activity above that of control cultures.

**Inhibition of Aggrecanase Activity in Conditioned Medium Obtained from Bovine Chondrocyte Agarose Cultures Stimulated with RA for 120 Hours**—The Western blot analysis of the inhibitory profile of bovine aggrecanase is shown in Fig. 4.
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**FIG. 3.** Time course of generation of BC-3 and BC-14 positive rAgg1mut digestion products using porcine chondrocyte cultures. Conditioned medium from a time course experiment (0–120 h) using porcine chondrocytes treated with or without IL-1α was incubated with rAgg1mut substrate and subjected to Western blotting. Panel A, aggrecanase-generated rAgg1mut fragments were visualized using monoclonal antibody BC-3; panel B, MMP-generated rAgg1mut fragments were visualized with monoclonal antibody BC-14.

**FIG. 4.** Inhibition of aggrecanase activity in cell-free conditioned medium from bovine chondrocytes exposed to RA. Western blot analysis using antibody BC-3 of rAgg1mut fragments produced after preincubation with no inhibitor (lane 1), 1,10-phenanthroline (lane 2), EDTA (lane 3), 1,10-phenanthroline and EDTA (lane 4), dichloroisoucumarin (lane 5), E64 (lane 6), and benzamidine (lane 7).

This shows aggrecanase-generated rAgg1mut digestion products with BC-3 epitope from RA-conditioned medium, which was used for comparison of enzyme activity with each inhibitor tested. Inhibition of aggrecanase activity was only achieved after preincubation of RA-conditioned medium with 1,10-phenanthroline alone, EDTA alone, or a combination of the two inhibitors mentioned earlier (Fig. 4, lanes 2–4). No inhibition was apparent with preincubation of the conditioned medium with the serine protease inhibitor 3,4-dichloroisoucumarin (Fig. 4, lane 5), the thiol protease inhibitor E64 (Fig. 4, lane 6), or the serine protease inhibitor benzamidine (Fig. 4, lane 7). In addition, aggrecanase activity in the conditioned medium was stable to freeze/thawing (results not shown). These data indicate that aggrecanase belongs to the metalloproteinase family of proteases.

**FIG. 5.** Determination of the limiting dilutions of aggrecanase activity (detected by antibody BC-3) and MMP activity (detected by antibody BC-14) in conditioned medium from porcine chondrocytes cultured in the presence of IL-1α. Panel A, Western blot analyses with monoclonal antibodies BC-3 and BC-14. Panel B, quantification using densitometric scanning of Western blots shown in panel A (n = 2). The limiting dilutions of conditioned media incubated with rAgg1mut substrate were equivalent to 250 μl (lanes 1 and 7), 125 μl (lanes 2 and 8), 62.5 μl (lanes 3 and 9), 31.25 μl (lanes 4 and 10), 15.6 μl (lanes 5 and 11), and 7.8 μl (lanes 6 and 12).

### Comparison of Aggrecanase and MMP Inhibition in IL-1α-Conditioned Medium from Porcine Chondrocyte Agarose Cultures by Specific Metalloprotease Inhibitors—Similarities and Differences in MMP and aggrecanase activities in the presence of specific metalloprotease inhibitors were tested by comparison of inhibition profiles generated by the catabolism of rAgg1mut using porcine conditioned medium from two separate IL-1α-treated culture experiments. Western blot analysis with antibody BC-3 (Fig. 5A, lanes 1–6) indicated a decrease in staining intensity detectable between 250 and 7.8 μl of medium. In contrast, Western blot analyses of medium with antibody BC-14 (Fig. 5A, lanes 7–12) indicated that MMP-generated fragments of rAgg1mut showed a detectable decrease in staining intensity between 125 and 7.8 μl of the medium digest. A separate standard curve of densitometric peak height versus dilution was generated for the two antibodies (Fig. 5B).

Conditioned medium was incubated with decreasing quantities of the chemically derived metalloproteinase inhibitors 1,10-phenanthroline and EDTA, the bacterially derived metalloproteinase inhibitor actinonin, and the physiological MMP inhibitors TIMP-1 and TIMP-2 to determine subtle differences in inhibition of aggrecanase versus MMP activities in our culture systems (Fig. 6). The percentage inhibition by each dose of these inhibitors as calculated from the densitometric standard curve (Fig. 5B) is shown in Table I. The results presented in Table I represent the average value from two separate inhibition experiments.

These analyses clearly demonstrated a significant dose-dependent inhibition of both aggrecanase and MMP activities by 1,10-phenanthroline and EDTA, and actinonin (p = < 0.001 for all analyses). However, there was no significant difference in the dose-dependent inhibition between aggrecanase and MMP activities by 1,10-phenanthroline or EDTA (p > 0.05 for both inhibitors). In contrast, inhibition of aggrecanase and MMP activities by actinonin showed a significant difference (p < 0.0001). Aggrecanase was significantly inhibited at all doses of actinonin; however, MMP activity was only significantly inhibited at the highest dose (500 μM) of actinonin.

There was a significant difference between the percentage inhibition of MMP and aggrecanase activities by both TIMP-1 and TIMP-2 (p = 0.048 and 0.0004, respectively). MMP activity was more than 90% inhibited by preincubation of the conditioned medium with 255 nM (7.5 μg/ml) TIMP-1 and 285 nM (10 μg/ml) TIMP-2. In contrast, preincubation with TIMP-1 at the same concentration only partially inhibited aggrecanase activ-
In contrast, porcine chondrocyte cultures showed evidence for MMP cleavage of the IGD substrate at the Asn341-Phe342 cleavage site. In contrast, porcine chondrocyte cultures showed evidence for cleavage at both the aggrecanase and MMP cleavage sites in the IGD substrate. Collectively, these results indicate that aggrecanase and MMP cleavage of the IGD substrate can occur alone or simultaneously.

Interestingly, MMP activity was present in porcine but not bovine control cultures. Furthermore, this MMP activity was increased in medium, only in cultures exposed to IL-1α but not those treated with RA. This result contrasts that seen for aggrecanase where both IL-1α and RA induced its activity. Collectively, these results indicate that induction of aggrecan catabolism by IL-1 and RA use different mechanisms. In addition, our results have shown that in bovine culture systems, MMPs are secreted as latent enzymes that can be activated by exposure to APMA or trypsin. In porcine culture medium the addition of APMA further increased the levels of MMP activity which were present. Importantly, for both bovine and porcine cultures, the addition of APMA to RA-conditioned medium showed no evidence for the presence of latent MMP activity on the IGD substrate. Collectively, these data substantiate further that MMP and aggrecanase activity are induced differentially and that exposure to RA specifically decreases IGD-degrading MMP activity. Furthermore, aggrecanase activity appears to be destroyed by conditioned media being exposed to APMA.

The uncoupling of aggrecanase and MMP activities on the catabolism of the IGD of aggrecan has been recently suggested (5, 6). Lark et al. (6) have proposed three possible mechanisms for catabolism of aggrecan within the IGD. They are: (i) primary cleavage at the aggrecanase site (Glu377-Ala378) followed by secondary cleavage of the remaining G1 aggrecan component at the MMP cleavage site (Asn341-Phe342); (ii) primary cleavage at the MMP site followed by a secondary cleavage at the aggrecanase site; and (iii) independent (uncoupled) cleavage at the two sites. Data from studies by Fosang et al. (9) of recombinant MMP-8 cleavage of aggrecan G1-G2 at the aggrecanase site supported mechanism (i) described above. However, more recently, conclusions from analyses by Fosang et al. (5) of aggrecan catabolites in synovial fluid from patients with arthritis support mechanism (iii) which involves independent

| Inhibitor     | Aggrecanase inhibition | MMP Inhibition |
|---------------|------------------------|---------------|
| 1,10-Phenanthrine | 100%                   | 97.5%         |
| 2.0 mM        | 100%                   | 96.5%         |
| 1.0 mM        | 95.5%                  | 95%           |
| 0.5 mM        | 73.5%                  | 80%           |
| 0.1 mM        | 40%                    | 68%           |
| EDTA          | 100%                   | 100%          |
| 10 mM         | 100%                   | 100%          |
| 5 mM          | 100%                   | 100%          |
| 1 mM          | 81.5%                  | 78%           |
| 0.1 mM        | 56%                    | 65%           |
| Actinonin     | 100%                   | 73.5%         |
| 500 μM        | 100%                   | 72.5%         |
| 100 μM        | 100%                   | 22.5%         |
| 10 μM         | 78.5%                  | 0%            |
| 1 μM          | 61.5%                  | 0%            |
| TIMP-1 255 nM | 41%                    | 92.5%         |
| TIMP-2 285 nM | 0%                     | 98%           |
cleavage by both classes of proteases. Our results conclusively demonstrate the independent cleavage of the IGD of aggrecan at these two cleavage sites. We presume that similar cleavage mechanisms are occurring in the IGD of native aggrecan.

This study is the first to report that exposure of chondrocytes to RA abolishes MMP catabolism of aggrecan in the IGD. This finding is consistent with the published down-regulation of MMP-1 activity in synoviocytes exposed to RA (22). We have also shown that soluble aggrecanase in conditioned medium does not occur in a latent form that is activatable by exposure to APMA or trypsin. Indeed, aggrecanase activity appears to be destroyed by APMA exposure similar to that described for membrane type 2 MMP but not other members of the MMP family (23). This study has not addressed the potential of other physiological mechanisms for aggrecanase activation e.g. plasmin, cathepsins (24–26).

Our analyses of the time course of proteolytic cleavage of the aggrecan IGD substrate also support the uncoupling of aggrecanase versus MMP activities. IL-1α induction of aggrecanase activity in porcine chondrocyte cultures occurs in a time-dependent fashion up to 96 h, which is similar to our previous reports for RA induction of aggrecanase in similar culture systems using bovine chondrocytes (16). In contrast, high levels of MMP activity were observed in both control and IL-1α-treated porcine cultures at 24 h (Fig. 3B). This activity then decreased at 48 h but increased with further time in culture, the increase being greatest in the IL-1α-exposed cultures. We considered the possibility that this MMP activity seen in the porcine cultures was caused by contamination from the collagenase used in the methods for cell isolation, but this activity was not present in bovine cultures prepared in the same manner. The decrease in MMP activity observed from 24 h to 48–72 h of culture may be associated with increased TIMP production by the chondrocytes over this time period.

Our observation of differences in the presence of aggrecanase activity alone in bovine culture systems versus both aggrecanase and MMP activities in porcine culture systems facilitated our studies of the effects of inhibitors of proteinases on these different activities. Aggrecanase activity in bovine culture systems was only inhibited by metal-chelating chemicals, indicating that it is a member of the metalloproteinase family similar to that concluded by several laboratories (27–30). Our studies utilizing the porcine culture system allowed us to study the effect of four different proteinase inhibitors on both aggrecanase and MMP activities (Fig. 6 and Table I). Statistically significant differences found in the inhibition profiles with actinonin suggest subtle differences in the active sites of the aggrecanase- versus MMP-related activities. Although the physiological inhibitors TIMP-1 and TIMP-2 show differences in their inhibition of aggrecanase, they both completely inhibit the MMP activity. As reported previously, TIMP-1 at a very high concentration (250 nM) partially inhibits aggrecanase activity, and this result led to the speculation that aggrecanase was a novel MMP (30). However, our observation showing a lack of inhibition of aggrecanase by TIMP-2 suggests that aggrecanase may be a member of another metalloproteinase family. The high concentration of TIMP-1 needed to inhibit aggrecanase activity suggests that this natural polypeptide is not an inhibitor of aggrecanase in vivo.

The possibility that the rAgg1mut substrate is cleaved simultaneously by both aggrecanase and MMPs was considered unlikely because such a mechanism of cleavage would result in BC-3 detection only, as the small fragment (32 amino acids) carrying the BC-14 epitope would be lost in dialysis. Furthermore, inhibition of MMP cleavage by TIMP-2 does not result in increased BC-3 positive staining, indicating that dual cleavage of even a portion of the rAgg1mut substrate is not occurring. Cleavage of the rAgg1mut substrate by other classes of proteinases which could result in interference with the detection of BC-3 and BC-14 reactive fragments is also unlikely although not ruled out in every experiment. Cleavage of the rAgg1mut substrate by other proteinases may be expected either to alter the size of BC-3 or BC-14 positive fragments or to change staining intensity. However, there was no alteration in BC-3 positive staining intensity or the size of the fragments in the presence of serine and cysteine proteinase inhibitors when compared with conditioned medium alone (Fig. 3). Similar results were obtained for BC-14 immunoreactivity using porcine conditioned medium (results not shown).

In summary, our data now clearly establish that catabolism of aggrecan in the IGD of the protein core by aggrecanase or MMPs occurs by two independent mechanisms as suggested recently by in vivo analyses by Lark et al. (6) and Fosang et al. (5). Recently, Tortorella et al. (31) demonstrated that cleavage of the core protein of aggrecan at the aggrecanase site by the reprotoxin atrorlysin C did not require prior cleavage at the MMP site. However this non-mammalian enzyme, like the two MMP enzymes MMP-8 and membrane type 1 MMP (9, 10), catalyzes cleavage of the aggrecan IGD at both Asn341-Phe342 and Glu373-Ala374. Thus no enzyme has yet been identified which cleaves only at the aggrecanase site. Our data of uncoupled and independent aggrecanase and MMP activities indicate that any enzyme that cleaves at both the aggrecanase and MMP sites cannot account for the aggrecanase activity studied in our experimental systems.

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