An aggrecan-degrading activity associated with chondrocyte membranes

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INTRODUCTION

Aggrecan is the most abundant of the proteoglycans (PGs) found in articular cartilage; it functions in load distribution in joints during movement. Structurally, aggrecan comprises a core protein made up of three globular domains, G1, G2 and G3, linked by two linear peptide chains. The short extended peptide region between G1 and G2 is called the interglobular domain (IGD) and is sensitive to proteolytic attack. The longer polypeptide segment between G2 and G3 forms the major glycosaminoglycan attachment region; these sulphated groups serve to hydrate the cartilage tissue, thus enabling the cartilage to resist compression under load [1].

Aggrecan catabolism involves a limited proteolysis of the core protein within the IGD, leading to the release of large chondroitin-carrying species into the synovial fluid [2,3]. This breakdown is up-regulated in pathological conditions such as rheumatoid and osteoarthritis, and can be modelled in vitro by the stimulation of cartilage explants with inflammatory cytokines such as interleukin 1α (IL-1α) [4,5]. N-terminal amino acid sequencing of aggrecan cleavage products has identified two main sites of cleavage within the IGD [6,7]. Cleavage between Asp-341 and Phe-342 has been attributed to the action of the matrix metalloproteinases (MMPs) on the IGD [8]; however, it is the cleavage between Glu-373 and Ala-374 that seems to be the predominant one in stimulated explants and in diseases such as rheumatoid arthritis. The identity of the enzyme responsible for this cleavage, named ‘aggrecanase’, is unclear. Turnover of aggrecan within the cartilage is mediated by the metabolically active cells of the extracellular matrix, the chondrocytes, and studies have shown that stimulation of these cells or cartilage explants with cytokines such as IL-1α results in cleavage at the aggrecanase site [9]. Recent results from this group [10] have shown that a combination of oncostatin M (OSM), a member of the IL-6 family of cytokines, and IL-1α is able to stimulate a large and rapid release of PG from cartilage explants. OSM is produced by T-cells and mononcytic cells; a number of cell types, including chondrocytes and synovial cells, possess receptors for this cytokine [11], possibly implicating it in the inflammatory processes associated with joint diseases such as rheumatoid arthritis.

Although recent reports have shown that aggrecanase activity can be found in the culture media of stimulated chondrocytes [12], we have investigated the possibility that aggrecanase activity might also be associated with the membranes of the chondrocytes.

A combination of OSM and IL-1α was used to stimulate the chondrocytes before membrane purification; aggrecanase activity was monitored with an antibody that specifically recognizes the new N-terminal sequence formed by the action of aggrecanase on its substrate.

MATERIALS AND METHODS

Materials

The monoclonal antibodies BC3 and BC14 were a gift from Dr. B. Caterson (University of Cardiff, Cardiff, U.K.). The polyclonal antibody R663 was supplied by Dr. D. Buttle (University of Sheffield, Sheffield, U.K.). Neprilysin and the inhibitor CH1707 were supplied by Chiroscience (Cambridge, U.K.). BB94 was a kind gift from Professor H. Nagase (University of Kansas Medical Center, Kansas City, KS, U.S.A.). Hylauroidase (Type I-S from bovine testes), trypsin (Type I-S from pig pancreas), collagenase (Type I) and bovine plasmin were obtained from Sigma Aldrich (Poole, Dorset, U.K.). Chondroitinase ABC lyase (protease-free), keratanase and CsCl were purchased from ICN Biomedicals (Thame, Oxon., U.K.). Dulbecco’s modified Eagle’s medium (DMEM) and Hanks balanced salt solution were from Gibco BRL (Paisley, Renfrewshire, U.K.). Alkaline phosphatase-conjugated pig anti-(rabbit IgG) was from DAKO (High Wycombe, Bucks., U.K.). Alkaline phosphatase-conjugated anti-(mouse IgG) was from Promega (Southampton, U.K.). The alkaline phosphatase conjugate substrate kit was purchased from Bio-Rad (Hemel Hempstead, Herts., U.K.), and the bicinchoninic acid protein assay kit was bought from Pierce and Warriner (Chester, U.K.).
Extraction and culture of bovine nasal chondrocytes
Chondrocytes were prepared from bovine nasal septum cartilage obtained within 24 h of slaughtering and stored at 4 °C. Chondrocytes were isolated from the cartilage by sequential enzymic digestion with 1 mg/ml hyaluronidase, 0.25% (w/v) trypsin and 3 mg/ml collagenase as described previously [13]. The cells were grown in monolayer culture in DMEM containing 25 mM Hepes and supplemented with 10% fetal calf serum, 2 mM glutamine, 20 i.u./ml nystatin, 100 i.u./ml penicillin, 50 µg/ml gentamicin and 100 µg/ml streptomycin. Chondrocytes up to passage 2 were used in subsequent membrane purification procedures, after stimulation with IL-1α (1 ng/ml) and oncostatin M (10 ng/ml) for 24 h. Concentrations of these two cytokines were chosen for their effects on glycosaminoglycan release when added to bovine nasal cartilage explants [10].

Chondrocyte membrane purification
Confluent, stimulated bovine nasal chondrocytes were harvested in Hanks balanced salt solution. The cells were pelleted by centrifugation and the pellet was then resuspended in 10 mM Tris/HCl, pH 7.4, containing 8.5% (w/v) sucrose, 1 mM N-ethylmaleimide, 1 mM PMSF and 10 µg/ml soybean trypsin inhibitor (lysis buffer). Cells were lysed by sonication and the plasma membrane fractionation was achieved by differential centrifugation based on the methods described by Mollenhauer et al. [14] and Cates et al. [15]. In brief, unlysed cells and cell nuclei were removed by centrifugation at 1700 g for 10 min at 4 °C. This centrifugation was repeated after resuspension of the pellet in half the original volume of lysis buffer, and the two supernatants were combined and centrifuged at 33000 g for 1.5 h at 4 °C. The pellet was resuspended in 1 ml of lysis buffer and the plasma-membrane-enriched fraction collected by sucrose-density-gradient centrifugation. In brief, a discontinuous gradient consisting of 5 ml layers of 40% and 17% (w/v) sucrose was prepared; 0.5 ml of the sample was layered on top and the tubes were centrifuged at 150000 g for 1.5 h at 4 °C in a Beckman SW41Ti rotor. Material from the 40%:/17% sucrose interface was collected, diluted in 10 mM Tris/HCl, pH 7.4, and re-centrifuged. The pellet was resuspended in the same buffer and stored at −20 °C. Membrane enrichment was monitored by assaying for the plasma membrane marker enzyme 5'-nucleotidase [16]. The material collected from this sucrose interface was typically shown to have a 7-10-fold enrichment in the 5'-nucleotidase activity and thus was used in subsequent assays as the chondrocyte membrane fraction. Protein concentration was determined by the Bradford assay.

Aggrecan preparation
Bovine nasal septum cartilage was cut into 2 mm³ pieces and added to 10 vol. of extraction buffer [4 M guanidinium chloride/50 mM sodium acetate (pH 6.0)/2 mM PMSF/5 mM benzamidine/HCl/10 mM EDTA/0.1 mM 6-aminoheptanoic acid] at 4 °C. The mixture was stirred for 48 h at 4 °C. After extraction, CsCl was added to give a final density of 1.45 g/ml. The solution was then centrifuged at 100000 g for 48 h at 10 °C in a Beckman Ti70 rotor. The monomeric PG fraction (D1) was recovered from the bottom 25% of the tubes and was dialysed exhaustively into 10 mM Tris/acetae, pH 7.4, before storage at −20 °C.

Aliquots of the D1 fraction were treated with chondroitinase ABC lyase at 2.5 m-units/mg of PG, and keratanase at 50 m-units/mg of PG, in the presence of proteinase inhibitors for 5 h at 37 °C to remove chondroitin sulphate and keratan sulphate chains. Samples were dialysed against 10 mM Tris/HCl, pH 7.4, and stored at −20 °C. This enzyme-treated aggrecan was subsequently used in all experiments as the aggrecanase substrate.

Antiserum preparation
A polyclonal antiserum, T767, was raised in rabbit against the amino acid sequence ARGSVILTVKGGC coupled to ovalbumin at the cysteine residue. This sequence represents the new N-terminal sequence on aggrecan catabolic products generated by the activity of aggrecanase. The serum was partly purified by ovalbumin-Sepharose chromatography to remove ovalbumin-specific antibodies, and stored in aliquots at −20 °C. Microtitre plates coated with immunizing peptide were used in an ELISA format to ensure antiserum specificity and to determine the dilution of antiserum used in subsequent immunoblotting experiments.

Assays of chondrocyte membranes with aggrecan substrate
A 300 µl portion of the aggrecan substrate (1 mg/ml protein) was incubated in the presence of 10 mM CaCl₂ with the membrane preparation (50 µg of protein) for 24 h at 37 °C, together with any inhibitors (see the Results section). Reactions were stopped by the addition of 5 x SDS sample buffer containing 2-mercaptoethanol, then boiled for 5 min. Samples were subjected to electrophoresis on 10 %, SDS/PAGE mini-gels by the method of Laemmli [17], and either stained with Coomassie Blue or transferred to nitrocellulose as described previously [18]. Nitrocellulose was blocked with 5% (w/v) non-fat powdered milk (Marvel) in Tris-buffered saline (TBS), then incubated overnight with antiserum diluted 1:400 in TBS containing 0.1%Tween 20 (TBST) containing 1% (w/v) Marvel. After five washes in TBST, blots were incubated for 1 h with pig anti-(rabbit IgG)-alkaline phosphatase conjugate diluted 1:500. Blots were then washed three times in TBST before colour development with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium. In experiments using the antibody against the MMP cleavage site (BC-14), this antibody was used diluted 1:500, and the secondary antibody, alkaline phosphatase-conjugated anti-(mouse IgG), was used at 1:7500 dilution [19].

In experiments investigating the effects of purified enzymes (stromelysin, neprilysin and plasmin) on the substrate, the appropriate amounts of each enzyme (see the Results section) were incubated with the substrate in the presence of calcium as above, before processing of the samples as described.

RESULTS
Incubation of chondrocyte membranes with the aggrecan substrate resulted in cleavage of the substrate (Figure 1a) and the generation of a number of immunoreactive bands (Figure 1b, lane 1). These included bands of approx. 150 kDa, together with a lower-molecular-mass band of approx. 66 kDa. Inclusion of 20 mM EDTA in the assay (lane 2) inhibited the generation of this immunoreactivity, and incubation of either the membranes (lane 3) or the substrate (lane 4) alone also prevented staining of the blot with the antibody. These results show that the generation of the neoepitope was mediated by a metalloproteinase located on the chondrocyte membrane. Identical results were obtained with two other antibodies raised against the aggrecanase neoepitope. Both the monoclonal antibody BC-3 [20] and a polyclonal antibody, R663, stained identical bands on Western blots, confirming the specificity of the antiserum used in these experiments. An aggrecan substrate that had not been treated with chondroitinase and keratanase before incubation with the...
chondrocyte membranes was also tested. After incubation with the chondrocyte membranes, endogenous enzyme activity was inactivated by boiling for 5 min; the samples were then treated with the chondroitinase and keratanase, dialysed into 50 mM Tris/HCl, pH 7.4, and subjected to electrophoresis and blotting as described in the Materials and methods section. The results (Figure 1c) showed labelling of high-molecular-mass bands by the antiserum, indicating that the generation of a product bearing the aggrecanase neoepitope had occurred before substrate deglycosylation. Although less staining of the 66 kDa band was observed than with the products derived from the pretreated aggrecan substrate, we suggest that this 66 kDa band might represent the product of a C-terminal cleavage of the aggrecanase-generated product. The enzyme responsible for this secondary cleavage might then be prevented from processing this native form of the aggrecan substrate owing to the presence of the charged chondroitin and keratan sulphate groups, with the cleavage site becoming accessible only after the removal of these groups.

That this aggrecan-cleaving activity was associated predominantly with a membrane fraction was demonstrated by incubation of the substrate with equivalent amounts of protein (50 μg) from the pellet (membrane-containing fraction) and the supernatant (cytosolic fraction) obtained after the 33000 g centrifugation. Immunoblotting of products of the reaction (Figure 2) indicated that, whereas intense staining was associated with the pellet fraction (lane 2), relatively little was seen in the supernatant fraction (lane 1), implying the presence of a membrane-associated aggrecanase activity. Levels of 5′-nucleotidase in the supernatant and pellet fractions were 1.6 units/mg of protein and 7 units/mg of protein respectively; thus the low levels of aggrecan-degrading activity associated with the supernatant fraction were consistent with the presence of small amounts of unsedimented plasma membrane remaining in this fraction. That the majority of the aggrecan-degrading activity was associated with a fraction enriched for 5′-nucleotidase implied that the activity was associated with the chondrocyte plasma membrane.

Substrate cleavage was dependent on stimulation of the chondrocytes before membrane purification, as shown in Figure 3. Cells were either incubated in DMEM alone for 24 h or with the combination of oncostatin M and IL-1α. Membranes from the control cells (Figure 3, lane 2) were unable to generate the amount of immunoreactivity seen with the membranes derived from the stimulated cells (lane 1), suggesting that there was little activity on the surface of unstimulated chondrocytes.

Incubation of the aggrecan substrate with purified stromelysin at an assay concentration of 10 μg/ml resulted in cleavage of the substrate (Figure 4a, lane 1). This cleavage was prevented by the addition of tissue inhibitor of matrix metalloproteinase (TIMP)-1 (Figure 4a, lane 2) or TIMP-2 (lane 3) to the assay mixture at concentrations of 100 units/ml; the undigested substrate was loaded in lane 4. No immunoreactivity to T767 was seen on Western blotting (results not shown). The ability of the stromelysin to cleave the aggrecan substrate was demonstrated by immunoblotting products of this reaction with an antibody
Figure 4 Stromelysin-mediated cleavage of aggrecan substrate
(a) Coomassie Blue-stained gel of products after incubation of substrate with 1 µg of stromelysin alone (lane 1) or together with TIMP-1 (lane 2) or TIMP-2 (lane 3). Lane 4 contained undigested substrate. (b) Western blot with antibody against MMP neoepitope (BC-14). Lanes are as in (a).

Figure 5 Digestion of aggrecan substrate by neprilysin and plasmin
Coomassie Blue-stained gel of products after incubation of substrate with 5 ng of neprilysin (lane 1) or 25 µg of plasmin (lane 2). Lane 3 contained plasmin alone (50 µg); lane 4 contained substrate alone.

Two other purified enzymes, neprilysin and plasmin, were tested for their ability to cleave the substrate at the aggrecanase site. Digestion of the substrate with 5 ng of recombinant soluble neprilysin (10 ng/ml) (Figure 5, lane 1), or 25 µg of bovine plasmin (50 µg/ml) (lane 2) yielded cleavage products; lane 3 contained plasmin alone and lane 4 undigested substrate (no neprilysin control was included as the levels used were below the limit of detection on the stained gel). The cleavage products from these two enzymes were not recognized by the aggrecanase neoepitope antibody (results not shown), implying that these enzymes were not responsible for the cleavage effected by the chondrocyte membrane preparation.

To verify the classes of enzyme responsible for the substrate cleavage, chondrocyte membranes and aggrecan substrate were incubated in the presence of class-specific proteinase inhibitors: 20 mM EDTA (metalloproteinase inhibitor; Figure 6, lane 2), 1 mM PMSF (serine proteinase inhibitor, lane 3), 1 µM pepstatin A (aspartic proteinase inhibitor, lane 4), 10 µg/ml trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64; lane 5) or 0.5 µg/ml leupeptin (both cysteine proteinase inhibitors, lane 6). A blot of the products (Figure 6) indicated that only EDTA was able to inhibit completely the membrane-mediated cleavage of the substrate, confirming the enzyme(s) responsible to be a metalloproteinase. E-64 did seem to have a partial inhibitory effect on generation of the 66 kDa fragment labelled by the antiserum. This could represent the inhibition of a cysteine proteinase activity responsible for a further C-terminal cleavage of the aggrecanase-generated product. Although leupeptin did not seem to inhibit generation of this product, it is possible that the enzyme responsible was unaffected by this compound; leupeptin and E-64 are known to differ in their abilities to inhibit some cysteine proteinases.

The naturally occurring inhibitors of the MMPs, TIMP-1 and TIMP-2, were also tested for their ability to inhibit the cleavage of the aggrecan substrate by the membrane preparations. TIMP-1 was used at 5 µg/ml (100 units/ml, approx. 179 nM) and TIMP-2 at 10 µg/ml (100 units/ml, approx. 455 nM). These concentrations would be sufficient to inhibit completely the proteolytic activity of the MMPs. Figure 7 demonstrates the inability of either TIMP-1 (lane 2) or TIMP-2 (lane 4) to inhibit...
the generation of immunoreactivity, suggesting that the MMP family is not involved in the membrane-mediated substrate cleavage.

A number of metalloproteinase inhibitors were also tested in this assay system (Figure 8), including phosphoramidon (lane 3), thiorphan (lane 4), CH1707 (lane 5) and BB94 (lane 6). Phosphoramidon is a phosphorus-containing peptide that is a specific and potent inhibitor of neutral metalloendopeptidases such as thermolysin and neprilysin. Thiorphan, a thiol-containing compound, was developed as a synthetic and potent inhibitor of neprilysin; it also inhibits angiotensin-converting enzyme. BB94 (batimastat) is a broad-spectrum MMP inhibitor with $K_c$ values in the low nanomolar range against purified MMPs. CH1707 is also active against the MMPs, but at higher concentrations (micromolar $K_c$ values). Each of these compounds was used at a final assay concentration of 1 $\mu$M. Figure 8 shows that of the compounds tested, only BB94, a hydroxamate-based metalloproteinase inhibitor, inhibited the generation of immunoreactivity similar to that seen with 20 mM EDTA (lane 2).

To investigate whether an activation of the enzyme activity was occurring during the 24 h incubation of membranes with substrate, the membranes were either pretreated with trypsin ($50 \mu$g/ml for 15 min at room temperature) which had been inactivated before substrate addition with 500 $\mu$g/ml soybean trypsin inhibitor, or incubated in the presence of 4-amino-phenylmercuric acetate (APMA; 0.66 mM). Immunoblotting products of the reaction after incubation for 3, 6, 12 or 24 h indicated that no activation of the enzyme activity had occurred with either treatment (results not shown). This might suggest either that the activity is expressed in an active form on the membranes or that a different activation process is required.

**DISCUSSION**

We have demonstrated for the first time the existence of an enzyme activity on the membranes of stimulated chondrocytes that is able to cleave an aggrecan substrate at the aggrecanase cleavage site. Early work by Dingle and Dingle [21] used a cavity organ-culture system to investigate the breakdown of PG within the cartilage matrix under controlled conditions. The addition of IL-1 to radiolabelled PG inserted within the cartilage cavity resulted in rapid breakdown of this soluble PG. However, if the PG was first bound in polyacrylamide beads before insertion into the cavity, IL-1 was unable to induce the breakdown even though this PG was shown to be accessible to soluble proteinases. This led to the conclusion that the enzyme responsible for the degradation might be restricted to the pericellular micro-environment of the stimulated chondrocyte. Work by Morales and Hascall [22] had also implicated the involvement of a chondrocyte membrane-bound proteinase in PG degradation. Their studies indicated a close correlation between the catabolism of PG and hyaluronate (the extracellular matrix component to which aggrecan monomers bind in the cartilage). It was proposed that the degradation of this complex in the cartilage might involve binding of the hyaluronate to the chondrocyte surface, removal of the PG from the hyaluronate backbone by a membrane-associated proteinase, and subsequent internalization of the hyaluronate and breakdown by lysosomal enzymes.

The study presented here would also support the hypothesis that aggrecanase activity can be localized on the chondrocyte membrane. Membrane fractions isolated from stimulated chondrocytes were able to cleave an aggrecan substrate, generating a number of fragments recognized by an antibody against the aggrecanase N-terminal neoepitope. The membrane fraction used in these experiments was enriched in a plasma membrane marker enzyme, 5'-nucleotidase, thus suggesting that the activity was localized to the plasma membrane. It was further shown that this activity was completely inhibited in the presence of EDTA, a metal chelator, but not inhibited by other class-specific proteinase inhibitors, implying that the enzyme belongs to the metalloproteinase family. Given the importance of the MMPs in the breakdown of collagen within the extracellular matrix, and the increased levels of these enzymes found in osteoarthritic and rheumatoid cartilage [23], it has been suggested that PG breakdown might also be mediated by one of the members of this family. So far a number of the MMPs have been tested for their ability to cleave aggrecan at the aggrecanase site [8,24,25], and although all have been shown to cleave at a second site within the IGD (between an asparagine residue and a phenylalanine residue), only one, MMP-8, is capable of effecting the aggrecanase cleavage [26]. Recent evidence [27] has, however, suggested that this enzyme is not aggrecanase because not only does MMP-8 cleave aggrecan preferentially at the MMP site, it also fails to generate the same pattern of aggrecan fragments as endogenously produced aggrecanase activity. Our results also suggest that the aggrecanase activity we have observed is not an MMP, as neither of two naturally occurring inhibitors of the MMPs, TIMP-1 and TIMP-2, was able to inhibit the generation of immunoreactive fragments.

A series of metalloproteinase inhibitors were also tested for their ability to inhibit this aggrecanase activity. Of those tested, only BB94, a small-molecular-mass hydroxamate-based compound, was able to inhibit the cleavage at the aggrecanase site. Although these compounds were initially designed to inhibit at the active site of the MMPs, their efficacy against other metalloproteinases is not fully known; indeed it has been shown that similar inhibitors are able to inhibit the activity of members of the reprolysin family of metalloproteinases [28]. The reprolysins are a family of snake venom proteins containing a metalloproteinase domain, and it has recently been shown that mammalian homologues of this family (named ADAMs) are expressed by a number of cell types. These mammalian enzymes are characterized by the presence of a transmembrane domain, and McKie et al. [29] have demonstrated that chondrocytes are able to express at least three of these proteins, one of which, named ADAM 12, is up-regulated at the mRNA level by stimulation with IL-1. A recently published paper by Tortorella et al. [30] has demonstrated that a reprolysin, atrolysin C, has the ability to cleave the IGD of aggrecan at both the MMP site and the aggrecanase site, and that the cleavage at the aggrecanase site might occur independently of the cleavage at the MMP site. Thus it is possible that the enzyme activity that we have observed in these experiments might be a member of this family, or belong to another group of the metalloproteinase family of enzymes.
Given the recent observations of Hughes et al. [12] indicating the presence of an aggrecanase activity in conditioned media from retinoic acid-stimulated bovine chondrocytes, it will be interesting to determine whether this activity represents a soluble form of the membrane-bound activity that we have described here. Many membrane proteins, e.g. angiotensin-converting enzyme and tumour necrosis factor, are known to occur also as soluble forms, and the soluble enzyme is often derived from the membrane-bound form by proteolysis mediated by a group of enzymes called ‘secretases’ or ‘sheddases’ [31]. Alternatively, the two enzyme activities might be independent of each other and represent individual members of an aggrecanase family.

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