Analysis of ADAMTS4 and MT4-MMP indicates that both are involved in aggrecanolysis in interleukin-1-treated bovine cartilage

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Summary

Objective: To investigate the mechanism of aggrecanolysis in interleukin-1 (IL-1)-treated cartilage tissue by examining the time course of aggrecan cleavages and the tissue and membrane content of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin type I motifs (ADAMTS4).

Methods: Articular cartilage explants were harvested from newborn bovine femoropatellar groove. The effects of IL-1 treatment with or without aggrecanase blockade were investigated by Western analysis of aggrecan fragment generation, ADAMTS4 species (p68 and p53), and MT4-MMP, as well as by realtime PCR (polymerase chain reaction) for ADAMTS4 and 5. Aggrecanase was blocked with mannosamine (ManN), an inhibitor of glycosylphosphatidylinositol anchor synthesis, and esculetin (EST), an inhibitor of MMP-1, MMP-3, and MMP-13 gene expression.

Results: IL-1 treatment caused a marked increase in MT4-MMP abundance in the tissue and medium. ADAMTS4 (p68) was abundant in fresh cartilage and this was retained in the tissue in untreated cartilage. IL-1 treatment for 6 days caused a marked loss of p68 from the cartilage and the appearance of p53 in the medium. Addition of either 1.35 mM ManN or 31–500 μM EST blocked IL-1-mediated aggrecanase cleavage and this was accompanied by nearly complete inhibition of the MT4-MMP increase, the p68 loss and the formation of p53. IL-1 treatment increased mRNA abundance for ADAMTS4 (~3-fold) and ADAMTS5 (~10-fold) but this was not accompanied by a marked change in enzyme protein abundance.

Conclusion: These studies support a central role for MT4-MMP in IL-1-induced cartilage aggrecanolysis and are consistent with the identification of p68 as the aggrecanase that cleaves within the CS2 domain, and of p53 as the aggrecanase that generates G1-NITEGE. Since the induction by IL-1 was not accompanied by marked changes in total ADAMTS4 protein, but rather in partial conversion of p68 to p53 and release of both from the tissue, we conclude that aggrecanolysis in this model system results from MT4-MMP-mediated processing of a resident pool of ADAMTS4 and release of the p68 and p53 from their normal association with the cell surface.

Key words: Aggrecan degradation, ADAMTS4, MT4-MMP, Mannosamine, Esculetin.

Introduction

Enhanced release of proteoglycans from the articular cartilage to the synovial fluid is a prominent feature of early and late osteoarthritis. In particular, aggrecan is the most abundant proteoglycan in cartilage tissue and provides much of the tissue’s compressive strength through electrostatic repulsion of its tightly packed glycosaminoglycan (GAG) chains. Excess degradation of aggrecan in the cartilage tissue thus contributes directly to the failure of the mechanical function of the joint.

Investigation of enzymatic degradation of aggrecan has led to the identification of specific cleavage sites generated by enzymes termed aggrecanases, which have since been identified as a sub-group (ADAMTS1,4,5,8,9,15) of the 19-member family of ADAMTS (a disintegrin and metalloproteinase with thrombospondin type I motifs) proteinases. It is now clear that degradation of aggrecan induced by major inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor is mediated by the action of the aggrecanases and not the matrix metalloproteinases (MMPs). In addition, the evidence for aggrecanase-mediated cleavage of aggrecan in cartilage tissue and synovial fluid from patients with osteoarthritis suggests that aggrecanase activity is predominantly or maybe exclusively responsible for the excess degradation of cartilage aggrecan which is a feature of human osteoarthritis.

Recently, the steps involved in processing of ADAMTS4 (aggrecanase-1) have been described in a human chondrosarcoma cell line stably transfected with ADAMTS4, and...
the aggregcanase activities of the processed forms characterized. To exhibit any aggregcanase activity, the ADAMTS4 proenzyme first requires intracellular furin-mediated removal of the N-terminal pro-domain, which generates the p68 form. While this form can degrade the C-terminal chondroitin sulfate (CS)-bearing region, it appears that for "destructive" cleavage of aggregcan in the interglobular domain (IGD), further C-terminal truncation of ADAMTS4 is required, producing the p53 and p40 forms of the proteinase. We have previously hypothesized that this C-terminal truncation requires a glycosylphosphatidylinositol (GPI)-linked MMP, since IGD cleavage is blocked by inhibitors of GPI anchor synthesis and by MMP inhibitors. This was recently supported by the finding that transfection of ADAMTS4-expressing cells with membrane type 4-MMP (MT4-MMP) (a GPI-anchored MMP) promoted C-terminal processing of ADAMTS4 to the p53 and p40 forms and acquisition of IGD cleavage activity.

However, there has been no detailed description of the processing of ADAMTS4 in native cartilage tissue. Initial studies of the protein forms present in normal and IL-1-stimulated cartilage have shown evidence for ADAMTS4 forms in the ranges 30–60 kDa and 37–46 kDa, and the effect of IL-1 treatment on levels of MT4-MMP protein in cartilage has not been reported.

We therefore examined aggregcan degradation in newborn bovine cartilage explants treated with IL-1 by Western analysis of aggregcan, ADAMTS4 and MT4-MMP in the cartilage tissue and conditioned medium. In addition, we examined the effects of two aggregcanase blockers, ManN and esculetin (EST). We hypothesized that this C-terminal truncation requires a glycosylphosphatidylinositol (GPI)-linked MMP, since IGD cleavage is blocked by inhibitors of GPI anchor synthesis and by MMP inhibitors. This was recently supported by the finding that transfection of ADAMTS4-expressing cells with membrane type 4-MMP (MT4-MMP) (a GPI-anchored MMP) promoted C-terminal processing of ADAMTS4 to the p53 and p40 forms and acquisition of IGD cleavage activity.

The mechanism for aggregcanase inhibition by EST is not clear but it appears to inhibit MMP gene expression, and thus allows investigation of the effects of aggregcanase blockade through what is likely a different mechanism than ManN. Finally, to investigate regulation of aggregcanase activity at the level of transcription, we measured the effect of IL-1 and ManN treatment on mRNA expression of ADAMTS4 and ADAMTS5 by real-time PCR analysis.

### Methods

#### Materials

Human recombinant IL-1α was from R&D Systems, Inc., Minneapolis, MN. Unless indicated, other reagents were from Sigma Chemical Co. (St. Louis, MO).

#### Cartilage Explant and Culture

Articular cartilage disks were obtained from the femoropatellar groove of one- to two-week-old calves, a model system that has been widely studied to examine the biological response pathways relevant to cartilage degradation in human osteoarthritis, using methods similar to those described in detail previously. In brief, 9-mm-diameter cylinders of full-thickness cartilage and bone were cored from the articular cartilage. After slicing off sufficient superficial cartilage to create a flat surface (usually less than 500 μm), the next two sequential 0.5-mm-thick slices were cut with a sledge microtome. From each of these slices, four cartilage disks (3 mm in diameter and 0.5 mm in thickness) were cut out with a dental punch. Groups of cartilage disks were incubated at 37 °C in an atmosphere of 5% CO₂, in wells containing 0.25 ml/disk of a serum-free culture medium (low-glucose DMEM [Dulbecco’s modified Eagle’s medium] [Gibco, Grand Island, NY] and 10 mM HEPES buffer, with 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B). Experimental treatments with IL-1 and aggregcanase blockers were begun on the day of harvest. With the exception of the kinetic analysis, these cultures were incubated without medium replacement for up to 6 days. Each experiment had four to eight treatment groups, allowing disks from different groups to be matched for their original location along the surface of the joint.

#### Biochemical Analysis of Proteoglycans

Tissue and medium were analyzed for composition of aggregcan fragments by Western blotting as previously described. Aggregcan remaining in the cartilage tissue was extracted in 4 M guanidine (1.2 ml per 75 mg wet weight) for 48 h at 4 °C in the presence of protease inhibitors. Aggregcan fragments in tissue extracts and conditioned medium were isolated by ethanol precipitation and deglycosylated. Portions of medium and tissue extracts (corresponding to 10 μg of GAG in each case) were loaded on 4–12% polyacrylamide gels (Novex) for Western analysis and probed with polyclonal antisera to the bovine G1 domain (G1-2), the G3 domain (antibody LEC-7, provided by Dr Kurt Doege) and the C-terminal neoeptopes TFKEEE1706 (anti-serum provided by Merck), TAGELE1520 (antisera provided by DuPont), and NITEGE192 (antibody JSCNT). Tissue (digested overnight with proteinase K) and conditioned medium were assayed for sulfated GAG content by reaction with dimethylmethylene blue (DMMB) dye.

#### Western Analysis of ADAMTS4 (Aggregcanase-1) and MT4-MMP

Pilot studies were done to optimize the extraction conditions for ADAMTS4 in cartilage. Cartilage (15 mg wet weight) was freeze-milled in a Biopulverizer (Biospec Products, Bartlesville, OK) and the powder was extracted for 20 h at 4 °C in three volumes (μl/mg wet weight) of (1) 50 mM Tris–HCl, pH 7.0; (2) 50 mM Tris–HCl, 100 mM NaCl, pH 7.0; (3) 50 mM Tris–HCl, 100 mM NaCl, 0.5% Nonidet P-40, pH 7.0; or (4) 4 M guanidine, 50 mM Tris–HCl, pH 7.0. Macromolecules in the 4 M guanidine extract were purified by Sephadex G50 and DE 52 as previously described for aggregcan fragments and the unbound (protein) and bound (proteoglycan) fractions were analyzed. Other cartilage samples were treated at 100 °C for 10 min in 50 mM Tris–HCl and 2.5% SDS. The results of the comparative extraction experiments (not shown) indicated that the highest yield of immunoreactive product was consistently obtained with extractant (3). Poor recoveries were seen with 4 M guanidine extracts and boiling in SDS. Therefore, all data shown in the present paper were obtained using extraction with 0.5% Nonidet P-40, 50 mM Tris, and 100 mM NaCl at pH 7.0, and dilution of 5–10 μl of the extracts in gel-loading buffer before SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). Conditioned medium from cartilage explants was concentrated by lyophilization, dialyzed, dried and dissolved in gel-loading buffer.

Western analysis of ADAMTS4 was done with two affinity-purified antibodies that exhibit different reactivities for the molecular forms of ADAMTS4. JSCVMA was raised...
to a peptide (Val\(^{394}-\text{Pro}^{403}\)) in the catalytic domain, and it preferentially reacts with the p53 form in tissue extracts. JSCYNH was raised to a peptide (Tyr\(^{690}-\text{Pro}^{693}\)) within the Cys-rich region and it reacts preferentially with the p68 form. All soluble samples were loaded on an equivalent tissue weight basis and separate blots were probed with JSCYNH or JSCVMA. For Western analysis of MT4-MMP (MMP-17), blots were probed with antibodies to MT4-MMP from Sigma (catalog #M3684).

**RESULTS**

**KINETICS OF AGGREGAN DEGRADATION IN Calf EXPLANTS TREATED WITH IL-1**

In preliminary experiments to describe the kinetics of IL-1-induced aggregan degradation in the newborn bovine cartilage tissue, we obtained cartilage disks with a defined cylindrical geometry and treated them with 1, 10 and 100 ng/ml IL-1\(\alpha\). At 2, 4, and 6 days the cultures were terminated and the proportion of total GAG remaining in the tissue as well as the equilibrium and dynamic compressive moduli were measured. This analysis (data not shown) revealed that for explants cut to 0.5 mm in thickness, the addition of IL-1 at 10 ng/ml generated a gradual catabolic response which was most appropriate for kinetic analysis over 6 days.

We therefore treated cartilage explants with 10 ng/ml for 6 days and terminated cultures at eight different times (0, 0.5, 1, 1.5, 2, 2.5, 3, 4.5, and 6 days), with removal of medium and replacement with fresh medium and IL-1 at days 2 and 4.5. To examine the composition of aggregan remaining in the tissue at these times, we performed a Western analysis with anti-aggrecan G1 domain on the tissue extracts [Fig. 1(A)]. The composition of the control tissue (maintained in culture without IL-1 for 6 days) and fresh tissue (maintained for 0 days) was primarily core species 1 (full-length aggregan) with a low abundance of species a, b, c and d, which are not generated by aggreganase action (see Fig. 2 for peptide notations). Early in the IL-1 treatment period (days 1 and 1.5), species a and b appeared to be eliminated from the tissue, whereas from days 2–6 there was an accumulation in the tissue of species 5 (G1-TAGELE) at about 250 kDa. Western analysis of these samples with anti-TAGELE and anti-TFKEEE confirmed that the abundance of these aggreganase-generated species (at about 200–250 kDa) was increased during days 2–6, in parallel with the appearance of the G1-reactive band(s) in that region of the gel (data not shown). Interestingly, the N-terminal product G1-NITEGE (species 6 doublet) was not observed until day 4.5 of culture, consistent with the common finding that IL-1-promoted aggreganase-mediated aggregan degradation occurs in a stepwise fashion, with the C-terminal region being degraded before cleavage of the IGD\(^9,16,29\).

This interpretation was supported by Western analysis of the medium collected over various periods during this
culture with an antibody to the G3 domain [Fig. 1(b)]. The “ladder” of G3-reactive species labeled as 7, 8, 9, and 10 are characteristic of aggrecanase-mediated C-terminal processing during the early period (days 0–3), whereas the relative paucity of these products in medium collected after day 4.5 is consistent with aggrecanase cleavage now being targeted to the IGD. This was verified by Western analysis of the medium for the G1 domain, which showed nearly undetectable G1-NITEGE release until day 4.5, and no evidence for higher-molecular-weight G1-bearing fragments (data not shown), as observed in this system previously30. An unidentified G3-reactive fragment at about 44 kDa was a major medium product at all time periods, and the identifications are taken from Oshita et al.31 and references therein. Note that these residue numbers differ from previous reports31,29 by a constant offset of 19 to conform with genome-based numbering of the leader sequence. Peptides identified with the numbers 5–10 represent aggrecanase-generated aggrecan species.

Fig. 2. Schematic of the structures for bovine aggrecan core protein peptides. The diagram shows the currently accepted structures for aggrecan core protein peptides derived from bovine cartilage explants before and after treatment with IL-1 (see Fig. 1). The sequences and residue numbers shown are taken from data in Hering et al.29 for bovine aggrecan (accession number AAB38524), and the identifications are taken from Oshita et al.31 and references therein. Since it was clear that an active aggrecanase was present in this system, and since it has been suggested that ADAMTS4 (rather than TS-1, 5, 8, 9 or 15) is mostly responsible for this activity4,9,32-35, we analyzed both tissue and medium for ADAMTS4 in control cultures, IL-1-treated cultures, and IL-1-treated cultures with the addition of 1.35 mM ManN. Consistent with our previous findings29, in this experiment GAG released from the cartilage to the medium increased from 24 ± 2 µg/disk in controls to 115 ± 6 µg/disk in IL-1-treated cultures, and this aggrecanase-mediated degradation was blocked by the addition of ManN (40 ± 3 µg/disk; all N = 4 disks per group).

Extracts of untreated control tissue at days 0 and 6, probed with JSCYNH (Fig. 3, tissue), contained a single major immunoreactive protein which was identified as the p68 form. Since this antibody reacts with the proenzyme in cell culture experiments, the absence of immunoreactive proenzyme here suggests that either the proenzyme does not accumulate in the tissue or it is poorly reactive in these extracts, perhaps due to the presence of masking proteins. After 6 days of IL-1 treatment, the p68 form was essentially eliminated from the tissue, a process which was markedly blocked by the inclusion of ManN. Analysis of the medium showed that under control conditions, some of the p53 form (and an unidentified fragment of about 30 kDa) was released from the tissue, unlike the p68 form, which remained associated with the tissue. In marked contrast, abundant p68 and p53 were released into the medium after treatment with IL-1 for 6 days; however, the inclusion of ManN returned this process to control levels.

ANALYSIS OF EXPLANT SYSTEM FOR MT4-MMP PROTEIN

To examine the possibility that GPI-anchored MT4-MMP is involved in processing of ADAMTS4 in bovine cartilage, the tissue and conditioned medium from this experiment were further examined by Western analysis for MT4-MMP (MMP-17) protein (Fig. 4). In fresh tissue (day 0), a trace amount of MT4-MMP protein was detected, whereas in control tissue and medium at 6 days it was not detectable. In contrast, analysis of tissue treated with IL-1 showed a marked increase in MT4-MMP abundance at ~65 kDa (a size consistent with previous observations in cells and
This increase in protein abundance was largely reversed on addition of ManN. Similarly, analysis of the collected conditioned medium showed an increased abundance of MT4-MMP protein in IL-1-treated cultures that was blocked by inclusion of ManN. The nature of the minor immunoreactive product at 80 kDa is unknown.

EFFECT OF EST ON IL-1-INDUCED CHANGES

EST (dihydroxycoumarin) has previously been shown to inhibit cartilage degradation induced by the combination of IL-1 and oncostatin M, but the mechanism is not clear. We therefore performed a parallel set of experiments, first demonstrating inhibition of IL-1-induced aggrecanase activity by EST, and then testing whether EST also reversed IL-1-induced changes in ADAMTS4 and MT4-MMP protein. Cartilage explants were incubated in the presence or absence of 10 ng/ml IL-1 and EST for 6 days, with doses from 0.31 μM to 500 μM EST. EST was solubilized in dimethylsulfoxide (DMSO) at a concentration sufficient to ensure that the final concentration of DMSO did not exceed 0.5% in any culture medium.

EST inhibited GAG loss from tissue in a dose-dependent manner (Fig. 5(A)), and had marked effects on both the abundance and distribution of ADAMTS4 and MT4-MMP proteins. Cartilage explants were incubated in the presence or absence of 10 ng/ml IL-1 and EST over a range of doses up to 500 μM. (A) The GAG released from the tissue to the conditioned medium showed dose-dependent inhibition of IL-1-induced GAG loss by EST (bars indicate mean ± s.e.m. for six replicates per group). Linear regression analysis of rank-transformed EST dose vs GAG loss showed a significant trend (P = 0.02 for N = 4 doses). (B) Western analysis for ADAMTS4 was performed on cartilage tissue extracts and conditioned medium by probing with JSCVMA. Treatment with IL-1 caused a prominent increase in medium abundance of p53 that was blocked by addition of 125 μM EST. (C) Western analysis for MT4-MMP in cartilage tissue and conditioned medium showed that IL-1 treatment resulted in a strong increase in MT4-MMP abundance, and this increase was blocked by addition of 125 μM EST.

REALTIME PCR ANALYSIS

To examine the possibility that the ManN blockade of IL-1 mediated aggrecanase activity in bovine cartilage explants could be due to transcriptional inhibition we carried out realtime PCR of ADAMTS4 and ADAMTS5 in this system. The data (Fig. 6) shows that for both aggrecanases there was a marked (~3-fold for ADAMTS4 and ~10-fold for ADAMTS5) increase in transcription 1 day after addition of 10 ng/ml IL-1 (P < 0.001 for both). There was no significant effect of ManN treatment alone, but ManN significantly inhibited the IL-1-induced increase in ADAMTS4 (P = 0.047) and showed the same trend for ADAMTS5 (P = 0.052).
shown to occur during autoproteolysis of ADAMTS4.\(^{15,18}\) MT4-MMP-mediated cleavage sites are the same as those at the attachment region.\(^{14,18}\) This p68 form can be presented on the cell surface in association with MT4-MMP\(^{18}\) where it is C-terminally cleaved by MT4-MMP to generate the p53 form, which can be found in association with syndecan-1,\(^{18}\) and which readily cleaves at the IGD site of aggrecan to destroy the biomechanical properties of the tissue.\(^{29}\) The MT4-MMP-mediated cleavage sites are the same as those shown to occur during autoproteolysis of ADAMTS4.\(^{15,18}\)

In the present work we have shown that in a cartilage explant, the IL-1-promoted and aggrecanase-mediated sequential cleavage of the C-terminal and IGD of aggrecan [Fig. 1(A, B)] is not accompanied by a marked change in the amount of ADAMTS4 protein but, in agreement with the studies of Pratta et al.\(^{38}\), it appears to be due to activation of an existing pool of enzyme [Figs. 3 and 5(B)]. The changes induced by IL-1 were in the p68 form and the p53 form [Fig. 3], both of which were found in the medium compartment after 6 days. In time-course experiments (not shown) the p68 form was found to be abundant at day 2 whereas the p53 form was only apparent after 6 days. Given that the p68 form is confined to C-terminal cleavage of aggrecan\(^{14}\), the kinetics of appearance of the p68 and p53 seen here are consistent with the finding that C-terminal cleavage occurred early in the process [Fig. 1(B)] and this was followed after about 4 days by IGD cleavage [Fig. 1(A)]. These data, on detergent extracts of cartilage tissue, show some differences from previous observations of increased abundance of guanidine-extractable ADAMTS4 at 46 kDa\(^{14}\) or at ~60 and 30 kDa\(^9\), which may be explained by variable recovery of ADAMTS4 forms depending on the extractant.

Of particular interest is the finding that both the p68 form and the p53 form are released into the culture medium, suggesting that their proposed cell surface associations with MT4-MMP and syndecan-1, respectively\(^{18}\), are saturable and/or reversible. Regarding the association of p68 with MT4-MMP, it is clear that MT4-MMP also increases in abundance in the presence of IL-1 (Fig. 4) and that a proportion of this newly synthesized GPI-anchored protein is released from the cell and matrix under IL-1 stimulation. While the p68—MT4-MMP complex can be released intact from human chondrosarcoma cells by treatment with phosphatidylinositol-specific phospholipase C\(^{18}\), it is unknown whether the two proteins remain associated after MT4-MMP release from the cell surface in the present system. Interestingly, Itoh et al.\(^{17}\) have observed in COS cells that the release of MT4-MMP from the cell surface is partially inhibited by a metalloproteinase inhibitor, suggesting that a member of the ADAM family of sheddases\(^{38}\) might also be involved in the aggrecanase cascade. Alternatively, membrane vesiculation during IL-1 treatment\(^{39}\) might provide an explanation for the release of the MT4-MMP.

While the p68 form is found in both the tissue and the medium, it appears that the p53 form is only abundant in the medium, suggesting that all of the newly formed p53 dissociates from the cell surface syndecan-1 at some time in the process. Again, while the association of p53 with the GAG chains of syndecan-1 in chondrosarcoma cell culture is stable to bi-directional immunoprecipitation protocols\(^{18}\), it is not known whether the two proteins remain associated after MT4-MMP release from the cell surface. The data reported also provide novel insight into the mechanism by which inhibitors of the aggrecanase pathway may operate. The findings that MMP inhibitors (such as TIMP-1, peptide hydroxamates, etc.) can partially block aggrecanolyis in tissue explants suggested early that either MMP inhibitors can directly inhibit the ADAMTS4...
In support of this, in the present study we have shown that EST, an inhibitor of MMP-1, 3, and 13 gene transcription, is also an inhibitor of aggrecanase activation (Fig. 2(A)), p53 ADAMTS4 formation (Fig. 2(B)), and MT4-MMP protein accumulation (Fig. 5(C)). Since the IC50 is about 50 μM for each of these three effects, it seems likely that the EST effect operates primarily by blocking induction of MT4-MMP synthesis by IL-1, which results in a lack of conversion of p68 to p53, and a consequent loss of destructive aggrecanolysis. The concentrations of EST required to block cartilage resorption were not due to cytotoxic effects. It has previously been reported that EST concentrations of up to 500 μM had no effect on chondrocyte viability by measurement of LDH (lactate dehydrogenase) release. In addition, we have shown in preliminary experiments that 500 μM EST does not affect expression of TIMP-1 and GAPDH mRNAs as measured by realtime PCR, indicating that transcription had not been universally altered, and suggesting that the inhibition by EST was not based on cytotoxic activity.

The other inhibitor studied here was ManN, which we have suggested exerts its effect by inhibition of the GPI anchor formation required for membrane insertion of MT4-MMP. The present data are consistent with this idea since 1.35 mM ManN markedly (~80%) inhibited the IL-1-induced production of MT4-MMP (Fig. 4) in the cartilage. This predictably blocked the IL-1-induced accumulation of p68 and p53 ADAMTS4 in the medium (Fig. 3). Interestingly, in the presence of ManN there is a complete blockade of IGD cleavage by p53; however, some C-terminal cleavage to G1-TAGLE is observed (data not shown). This is consistent with the idea that the pre-existing p68 would likely be active in the presence of ManN, since it would not require further GPI anchor formation.

Finally, we show here (Fig. 6) that IL-1 treatment of bovine chondrocytes in cartilage explants results in major upregulation of mRNA for both ADAMTS4 (3-fold) and ADAMTS5 (10-fold). This marked increase in transcriptional activity for these proteinases was not accompanied by major changes in the abundance of these proteins (data not shown for ADAMTS5). This is consistent with the general view that control of aggrecanolysis in cartilage by inflammatory mediators such as IL-1, is initiated by proteinase processing on the cell surface and release of active enzyme into the matrix. Presumably prolonged exposure to such mediators in joint diseases, with an attendant upregulation of transcription, generates a continuous supply of newly synthesized enzyme, which will maintain the activity of this degradative cascade.

The upregulation of ADAMTS5 mRNA observed here is not necessarily indicative of an increase in ADAMTS5 activity, but emphasizes that while the data presented here has focused on activation of ADAMTS5, other family members may also play a role in aggrecanolysis. In particular, the recent findings in mice that the IL-1-induced aggrecanase pathway to G1-NITEGE formation is apparently not blocked by gene deletion of ADAMTS15 or ADAMTS5 suggest that further analysis of the expression and activation of the individual aggrecanases (ADAMTS1, 4, 5, 8, 9, 15) will be needed to fully define the role of MT4-MMP in the aggrecanase associated with human osteoarthritis.

As a general comment, it is important to note that the effectiveness of ManN in this process should not be interpreted as suggesting the use of ManN in place of glucosamine for therapy of human osteoarthritis. ManN, unlike glucosamine, is a potent inhibitor of GPI-anchor synthesis, which is necessary for the anchoring and function of multiple essential cell-surface proteins. ManN ingestion is therefore likely to have profound side effects, which should preclude its use as a therapeutic.

Our evidence for the involvement of MT4-MMP in aggrecanolysis in cartilage tissue culture also has implications for the interpretation of MMP inhibitor studies. For example, some “broad-spectrum” MMP inhibitors reportedly do not inhibit aggrecanase activity. However, it is not clear whether all broad-spectrum MMP inhibitors are active against MT-MMPs, which were not characterized until 1999, and may have some unique features in the structure of their catalytic domains. It is therefore intriguing that CGS20723A, a broad-spectrum MMP inhibitor that potently inhibits the catalytic domain of MT4-MMP in solution, does not appear to inhibit overall IL-1-induced GAG loss in tissue culture, but has been reported to protect against loss of pericellular GAG staining. This could be explained if loss of pericellular GAG staining is due to p53 generated by MT4-MMP, whereas more generalized aggrecanolysis in the intercellular matrix is due to p68 and therefore confined to the C-terminal of aggrecan. Thus, the requirement for MT4-MMP for aggrecanase inhibition suggested by the present data may not extend to all experimental situations, and the development of specific small-molecule MT-MMP inhibitors may in future be useful for testing these hypotheses.

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