Resistance of small leucine-rich repeat proteoglycans to proteolytic degradation during interleukin-1-stimulated cartilage catabolism

Robert SZTROLOVICS*, Robert J. WHITE†, A. Robin POOLE*‡, John S. MORT*‡ and Peter J. ROUGHLEY†‡¹

*Joint Diseases Laboratory, Shriners Hospital for Children, 1529 Cedar Avenue, Montreal, Quebec, Canada H3G 1A6, †Genetics Unit, Shriners Hospital for Children, 1529 Cedar Avenue, Montreal, Quebec, Canada H3G 1A6, and ‡Division of Surgical Research, Department of Surgery, McGill University, Montreal, Quebec, Canada H3G 1A4

A bovine nasal-cartilage culture system has been utilized to analyse the catabolic events occurring in response to interleukin-1 β over a 14-day period. An early event following the start of interleukin-1 treatment was the release of glycosaminoglycan into the culture medium. This release was accompanied by the appearance in the tissue, and shortly thereafter also in the culture media, of a globular domain (G1)-containing aggrecan degradation product generated by the action of aggrecanase. Link protein was also released from the cartilage with a similar timeframe to that of the G1 fragment, although there was no evidence of its proteolytic degradation. By comparison with aggrecan, the small leucine-rich repeat proteoglycans decorin, biglycan and lumican showed a resistance to both proteolytic cleavage and release throughout the culture period. In contrast, fibromodulin exhibited a marked decrease in size after day 4, presumably due to proteolytic modification, but the major

degradation product was retained throughout the culture period. Also in contrast with the early changes in the components of the proteoglycan aggregate, type II collagen did not display signs of extensive degradation until much later in the culture period. Collagen degradation products compatible with collagenase action first appeared in the medium by day 10 and increased thereafter. These data demonstrate that the leucine-rich repeat proteoglycans are resistant to proteolytic action during interleukin-1-stimulated cartilage catabolism, compared with aggrecan. This resistance and continued interaction with the surface of the collagen fibrils may help to stabilize the collagen fibrillar network and protect it from extensive proteolytic attack during the early phases of cartilage degeneration.

Key words: aggrecan, decorin, biglycan, fibromodulin, lumican.

INTRODUCTION

The hallmark feature of osteoarthritis and inflammatory joint disease is the progressive destruction of articular cartilage, thereby compromising joint function [1]. This is accompanied by the degradation of the major components of the cartilage extracellular matrix, including aggrecan and type II collagen [2]. Aggrecan is a large aggregating proteoglycan possessing a multi-domain structure [3]. At and near the N-terminus there are two globular domains, termed G1 and G2, respectively, separated by a short interglobular domain (IGD). G1 mediates the interaction of aggrecan with hyaluronic acid to form proteoglycan aggregates. Each interaction is stabilized by a link protein [4], and serves to anchor aggrecan in the tissue. Following G2 is the extended glycosaminoglycan (GAG)-attachment region, whose many keratan sulphate and chondroitin sulphate chains confer on cartilage its characteristic osmotic properties which are responsible for tissue hydration and resistance to compression [5]. The extreme C-terminus of aggrecan bears another globular domain, termed G3.

During aging and particularly in joint disease, aggrecan undergoes extensive proteolytic processing. This results in the loss of non-aggregating aggrecan fragments into the synovial fluid [6] and the accumulation of hyaluronate-binding degradation products in the tissue [7]. The use of anti-neoepitope antibodies recognizing specific aggrecan degradation products [8], as well as their characterization by N-terminal protein

sequencing [9,10], have identified two major sites of aggrecan proteolysis within the IGD between G1 and G2. One site is termed the matrix metalloproteinase (MMP) site, since several members of this proteinase family are able to cleave it *in vitro* [11]. The second site is termed the aggrecanase site and is cleaved by a proteinase whose identity has not yet been ascertained. Cartilage explant and chondrocyte cell cultures have been used to examine the proteolytic cleavage of aggrecan at these major sites following exposure to catabolic factors such as interleukin-1 (IL-1) [12] and retinoic acid [13]. These studies have indicated that the aggrecanase site is predominantly involved in response to these factors and that the degradation of aggrecan is an early event in cartilage catabolism.

Collagen is another important component of the cartilage extracellular matrix, providing the tissue with its tensile strength. A key event in the breakdown of type II collagen in cartilage is cleavage at the intrahelical collagenase site by interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) or collagenase-3 (MMP-13) [14,15]. Cleavage at this site results in the subsequent unwinding of the triple helix and susceptibility to further proteolytic attack, and may thus be important in tissue modelling and extracellular-matrix turnover. Collagen degradation initiated by collagenase cleavage may also play a role in arthritis. Collagenases are expressed and synthesized by different cell types present in the joint, including synovial cells and chondrocytes, in response to catabolic factors such as IL-1 [16], and they have also been detected in the synovial fluid of patients

Abbreviations used: DMEM, Dulbecco's modified Eagle medium; F(ab')₂, pepsin-generated fragment of immunoglobulin bearing the antigen-binding sites; GAG, glycosaminoglycan; IGD, interglobular domain; IL-1, interleukin-1; LRR-PG, leucine-rich repeat proteoglycan; MMP, matrix metalloproteinase; MMP-C and AGG-C, C-terminal epitopes generated by cleavage at the major aggrecan IGD, MMP and aggrecanase sites respectively.

¹ To whom correspondence should be sent, at the Genetics Unit address (e-mail proughley@shriners.mcgill.ca).

with osteoarthritis [17] and rheumatoid arthritis [18]. The use of antibodies to epitopes exposed only after collagen unwinding has shown that there is increased collagen denaturation in osteoarthritis [19]. Furthermore, the degree of collagen denaturation correlates with the extent of collagenase cleavage [15].

Although aggrecan and type II collagen are the major components of the extracellular matrix of articular cartilage, there are other matrix constituents which also play important functional roles. Of particular interest are the leucine-rich repeat proteoglycans (LRR-PGs), a family which includes the dermatan sulphate proteoglycans, decorin and biglycan, and the keratan sulphate proteoglycans, fibromodulin and lumican [20]. These molecules have the ability to interact with fibrillar collagens and are thought to be important in stabilizing fibril interactions [21–24]. Furthermore, their localization to fibril surfaces suggests that the LRR-PGs may provide collagen fibrils with some steric protection from the proteolytic environment present in degenerating cartilage if they show a relative resistance to proteolytic damage [25,26]. Despite the considerable amount of information on aggrecan catabolism in cartilage, little is known about the susceptibility of the LRR-PGs to proteolytic attack. This report describes a study in which cartilage explant cultures have been utilized to examine the effects of IL-1-stimulated catabolism on the degradation of aggrecan, type II collagen and the LRR-PGs with the purpose of determining how these events are interrelated. Bovine nasal cartilage was chosen for this study because of its ready availability and known susceptibility to matrix degradation induced by IL-1.

MATERIAL AND METHODS

Bovine cartilage explant cultures

Bovine nasal septal cartilages were obtained from mature animals at a local abattoir and transported to the laboratory for immediate use within 4 h of death. The tissue was dissected free of adherent connective tissue and the surrounding perichondrium was removed. Subsequently the cartilage was washed in Dulbecco's modified Eagle medium (DMEM) containing high concentrations of antibiotics, cut into blocks of approx. 5 mm³, which were divided into aliquots, weighed, and placed in 24-well culture plates (approx. 200 mg wet weight of tissue per well). It was then precultured in a 5% CO₂ incubator for 24 h in DMEM containing 44 mM sodium bicarbonate, 25 mM Hepes, 100 units/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 10 µg/ml BSA. This was followed by a 14-day period in which the tissue was cultured with the same medium supplemented with 5 ng/ml recombinant human IL-1β (R&D, Minneapolis, MN, U.S.A.). Since the weight of tissue differed between wells, the amount of medium added throughout the cultures was proportional to wet weight (1 ml of medium/100 mg of tissue). At 2-day intervals during the 14-day period, culture media were collected and fresh medium containing IL-1β was added. In addition, enough wells were set up so that tissue could also be harvested at these time points, including day 0 (the day on which IL-1β treatment commenced). Harvested media and tissues were stored at –20 °C until analysis.

Preparation and glycosidase treatment of tissue extracts

Frozen tissues were thawed, and excess moisture was removed by blotting. They were then weighed and extracted with 20 vol. (v/w) of 4 M guanidinium chloride/100 mM sodium acetate (pH 6.0), containing 1 mM disodium EDTA, 1 mM PMSF, 1 mM iodoacetamide and 10 µg/ml pepstatin A, for 48 h at 4 °C

[27]. The extracts were centrifuged for 5 min at 4 °C to remove particulate matter, and the supernatants were dialysed for 48 h at 4 °C against 10 mM sodium acetate, pH 6.0. During dialysis, extract volumes increased by approx. 15% of initial volumes. Before subsequent immunoblot analysis, extracts were treated with chondroitinase ABC (ICN, Montreal, Quebec, Canada). The enzyme was added at a final concentration of 0.1 unit/ml, and the incubation was performed at 37 °C for 4 h. For immunoblot analyses with the anti-aggrecan antibodies, chondroitinase ABC digestion was terminated by heating at 100 °C for 10 min, and the extracts were then incubated with 0.01 unit/ml keratanase II (Seikagaku, Ijamsville, MD, U.S.A.) at 37 °C overnight [28]. After glycosidase digestion, the samples were prepared for electrophoresis by the addition of 1 vol. of 2×SDS/PAGE sample buffer containing 10% β-mercaptoethanol [29].

SDS/PAGE and immunoblotting

All electrophoresis samples were denatured at 100 °C for 3 min and analysed on SDS/10% -polyacrylamide gels [29]. Following electrophoresis, resolved proteins were transferred to nitrocellulose (BioRad, Mississauga, ON, Canada) in Towbin buffer [30] at 100 V for 2 h in a TransBlot cell (Bio-Rad). The membranes were then blocked overnight in 3% BSA in Tris-buffered (10 mM, pH 8.0) saline. Immunodetection of the LRR-PGs (biglycan, decorin, fibromodulin and lumican), aggrecan degradation products and link protein was performed utilizing first-step specific anti-peptide antibodies and a second-step alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibody (Promega, Madison, WI, U.S.A.) or anti-(mouse IgG) (Sigma, St. Louis, MO, U.S.A.) antibody [31]. The colour was developed using Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate.

Source of antibodies for immunoblotting

Rabbit antibodies specific for the G1-containing aggrecan degradation products generated by cleavage at the IGD metalloproteinase and aggrecanase sites have previously been described [28]. These anti-neoepitope antibodies (MMP-C and AGG-C) were raised against synthetic peptides corresponding to the new C-termini created upon cleavage at these sites, and have been shown to be highly specific for the respective cleaved sequences relative to the uncleaved IGD sequence. In addition to the anti-neoepitope antibodies, a general anti-G1 antibody was raised against a mixture of peptides whose sequences were derived from different regions of the G1 domain [28]. The mouse monoclonal antibody used to detect link protein, 8A4, has previously been described [32]. Rabbit antibodies specific for biglycan, decorin, fibromodulin and lumican were raised using synthetic peptides corresponding to the C-termini of their respective core proteins. The antibodies specific for biglycan and fibromodulin have already been described [31,33]. Those specific for decorin and lumican were prepared using the same approach. Briefly, the peptides were synthesized on an Applied Biosystems model 431A peptide synthesizer, purified by HPLC and coupled to ovalbumin through a cysteine residue added at their N-termini utilizing *N*-hydroxysuccinimidyl bromoacetate [34]. The conjugates were injected into rabbits for the production of antisera, and specific immunoglobulins were purified by affinity chromatography using their respective immobilized peptides [28].

GAG assay

Sulphated GAG was measured in media and tissue extracts utilizing the dimethylMethylene Blue dye-binding colorimetric assay modified for use in a microwell format [35]. Samples of

media or dialysed guanidinium chloride extract from uncultured cartilage (10 or 20 μ l) were combined with assay reagent (46 μ M dimethylmethylene Blue/40 mM glycine/40 mM sodium chloride, pH 3.0) to a final volume of 200 μ l in a 96-well plate. The absorbances were immediately read at 530 nm on a BioTek Instruments Elx808 reader (Winooski, VT, U.S.A.), and the data were processed with the KC4 Kineticalc software (BioTek Instruments).

Analysis of type II collagen cleavage by collagenase

Culture media were assayed for the C-terminus of the primary collagenase cleavage site using the COL2-3/4C rabbit antibody in an inhibition ELISA assay [15]. Briefly, aliquots of culture media (600 μ l) were dried in a Savant Speedvac and reconstituted in 200 μ l of water. Samples of media or of peptide standard (containing the immunizing neopeptide) were preincubated with F(ab')₂ for 1 h at 37 °C, and the mixtures were then added to plates pre-coated with a neopeptide-containing peptide-BSA conjugate. Binding of the F(ab')₂ fragment was detected using an alkaline phosphatase-conjugated goat anti-(rabbit IgG) second antibody (Sigma) and an NADPH/iodonitrotetrazolium/diaphorase violet amplification system (Gibco-BRL, Mississauga, ON, Canada). Percentage inhibition of F(ab')₂ binding was calculated for samples and standards, and the levels of the COL2-3/4C neopeptide in culture media were expressed as concentration equivalents of the neopeptide-containing peptide standard.

RESULTS

GAG release and aggrecan degradation

In order to assess the extent of proteoglycan release from bovine nasal cartilage stimulated with IL-1 β , culture media were assayed for GAG. Tissue GAG was released early in the culture period, with the vast majority of total tissue GAG lost in the first 4 days, and thereafter there was little further loss (Figure 1). The nature of the molecular process causing proteoglycan release was investigated by Western blotting using anti-neopeptide antibodies specific for aggrecan degradation products containing the N-terminal G1 domain resulting from proteolytic cleavage at the major MMP (MMP-C) or aggrecanase (AGG-C) sites within the interglobular domain. Western-blot analysis of tissue extracts revealed that neither neopeptide was present at detectable levels in uncultured bovine nasal cartilage and in tissue subjected only to preculture without IL-1 β . While the MMP-C neopeptide

Figure 1 Effect of IL-1 β on GAG release from bovine nasal cartilage

The tissue was cultured with 5 ng/ml IL-1 β for 14 days. Media were collected at 2-day intervals and analysed for GAG content. The values obtained are reported as a percentage of the GAG extractable from uncultured tissue.

Figure 2 Western-blot analysis of the aggrecanase-generated aggrecan G1 domains in extracts and culture media of IL-1 β -treated bovine nasal cartilage

Tissue extracts (A) and media (B) were analysed using the AGG-C anti-neopeptide antibody specific for the G1-containing aggrecan degradation product generated by aggrecanase action. Extracts were analysed for tissues cultured with IL-1 β for 0–14 days and the media were analysed at 2-day intervals. The arrows on the right indicate the migration positions of molecular-mass markers (shown in kDa).

continued to be undetected throughout the culture period (results not shown), the AGG-C neopeptide was detected in tissue extracts by day 2 (Figure 2A), appearing as a doublet with a molecular-mass range of approx 70–80 kDa. The neopeptide remained at comparable levels until day 6, but subsequently decreased in abundance. This loss was apparently due to the release of the neopeptide into the culture media with a temporal pattern which mirrored its presence in the tissue, but with a slight lag period (Figure 2B). Cleavage at the aggrecanase site appeared to be the major proteolytic event causing dissociation of the GAG-attachment domain from the G1 domain, since blotting analysis of both tissue extracts and culture media with a general anti-G1 antibody revealed only degradation products comigrating with those detected with the AGG-C antibody (results not shown). These data indicate that adult bovine cartilage stimulated with IL-1 β undergoes rapid aggrecanase-mediated aggrecan degradation and that the GAG-containing fragments and G1 domain are both subsequently lost from the tissue, but at different rates.

Analysis of link protein

It was also of interest to determine whether the catabolic effects of IL-1 β on the proteoglycan aggregate were specific to aggrecan or whether the other protein component, link protein, also

Figure 3 Western-blot analysis of link protein in extracts and culture media of IL-1 β -treated bovine nasal cartilage

Tissue extracts (A) and media (B) were analysed using the 8A4 antibody specific for link protein. Extracts were analysed for tissues cultured with IL-1 β for 0–14 days, and the media were analysed at 2-day intervals. The arrows on the right indicate the migration positions of molecular-mass markers (shown in kDa).

underwent proteolysis and/or loss. Thus tissue extracts and culture media were analysed by Western blotting using an antibody specific for link protein. The analyses demonstrated that while IL-1 β treatment did not result in the appearance of link-protein degradation products during the culture period, it resulted in a dramatic loss of link protein from the tissue (Figure 3A), in a manner similar to the aggrecanase-generated G1 domain of aggrecan. The loss of link protein from the tissue was accompanied by its appearance in the medium (Figure 3B), also in a pattern similar to that for the G1 domain. The concomitant release of aggrecan degradation products and link protein suggests the involvement of a common catabolic mechanism. It is noteworthy that the proteolytic fragments of link protein (with molecular masses of approx. 30 kDa) already present in the tissue prior to IL-1 β treatment were subsequently lost from the tissue but were not detected in the culture media, reflecting their further degradation.

Analysis of LRR-PGs

Having demonstrated that aggrecan degradation occurs early in IL-1-stimulated cartilage catabolism, it was of interest to determine whether the LRR-PGs biglycan, decorin, fibromodulin and lumican are also subjected to a similar proteolytic attack. Thus these small proteoglycans were studied by Western-blot analysis of tissue extracts, using antibodies specific for the C-termini of their respective core proteins. The dermatan sulphate

Figure 4 Western-blot analysis of biglycan and decorin in extracts of IL-1 β -treated bovine nasal cartilage

Tissue extracts were analysed using antibodies specific for the respective core proteins of biglycan (A) and decorin (B). Extracts were analysed for tissues cultured with IL-1 β for 0–14 days. The arrows on the right indicate the migration positions of molecular-mass markers (shown in kDa).

proteoglycans biglycan and decorin did not exhibit any changes in structure or abundance throughout the culture period, indicating that they are relatively resistant to the degradative processes affecting aggrecan and link protein (Figures 4A and 4B). Similar to biglycan and decorin, the keratan sulphate proteoglycan lumican also did not exhibit any signs of proteolytic damage or tissue loss during the culture period (Figure 5A). In contrast, the other keratan sulphate proteoglycan studied, fibromodulin, displayed a clear change in structure characterized by the appearance of an additional component (approx. 50 kDa) by day 4 of the culture period (Figure 5B). The higher-molecular-mass component present at all time points (approx. 60 kDa) is likely the intact glycoprotein form of fibromodulin lacking keratan sulphate. Thus the lower-molecular-mass component present by day 4 must be a degradation product resulting from the proteolytic cleavage of fibromodulin near its N-terminus, since the antibody used in Western blotting recognizes the C-terminal extremity of the core protein. The analysis of culture media did not reveal extensive release of any of the LRR-PGs from the cultured tissues (results not shown).

Degradation of type II collagen

Another important event in cartilage catabolism is the degradation of type II collagen, a matrix component with which the

Figure 5 Western-blot analysis of lumican and fibromodulin in extracts of IL-1 β -treated bovine nasal cartilage

Tissue extracts were analysed using antibodies specific for the respective core proteins of fibromodulin (A) and lumican (B). Extracts were analysed for tissues cultured with IL-1 β for 0–14 days. The arrows on the right indicate the migration positions of molecular-mass markers (shown in kDa).

Figure 6 Analysis of collagenase cleavage epitope release from IL-1 β -treated bovine nasal cartilage

Culture media were collected at 2-day intervals from tissue treated for 14 days with IL-1 β . The media were analysed by competitive inhibition ELISA for the presence of the COL2-3/4C neopeptide generated by collagenase-mediated cleavage of type II collagen. The values are reported as concentration equivalents of a peptide containing the COL2-3/4C neopeptide.

LRR-PGs interact. Thus we determined the time during the culture period when collagen degradation occurred. This was assessed by measuring the generation and release of a type II

collagen neopeptide (COL2-3/4C), following collagenase-mediated cleavage. The levels of the COL2-3/4C neopeptide in culture media were low during the first 8 days of the culture period (Figure 6), then started to increase by day 10 and continued to do so until the end of the culture period. Although these data do not reveal the time at which the initial collagenase-mediated cleavage occurs, they indicate that further collagen degradation resulting in the release of the COL2-3/4C neopeptide occurs much later in the culture period than aggrecan degradation and somewhat less delayed with respect to the fibromodulin modification.

DISCUSSION

The present paper describes the use of a bovine nasal-cartilage system to examine the degradative events occurring during IL-1 β -stimulated catabolism. The effects on the major structural components of cartilage extracellular matrix, aggrecan and type II collagen, have been investigated, together with the LRR-PGs, whose functions have not yet been firmly established, but which may have important roles in matrix organization and integrity. The results suggest that the molecular events following IL-1-stimulated bovine nasal cartilage degradation can be divided into three temporal phases. The first phase immediately following the initiation of IL-1 treatment (days 0–4) is characterized by aggrecanase-mediated aggrecan proteolysis and a rapid loss of the resulting GAG-containing fragments. The second phase (days 4–10) is characterized by the continued progressive loss of the aggrecan G1 fragment and link protein and the proteolytic modification of the collagen-binding LRR-PG fibromodulin. The final phase (days 10–14) is characterized by the release from the tissue of the COL2-3/4C neopeptide, indicative of proteolytic degradation of collagenase-cleaved type II collagen. These events occur independently of detectable cleavage of the other LRR-PGs. While other investigators have shown that there is a lag between aggrecan and collagen degradation [36], this is the first report relating the small leucine-rich proteoglycans to these events.

The concomitant release of aggrecan G1 domains and link protein from the tissue suggests that a common mechanism may be involved, such as hyaluronic acid depolymerization. Although hyaluronidases could be possible mediators of this hyaluronate degradation, these enzymes have yet to be identified in the extracellular matrix of cartilage, and free radicals are the more likely candidates, having been shown to be produced in cartilage treated with IL-1 [37]. Such an action of free radicals would have a very detrimental effect on tissue function, releasing both intact and cleaved aggrecan molecules from the extracellular matrix. It is noteworthy that whereas aggrecan is acutely sensitive to IL-1-stimulated proteolysis, mediated by aggrecanase, intact link protein shows no signs of overt proteolytic damage. There is no evidence of an increase in abundance of the link-protein degradation products already present in the tissue prior to IL-1 treatment. However, the more extensive link-protein degradation products seen in the tissue are not observed in the culture media, indicating that these forms of fragmented link protein are more susceptible to proteolysis.

The apparent resistance to proteolytic degradation of the LRR-PGs, other than fibromodulin, by comparison with aggrecan can be explained in different ways. Firstly, it is possible that the LRR-PGs examined in this study do not possess sites for efficient cleavage by aggrecanase, the predominant proteolytic activity generated in this tissue-culture system that is able to cleave aggrecan. The identification of several putative aggrecanase cleavage sites within the aggrecan molecule has allowed

the assessment of the structural requirements of this enzyme. Apart from the apparent requirement for a glutamic acid residue on the N-terminal side of the scissile peptide bond, there seem to be no other striking structural requirements except for perhaps a small non-polar residue in the P₁' position. An examination of the amino acid sequences of the LRR-PGs reveals many possible occurrences of this consensus sequence in all four molecules. Thus the resistance of the LRR-PGs to aggrecanase-mediated proteolysis seemingly cannot be explained by a lack of potential cleavage sites. Likewise, the reason for the eventual proteolytic cleavage of only fibromodulin is not readily explained, since its N-terminus is not unique in containing potential aggrecanase cleavage sites. It is also noteworthy that decorin has been demonstrated to be resistant to degradation by interstitial collagenase in solution [38]. This is consistent with the present data, which show the resistance of decorin to degradation even during a period of cartilage degradation characterized by evidence of collagenase action.

A second possible explanation for LRR-PG resistance to degradation is that they are protected from degradation due to their microenvironment in the extracellular matrix. This may be due to their interaction with collagen fibrils or other extracellular-matrix components, thereby limiting protease access due to a steric effect. Alternatively, the LRR-PGs may be segregated from the proteases activated following IL-1 treatment. This can be envisaged to occur if the activated proteases were localized to the pericellular matrix, whereas the bulk of the collagen-bound LRR-PGs would be present in the territorial and interterritorial regions of the matrix. However, the latter scenario is rather unlikely in view of the extent of aggrecan degradation taking place in this culture system and the distribution of aggrecan throughout the cartilage.

Finally, one could argue that degradation products of the LRR-PGs may not be detectable by an antibody whose epitope resides at the extreme C-terminus of the core protein, if cleavage were to occur solely within or adjacent to this site. There is only one report of processing involving this site, which occurs in mature adult human patellar cartilage [39], but there is no evidence for such processing in bovine cartilage at any age. In younger human cartilage, the only evidence for proteolytic processing in any LRR-PG is that reported in biglycan [31], which occurs at the N-terminus of its core protein [40]. Furthermore, it is difficult to envisage why proteolytic processing of LRR-PGs should be restricted to only a single site and one might then expect some degradation products to be detectable with the antibodies being used. In addition, the absence of LRR-PG loss from the tissue during the culture period would suggest that proteolytic processing of the LRR-PGs is not a prominent event, as any degradation would require replacement by the synthesis of additional proteoglycan, which is expected to be down-regulated in the presence of IL-1 [41].

Although three LRR-PGs are resistant to degradation, fibromodulin does undergo limited proteolysis following IL-1 treatment, albeit well after aggrecan has been extensively degraded. Since the cleavage of fibromodulin results in the loss of its N-terminal domain, it might be expected to have an effect on its function in the extracellular matrix. However, such an effect is difficult to identify, since the functions of this domain have yet to be ascertained. One possible property which could be affected by proteolytic attack is the binding to collagen fibrils. However, the onset of fibromodulin cleavage is accompanied by only minimal release of the degradation product into the culture medium, suggesting that collagen binding is not significantly affected by the cleavage. Degradation causing the release of collagenase-cleaved collagen molecules occurs only after the onset of fibro-

modulin cleavage. Therefore it is possible that fibromodulin, and possibly other members of the LRR-PG family, could serve to protect the collagen-fibril network from proteolysis, either by sterically limiting the access of collagenases or by preventing subsequent denaturation and limiting the attack by gelatinases. Different LRR-PGs, in particular decorin and fibromodulin, have previously been shown to bind to distinct regions of the collagen fibril [42]. It is noteworthy that the keratan sulphate proteoglycans have been shown to localize to the a and c bands of type I collagen fibrils [43], and these encompass the region of the collagen molecule adjacent to the collagenase cleavage site. Thus one could speculate that the proteolytic cleavage of fibromodulin may induce a conformational change that could provide access to the collagenase cleavage site without completely abrogating binding of the proteoglycan to the fibril. Release of collagen degradation products would not be an immediate consequence of such cleavage, but would lag behind, depending on the rate at which gelatinases or other proteinases could attack the collagen following its denaturation adjacent to the collagenase cleavage site. Rapid collagen damage is likely to require proteinases other than aggrecanase, and could be associated with the proteolytic environment found in the arthritic degeneration of cartilage.

We thank Ms. M. Ionescu for technical assistance and Ms. J. Wishart for preparing the Figures. This work was supported by the Arthritis Society of Canada and the Shriners of North America.

REFERENCES

- Buckwalter, J. A. and Mankin, H. J. (1997) *J. Bone Joint Surg.* **79A**, 612–613
- Poole, A. R., Alini, M. and Hollander, A. P. (1995) in *Mechanisms and Models in Rheumatoid Arthritis* (Henderson, B., Edwards, J. C. W. and Pettipher, E. R., eds.), pp. 163–204, Academic Press, New York
- Hardingham, T. E. and Fosang, A. J. (1992) *FASEB J.* **6**, 861–870
- Neame, P. J. and Barry, F. P. (1993) *Experientia* **49**, 393–402
- Hascall, V. C. (1988) *ISI Atlas Sci. Biochem.* **1**, 189–198
- Witter, J., Roughley, P. J., Webber, C., Roberts, N., Keystone, E. and Poole, A. R. (1987) *Arthritis Rheum.* **30**, 519–529
- Roughley, P. J., White, R. J. and Poole, A. R. (1985) *Biochem. J.* **231**, 129–138
- Hughes, C. E., Caterson, B., Fosang, A. J., Roughley, P. J. and Mort, J. S. (1995) *Biochem. J.* **305**, 799–804
- Sandy, J. D., Flannery, C. R., Neame, P. J. and Lohmander, L. S. (1992) *J. Clin. Invest.* **89**, 1512–1516
- Flannery, C. R., Lark, M. W. and Sandy, J. D. (1992) *J. Biol. Chem.* **267**, 1008–1014
- Fosang, A. J., Neame, P. J., Last, K., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1992) *J. Biol. Chem.* **267**, 19470–19474
- Sandy, J. D., Neame, P. J., Boynton, R. E. and Flannery, C. R. (1991) *J. Biol. Chem.* **266**, 8683–8685
- Ilic, M. Z., Handley, C. J., Robinson, H. C. and Mok, M. T. (1992) *Arch. Biochem. Biophys.* **294**, 115–122
- Martel-Pelletier, J. and Pelletier, J. P. (1996) *Br. J. Rheumatol.* **35**, 818–820
- Billinghurst, R. C., Dahlberg, L., Ionescu, M., Reiner, A., Bourne, R., Rorabeck, C., Mitchell, P., Hambor, J., Diekmann, O., Tschesche, H. et al. (1997) *J. Clin. Invest.* **99**, 1534–1545
- Poole, A. R. (1997) in *Arthritis and Allied Conditions: a Textbook of Rheumatology* (Koopman, W. J., ed.), pp. 255–308, Williams and Wilkins, Baltimore
- Lohmander, L. S., Hoerrner, L. A. and Lark, M. W. (1993) *Arthritis Rheum.* **36**, 181–189
- Clark, I. M., Powell, L. K., Ramsey, S., Hazleman, B. L. and Cawston, T. E. (1993) *Arthritis Rheum.* **30**, 372–379
- Hollander, A. P., Pidoux, I., Reiner, A., Rorabeck, C., Bourne, R. and Poole, A. R. (1995) *J. Clin. Invest.* **96**, 2859–2869
- Iozzo, R. V. (1997) *Crit. Rev. Biochem. Mol. Biol.* **32**, 141–174
- Vogel, K. G., Paulsson, M. and Heinegård, D. (1984) *Biochem. J.* **223**, 587–597
- Hedborn, E. and Heinegård, D. (1989) *J. Biol. Chem.* **264**, 6898–6905
- Schönherr, E., Witsch-Prehm, P., Harrach, B., Robenek, H., Rauterberg, J. and Kresse, H. (1995) *J. Biol. Chem.* **270**, 2776–2783
- Rada, J. A., Cornuet, P. K. and Hassell, J. R. (1993) *Exp. Eye Res.* **56**, 635–648
- Scott, J. E. (1992) *FASEB J.* **6**, 2639–2645
- Scott, J. E. (1996) *Biochemistry* **35**, 8795–8799

-
- 27 Roughley, P. J. and White, R. J. (1980) *J. Biol. Chem.* **255**, 217–224
- 28 Sztrolovics, R., Alini, M., Roughley, P. J. and Mort, J. S. (1997) *Biochem. J.* **326**, 235–241
- 29 King, J. and Laemmli, U.K. (1971) *J. Mol. Biol.* **62**, 465–477
- 30 Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4350–4354
- 31 Roughley, P. J., White, R. J., Magny, M.-C., Liu, J., Pearce, R. H. and Mort, J. S. (1993) *Biochem. J.* **295**, 421–426
- 32 Caterson, B., Christner, J. E., Baker, J. R. and Couchman, J. R. (1985) *Fed. Proc. Fed. Am Soc. Exp. Biol.* **A44**, 386–393
- 33 Roughley, P. J., White, R. J., Cs-Szabó, G. and Mort, J. S. (1996) *Osteoarthritis Cartilage* **4**, 153–162
- 34 Bernatowicz, M. S. and Matsueda, G. R. (1986) *Anal. Biochem.* **155**, 95–102
- 35 Hollander, A. P., Atkins, R. M., Eastwood, D. M., Dieppe, P. A. and Elson, C. J. (1991) *Clin. Exp. Immunol.* **83**, 52–57
- 36 Kozaci, L. D., Buttle, D. J. and Hollander, A. P. (1997) *Arthritis Rheum.* **40**, 164–174
- 37 Tikku, M. L., Liesch, J. B. and Robertson, F. M. (1990) *J. Immunol.* **145**, 690–696
- 38 Imai, K., Hiramatsu, A., Fukushima, D., Pierschbacher, M. D. and Okada, Y. (1997) *Biochem. J.* **322**, 809–814
- 39 Witsch-Prehm, P., Miehke, R. and Kresse, H. (1992) *Arthritis Rheum.* **35**, 1042–1052
- 40 Roughley, P. J., White, R. J. and Mort, J. S. (1996) *Biochem J.* **318**, 779–784
- 41 Von den Hoff, H., de Koning, M., van Kampen, J. and van der Korst, J. (1995) *J. Rheumatol.* **22**, 1520–1526
- 42 Hedbom, E. and Heinegård, D. (1993) *J. Biol. Chem.* **268**, 27307–27312
- 43 Scott, J. E. (1988) *Biochem. J.* **252**, 313–323
-

Received 21 September 1998/11 January 1999; accepted 10 February 1999