Cleavage of Native Cartilage Aggrecan by Neutrophil Collagenase (MMP-8) Is Distinct from Endogenous Cleavage by Aggrecanase*

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Cleavage of aggrecan core protein at the Glu373-Ala374 site by the unidentified enzyme, “aggrecanase,” is thought to play an important role in cartilage degradation. To examine aggrecan cleavage by MMP-8 at this aggrecanase site, we evaluated the release of fragments with the N terminus ARGSVIL from freeze-thawed bovine nasal cartilage using the monoclonal antibody BC-3. Recombinant human MMP-8 catalytic domain cleaved native aggrecan in a concentration-related manner between 0.2 and 2 μg/ml, with complete release of glycosaminoglycan at 2 μg/ml or greater. Cleavage at the aggrecanase site was observed only at MMP-8 concentrations resulting in complete release of glycosaminoglycan from the cartilage, suggesting that preferential cleavage occurs at a different site. Time course studies indicated that only following depletion of substrate containing the preferred clip site did MMP-8 rapidly cleave at the aggrecanase site. Finally, MMP-8 resulted in a different pattern of BC-3-reactive fragments from that produced by endogenous aggrecanase in live cartilage, and SA751 (N-(1(R)-carboxyethyl)-O-methyltyrosine, N-methylamide), a potent inhibitor of MMP-8 (Kᵢ = 2 nM) which was effective in blocking cleavage by MMP-8 at the aggrecanase site with an IC₅₀ in the nanomolar range, did not prevent aggrecan degradation or specific cleavage at this site by endogenously generated aggrecanase at concentrations up to 100 μM. Taken together these data suggest that MMP-8 does not represent cartilage aggrecanase.

Aggrecan provides cartilage with its properties of compressibility and elasticity and is the first cartilage matrix component to undergo measurable loss in arthritis. The aggrecan molecule is composed of two N-terminal globular domains, G1 and G2, which are separated by an approximately 150 residue interglobular domain (IGD), followed by a long central glycosaminoglycan (GAG) attachment region and a C-terminal globular domain, G3 (1–2). These aggrecan monomers interact through the G1 domain with hyaluronic acid and link protein to form large molecular weight aggregates that are trapped within the cartilage matrix (3–5). Normal turnover as well as loss in arthritic conditions involves proteolytic cleavage of the aggrecan core protein within the IGD, releasing a large C-terminal GAG-containing aggrecan fragment which diffuses out of the cartilage matrix and an N-terminal G-1 fragment which remains bound to hyaluronic acid and link protein within the matrix. However, the proteinases responsible for the normal turnover and pathological loss of aggrecan have not been identified.

Studies indicate two major sites of proteolytic cleavage within the IGD between amino acid residues Asn341–Phe342 and Glu373–Ala374. G-1 fragments with the former cleavage site have been identified within articular cartilage bound to hyaluronic acid (6). However, C-terminal fragments with the latter cleavage site have been identified in synovial fluid of patients with both osteoarthritis (7) and inflammatory joint disease (8) and in the media from cartilage explant and chondrocyte cultures (9–13) stimulated with interleukin-1 or retinoic acid, suggesting that cleavage at this site may play an important role in cartilage degradation. Many matrix metalloproteinases (MMP-1, -2, -3, -7, -8, and -9) have been shown to cleave in vitro at the Asn341–Phe342 bond (6). However, attempts to generate cleavage at the Glu373–Ala374 site with a number of purified proteinases (6, 14–17) have been unsuccessful, indicating that this cleavage is the result of a novel, yet unidentified, proteinase given the name “aggrecanase” based on its ability to cleave the aggrecan core protein.

In vitro studies, using a purified G1-G2 substrate, demonstrated the ability of native and recombinant MMP-8 to cleave at the Glu373–Ala374 aggrecanase site, although the preferential cleavage site was the Asn341–Phe342 bond (18). The aggrecanase activity of MMP-8 was detected only at high enzyme concentrations (160 μg/ml) or in the presence of polyethylene glycol (PEG) 4000. The authors hypothesized that PEG 4000 acted by increasing the local concentration of enzyme by an excluded volume effect similar to the effect of aggrecan in cartilage. Recent studies have shown that MMP-8 is not a unique gene product of neutrophils, but is expressed by chondrocytes in normal human articular cartilage, and that this expression is up-regulated in response to IL-1 stimulation in explant culture (19). In addition, MMP-8 message in human osteoarthritic cartilage has been shown to be elevated as compared with that in normal cartilage (20). These data open the possibility that MMP-8 may represent the cartilage aggrecanase.

To determine whether MMP-8 is capable of cleaving aggreg- can at the Glu373–Ala374 aggrecanase site and the requirements and preference for this cleavage under conditions mimicking those occurring in situ within the cartilage matrix, we evaluated the release of fragments with the N terminus Ala374 from native aggrecan substrate in freeze-thawed bovine nasal cartilage slices using the monoclonal antibody BC-3 (21), which is specific for the N-terminal neoepitope generated by cleavage at the aggrecanase site. In this paper, we report that MMP-8...
cleaves the native aggrecan substrate at the aggrecanase site at 50–100-fold lower concentrations than those required using a purified G1-G2 substrate, consistent with the possibility that this protease could contribute to cleavage at the aggrecanase site. However, MMP-8 preferentially cleaves aggrecan at a site distinct from the aggrecanase site and produces a pattern of cleavage fragments with the Ala274 N terminus which differs from that produced by endogenously generated aggrecanase in IL-1-stimulated cartilage explant cultures. Finally, we show a potent inhibitor of MMP-8-mediated cleavage at the aggrecanase site to be ineffective in blocking cleavage at this site in response to endogenously generated aggrecanase, indicating that MMP-8 is not the endogenous cartilage aggrecanase.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were from Life Technologies, Inc. (Grand Island, NY). The IL-1 used was a soluble, fully-active recombinant human IL-1β produced as described previously (22). The specific activity was 1 × 10^6 units/mg of protein, with 1 unit being defined as the amount of IL-1 that generated half-maximal activity in the thymocyte proliferation assay. The C-terminally truncated form of human neutrophil proMMP-8 (proMMP-8) (22) was expressed as detergent-soluble homogeneity. ProMMP-8 was activated by (4-aminophenyl)mercuric acetate, and APMA was removed by dialysis against enzyme buffer prior to use. Full-length human fibroblast proMMP-3 was expressed in baculovirus and purified to homogeneity. Antibody BC-3 (18), which recognizes the new N terminus, ARGSSV, on aggrecan fragments produced by aggrecanase, was provided by Dr. Bruce Caterson (University of Wales, Cardiff, UK); Chondroitinase ABC lyase (Proteus vulgaris) (EC 4.2.2.4), keratanase (Pseudomonas sp.) (EC 3.2.1.103), and keratanase II (Bacillus sp.) were from Seikugaku (Kogyo, Japan). XOG076 (7-aza-2-phenylbenzothiazol-3-one), an inhibitor of proMMP-3 activation (23), and SA751 (N-(1(R)-carboxyethyl)-(4-phenyl-3-butynyl) glycyl-(O)-methylthiophosphate, N-methylamide), a selective, potent inhibitor of MMP-8 were synthesized at DuPont Merck (Wilmington, DE). SA751 has a K of 2 μM against MMP-8 and a K of >10,000 μM against MMP-3 and MMP-1.

Cartilage Preparation—Bovine nasal cartilage (BNC) septa were removed from bovine noses obtained fresh at the time of slaughter. Uniform cartilage disks (1 mm thick, 8 mm in diameter) were prepared. Prior to organ culture studies, disks were equilibrated in tissue culture for at least 3 days in DMEM supplemented with 5% heat-inactivated fetal calf serum, penicillin, streptomycin, amphotericin B, and neomycin (100 IU/ml, 100 μg/ml, 0.25 μg/ml, and 50 μg/ml, respectively).

Cartilage Cultures—Cartilage pieces were cultured as described previously (24). Briefly, following equilibration, cartilage disks were cut at 37 °C in an atmosphere of 95% air, 5% CO2. Included as a “blank” for each group. Cultures were incubated for 40 h experimental conditions. Eight replicates per treatment group were run with at least two replicates per treatment group.

Enzyme Digestion—Digests were carried out in 200 μl of 100 mM Tris/HCl buffer, pH 7.5, containing 100 μM NaCl and 10 mM CaCl2. Free-thawed NC (approximately 10 μg/ml) was weighed and then incubated with recombinant human catalytic domain MMP-8 at 37 °C for the times indicated, quenched with EDTA, and the incubation buffer frozen for analysis of total aggrecan cleavage by monitoring release of sulfated GAG and for analysis of fragments with the new N terminus, ARGSSVIL, formed by specific cleavage at the aggrecanase site, by BC-3 Western blot analysis. To determine the amount of aggrecan remaining in the cartilage following incubation with MMP-8, the cartilage pieces were digested with papain, and the digests were analyzed for GAG.

To address the concern that the exogenously added MMP-8 may activate latent proteases, such as prostromelysin, present in the cartilage, which could result in some initial cleavage of the aggrecan, two control experiments were done. 1) Human recombinant prostromelysin (proMMP-3) was incubated in the presence or absence of 5 μg/ml MMP-8 for 24 h at 37 °C and then evaluated by Western analysis for the generation of mature, active stromelysin. 2) MMP-8 was incubated with freeze-thawed bovine nasal cartilage for 48 h in the absence or presence of 100 μg/ml XOG076 (7-aza-2-phenylbenzothiazol-3-one), a compound which inhibits pro-MMP activation but does not inhibit the active enzyme (23). Release of both GAG and BC-3 fragments from the cartilage were monitored.

Glycosaminoglycan Assay—Sulfated GAG released into the buffer or culture media and in the cartilage digestes was monitored by the amount of polyanionic material reacting with 1,9-dimethylmethyline blue (25), using shark chondroitin sulfate as a standard. Results are reported as μg of GAG per mg wet weight cartilage or as percent of total cartilage GAG released.

Analysis of Aggrecan Catabolites—For analysis of aggrecan fragments generated by specific cleavage at the Glu274-Ala274 site, proteoglycans, and proteoglycan metabolites were enzymatically deglycosylated with chondroitinase ABC (0.1 units/10 μg of GAG) for 2 h at 37 °C and then digested with 10 μg/ml keratanase II (0.002 units/10 μg of GAG) for 2 h at 37 °C in buffer containing 50 mM sodium acetate, 0.1 M Tris/HCl, pH 6.5 (21). The digestes were monitored by measuring the decrease in dimethylmethyline blue reactivity. After digestion, the samples were precipitated with 5 volumes of acetone and reconstituted in an appropriate volume of SDS-PAGE sample buffer.

Western Blot Analysis—Equivalent amounts of GAG from each sample were loaded on 4–12% gradient gels and then separated by SDS-PAGE under nonreducing conditions, transferred overnight to nitrocellulose, and immunolocalized with 1:1000 dilution of the monoclonal antibody BC-3 (18). Subsequently, membranes were incubated with goat anti-mouse IgG alkaline phosphatase conjugate and aggrecan catabolites visualized by incubation with the appropriate substrate (Promega Western blot alkaline phosphatase system) for 10–30 min to achieve optimal color development. BC-3-reactive aggrecan fragments were then quantified by scanning densitometry. Overnight transfer resulted in complete transfer of high and medium weight standards, and the densitometric response was found to be linear over the density range required for the blots, as determined by loading increasing amounts of BC-3-reactive material.

RESULTS

Effect of MMP-8 Concentration on Native Cleavage—MMP-8 cleaved native aggrecan in freeze-thawed bovine nasal cartilage at the Glu274-Ala274 aggrecanase site as detected by BC-3 Western blot analysis (Fig. 1A) following a 48-h incubation. Five prominent BC-3 reactive bands were observed, a doublet at 150 kDa, a single band at 100 kDa, and a doublet at 65 kDa. With as low as 2 μg of MMP-8/ml, cleavage was detected at this site, with total band intensity increasing through 20 μg/ml. However, the banding pattern shifted with increasing enzyme concentration, indicating that at higher concentrations, the larger fragments were further cleaved at the C terminus resulting in conversion to fragments represented by the doublet at 65 kDa. At concentrations of 50 μg/ml and above, these bands at 65 kDa decreased in intensity, suggesting that they were further degraded to smaller fragments which ran off the gel. Evaluating the concentration of MMP-8 required for maximum generation of the various bands indicated that generation of the 150-kDa doublet and 100-kDa band was maximal with 5 μg/ml while generation of the doublet at 65 kDa was maximal at 20 μg/ml.

GAG analysis indicated that MMP-8 cleaved aggrecan in freeze-thawed bovine nasal cartilage in a concentration-related manner between 0.2 and 2 μg/ml, with complete release of GAG from the cartilage with 2–50 μg of MMP-8/ml (Fig. 1B). The possibility that the exogenously added MMP-8 was activating latent MMPs, such as MMP-3, present in the cartilage matrix, in which turn caused the initial cleavage and GAG release.
Effect of an MMP-8 Inhibitor on Aggrecan Cleavage at the Glu\textsuperscript{373}-Ala\textsuperscript{374} Aggrecanase Site—SA751, a potent, selective inhibitor of MMP-8 (MMP-8, $K_i = 2 \text{ nM}$; MMP-3, $K_i = >10,000 \text{ nM}$; MMP-1, $K_i = >10,000 \text{ nM}$), blocked MMP-8 cleavage of native aggrecan in freeze-thawed BNC at the aggrecanase site as monitored by BC-3 Western blot analysis (Fig. 5A) with an $IC_{50}$ as determined from the concentration-response curve (Fig. 5B) to be 0.13 $\mu\text{M}$. In these same experiments, higher concentrations of inhibitor were required to block total aggrecan breakdown as monitored by release of GAG with the $IC_{50}$ estimated to be 0.50 $\mu\text{M}$ (data not shown). In contrast, this inhibitor was ineffective at concentrations up to 100 $\mu\text{M}$ in blocking proteoglycan breakdown as monitored by MMP-8 cleavage from IL-1-stimulated bovine nasal cartilage (Fig. 6A) and had no effect on cleavage at the Glu\textsuperscript{373}-Ala\textsuperscript{374} bond by the endogenously generated aggrecanase in this system as evaluated by BC-3 Western analysis (Fig. 6B).

DISCUSSION

MMP-8 cleaves native, freeze-thawed cartilage aggrecan at the Glu\textsuperscript{373}-Ala\textsuperscript{374} aggrecanase site at concentrations as low as

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mM, which is ~50-fold lower than that reported to be required using a purified G1-G2 substrate (18). Studies using purified G1-G2 substrate also demonstrated the ability of PEG to dramatically lower the amount of MMP-8 required for cleavage at this site from 160 to 10 μM, and the authors suggest that this is due to the water exclusion properties of the PEG, which mimic the effect of aggrecan in cartilage. Our data illustrating cleavage of native aggrecan at low MMP-8 concentrations would support this hypothesis, particularly in light of the relative lack of effect of PEG when the native cartilage aggrecan substrate is used, and suggest that the physical environment and substrate presentation in cartilage may be important in determining enzyme cleavage.

Although synovial fluid levels of MMP-8 have not been determined, MMP-3 and MMP-1 have been shown to be present in synovial fluids of patients with arthritis at concentrations of 2–40 μM (26, 27), within the concentration range where we have demonstrated that MMP-8 is capable of cleaving at the aggrecanase site. Taken together with reports that human articular chondrocytes are capable of expressing MMP-8 and that elevated levels are present in osteoarthritic cartilage (19–20), our data open the possibility that MMP-8 could contribute to cleavage of aggrecan at the aggrecanase site in arthritic joints.

However, we found that MMP-8 preferentially cleaves the native aggrecan substrate at a site distinct from the aggrecanase site, likely the MMP site, to release GAG, similar to the results obtained with the purified G1-G2 substrate (18). Thus, presentation of the substrate in the native form does not affect the site at which MMP-8 preferentially cleaves aggrecan. Therefore, it is improbable that MMP-8 would preferentially cleave at the aggrecanase site within the cartilage matrix in vivo. Since only the catalytic domain of MMP-8 was used in our studies, the absence of the hemopexin domain could, in principle, influence the substrate specificity of the enzyme. However,
since similar substrate specificity was observed using full-length MMP-8 with purified G1-G2 aggrecan substrate (18), this possibility is unlikely.

Time course studies indicate that complete cleavage at the preferred site is not required for cleavage to occur at the aggrecanase site since low levels of BC-3-reactive fragments are generated early on in the time course, prior to depletions of the preferred substrate (i.e. aggrecan containing the Asn341-Phe342 bond). However, only following depletion of substrate containing this preferred clip site (as indicated by complete loss of GAG from the matrix that occurs in response to the initial clip of the core protein) does MMP-8 rapidly cleave at the aggrecanase site.

MMP-8 cleavage of aggrecan also yields a different pattern of BC-3 reactive fragments than that obtained with the in situ induction of aggrecanase activity by stimulation of live bovine nasal cartilage with IL-1. Although several bands of BC-3-reactive aggrecan fragments were seen at 64, 92, and 120 kDa in response to both endogenously generated aggrecanase and to MMP-8, the high molecular mass band at 230 kDa, which represents the C-terminal aggrecan fragment formed by initial cleavage within the interglobular domain, was only generated by endogenously generated aggrecanase. This band was not produced on cleavage by MMP-8 at any of the times or concentrations evaluated. These data suggest that, while the cartilage aggrecanase appears to cleave initially at the Glu373-Ala374 bond to form a BC-3-reactive product, SA751, a potent MMP inhibitor selective for MMP-8 over MMP-3 or MMP-1 (MMP-8, $K_i = 2 \text{ nM}$; MMP-3, $K_i = >10,000 \text{ nM}$; MMP-1, $K_i = >10,000 \text{ nM}$) was effective in blocking specific cleavage of native aggrecan substrate at the Glu373-Ala374 aggrecanase site by MMP-8 and an IC$_{50}$ of 0.13 mM. In these same experiments, higher concentrations of inhibitor were required to block total aggrecan breakdown as monitored by release of GAG, which apparently occurs through cleavage at a site distinct from the aggrecanase site. This is consistent with preferential cleavage by MMP-8 occurring at a site other than the aggrecanase site.

We have previously demonstrated that BB-16 ((2S,3R)-2-methyl-3-(2-methylpropyl)-1-(N-hydroxy)-4-(O-methyl)-1-tyrosine-N-methylamide), a potent, nonselective hydroxamate MMP inhibitor (MMP-8, $K_i = 0.7 \text{ nM}$; MMP-3, $K_i = 1 \text{ nM}$; MMP-1, $K_i = 0.05 \text{ nM}$), was effective in blocking IL-1-induced proteoglycan breakdown and cleavage at the aggrecanase site in bovine nasal cartilage, suggesting that aggrecanase may be a zinc metalloproteinase or be activated by a member of this family of proteases. However, SA751, a potent inhibitor of MMP-8 which is effective in blocking cleavage of native aggrecan by MMP-8 with an IC$_{50}$ in the nanomolar range, does not prevent proteoglycan degradation or specific cleavage at the Glu373-Ala374 aggrecanase site in response to endogenously generated aggrecanase even at concentrations up to 100 mM. Thus MMP-8 does not appear to be responsible for cleavage at the aggrecanase site by enzyme generated in situ by cartilage stimulated with IL-1.

In summary, we have shown that MMP-8 is capable of cleaving the native aggrecan at the aggrecanase site at concentrations that may theoretically be present within the cartilage matrix. However, we have also demonstrated that 1) MMP-8 cleaves aggrecan preferentially at a site distinct from the aggrecanase site, 2) rapid cleavage at the aggrecanase site in response to MMP-8 occurs only following depletion of substrate with the preferential cleavage site, 3) the 230-kDa band of BC-3-reactive protein representing the primary aggrecanase-generated proteoglycan fragment in IL-1-stimulated cartilage degradation was not produced upon cleavage of native aggrecan by MMP-8, and 4) a potent MMP inhibitor was ineffective in blocking cleavage at the Glu373-Ala374 bond by endogenously generated aggrecanase. Taken together, these data suggest that MMP-8 does not represent the cartilage aggrecanase.

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