Hyaluronate degradation as an alternative mechanism for proteoglycan release from cartilage during interleukin-1β-stimulated catabolism

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Data presented previously suggest that release of components of the cartilage matrix, in response to catabolic agents, cannot be accounted for by proteolytic mechanisms alone. In the present study, the release of glycosaminoglycan-containing components from bovine nasal cartilage cultured in the presence of interleukin-1β, and from bovine nasal, fetal bovine epiphyseal and adult human articular cartilage cultured in the presence of retinoic acid, was accompanied by the loss of link protein and hyaluronate into the culture medium. Chromatographic analysis of the released hyaluronate showed it to be markedly reduced in size relative to that extracted from the corresponding tissue. It is proposed that, under stimulation by catabolic agents, two independent, but concurrent, mechanisms act to promote the release of aggrecan from the cartilage matrix. First, proteolytic cleavage of the aggrecan core protein results in the production of glycosaminoglycan-containing fragments that are free to diffuse from the tissue. Secondly, cleavage of hyaluronate renders portions of the proteoglycan aggregate small enough so that complexes of aggrecan (or fragments containing its G1 domain) and link protein are released from the tissue. It is likely that both mechanisms contribute to cartilage metabolism in normal physiology and pathology.

Key words: aggrecan, bovine, human, link protein, retinoic acid.

INTRODUCTION

The large aggregating proteoglycan, aggrecan, performs a critical role in articular cartilage because of its capacity to interact with large quantities of water [1]. Its localization within the type II collagen network allows cartilage to undergo reversible deformation in response to compressive loading, due to the redistribution of its water content. Retention of aggrecan within the collagen network is achieved by the non-covalent association of a large number of aggrecan subunits with a continuous hyaluronate filament. This interaction is stabilized by a small glycoprotein, link protein, which binds both to the aggrecan N-terminal globular (G1) domain and to hyaluronate [2]. The extremely large size of the resulting proteoglycan aggregate is believed to be responsible for its retention within cartilage.

Aggrecan is a modular glycoprotein which consists of a series of domains that serve different functions [3]. The G1 domain mediates protein–protein interaction and binding to hyaluronate. It is connected to a second globular domain (G2) (with no presently known function [4]) through an extended region termed the interglobular domain (IGD), which is the target for cleavage by many proteases [5]. Regions containing keratan sulphate and chondroitin sulphate follow the G2 domain. The first region contains sites for substitution with keratan sulphate, and the adjacent region (CS1) contains chondroitin sulphate substitution sites. A subsequent third region (CS2) has a lower density of chondroitin sulphate substitution sites. Finally, the aggrecan C-terminus contains a third globular domain (G3), which is important for secretion of aggrecan from the cell [6].

It is well established that, following treatment of cartilage in culture with catabolic agents, such as interleukin-1β (IL-1β) or retinoic acid, material containing sulphated glycosaminoglycans (GAGs) is released into the culture medium [7]. It was also generally believed that the majority of the released GAG-containing material consists of proteolytically derived fragments of aggrecan cleaved at various points along the CS1 and CS2 domains and the IGD [8,9]. A consequence of this degradative pathway is that the aggrecan G1 domain would be expected to be retained in the cartilage matrix due to its interaction with hyaluronate, this complex being stabilized through the interaction of both of these components with link protein. However, recent studies have shown that, in some situations, intact aggrecan or G1-containing fragments are released from cartilage, suggesting that alternative release mechanisms are operative [9a]. Since aggrecan retention is thought to be dependent on the integrity of hyaluronate, the possibility of the cleavage and release of hyaluronate from different types of cartilage stimulated with retinoic acid or IL-1β was investigated, and its association with link protein and aggrecan or its degradation products was determined.

Here we show that, in systems where high levels of aggrecan loss occur, there is also a concomitant release of link protein and hyaluronate in a cleaved form, suggesting that fragmentation of this cartilage component may also represent a mechanism for proteoglycan loss from this tissue.

MATERIALS AND METHODS

Cartilage explant cultures

Adult bovine nasal septum cartilage and fetal femoral epiphyseal cartilage were obtained at a local abattoir. Adult human femoral

Abbreviations used: CS1 and CS2, the first and second chondroitin sulphate-containing regions of the aggrecan core protein; G1, G2 and G3, the three globular domains in the aggrecan core protein (N- to C-terminal orientation); GAG, glycosaminoglycan; IGD, interglobular domain (between the aggrecan G1 and G2 domains); IL-1β, interleukin-1β.

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condylar cartilage was obtained at autopsy from individuals with no history of joint disease. Cartilage was taken with the permission of the chief pathologist from individuals where the next of kin had given consent for a complete autopsy. The cartilage samples were cultured as described in the accompanying paper [9a] in Dulbecco's modified Eagle's medium buffered with 44 mM sodium bicarbonate and 25 mM Hepes and containing 10 mg/ml BSA at a tissue/medium ratio of 1:10 (w/v). Tissue samples, in duplicate, were precultured for 24 h, followed by a 6-day period during which the same medium was supplemented with 5 ng/ml recombinant human IL-1β (R&D, Minneapolis, MN, U.S.A.), 1 μM all-*trans*-retinoic acid (Eastman Kodak, Rochester, NY, U.S.A.) or no additions. Culture media were collected at 2-day intervals and replaced with fresh medium containing IL-1β, retinoic acid or no factor.

Preparation of tissue extracts

Tissue was extracted for 48 h at 4 °C with 20 vol. (v/w) of 4 M guanidinium chloride and 100 mM sodium acetate (pH 6.0) containing 1 mM disodium EDTA, 1 mM PMSF, 1 mM iodoacetamide and 10 μg/ml pepstatin A [10]. The extracts were centrifuged at 3000 ***g*** 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).

For analysis of total tissue hyaluronate, cartilage (50–100 mg) was digested with proteinase K (0.5 mg/100 mg of cartilage) in 0.1 M Tris/HCl, pH 7.6 at 56 °C overnight. Proteinase K was then inactivated by incubation at 100 °C for 20 min.

Hyaluronate assay

Hyaluronate (from human umbilical cord; Sigma, St. Louis, MO, U.S.A.) used as the standard was purified by a modification [11] of the method of Cleland and Sherblom [12]. Hyaluronate was quantified by a modification [13] of the competitive binding assay described by Goldberg [14]. First, 96-well plates (Immulon-2; Nunc) were coated with hyaluronate. Samples were incubated for 4 h with bovine nasal cartilage aggrecan, transferred to the hyaluronate-coated plates and incubated overnight at 4 °C. Bound aggrecan was determined using a monoclonal antibody to keratan sulphate (5D4; ICN, Montreal, Canada), followed by goat anti-(mouse Ig) serum conjugated to alkaline phosphatase and colour development.

To eliminate competition by aggrecan or other hyaluronate-binding proteins present in the sample, culture media and cartilage extracts were reduced and alkylated prior to analysis. Pooled culture medium (0.5 ml) was evaporated to dryness using a Speed Vac. The pellets were resuspended in 0.5 ml of 4 M guanidinium chloride/50 mM Tris/HCl, pH 7.35, containing 5 mM dithiothreitol and incubated at 40 °C for 4 h. Iodoacetamide was then added to a final concentration of 15 mM and the solution was allowed to stand at 25 °C overnight. The samples were dialysed into PBS for hyaluronate determination, or into column buffer for Sepharose CL-2B chromatography.

Size-exclusion chromatography

Pooled medium from days 2, 4 and 6 of culture (1 ml) was analysed on a Sepharose CL-2B column (116 cm × 1 cm) in 0.2 M sodium acetate, pH 5.5, at a flow rate of 6 ml/h. Column fractions were assayed for GAG content using the Dimethylmethylene Blue method [15], and for link protein by SDS/PAGE and immunoblotting. In addition, pooled medium was also analysed following reduction and alkylation, as described above. Fractions were assayed for hyaluronate content as described above, except that samples were in column buffer. This did not affect the performance of the hyaluronate assay.

Density-gradient centrifugation

Sepharose CL-2B column fractions containing link protein were pooled and caesium chloride was added to give a final density of 1.40 g/ml [10]. The tubes were centrifuged at 100000 ***g*** for 48 h and the gradients were fractionated, dialysed into sample buffer, and analysed for link protein content by SDS/PAGE and immunoblotting.

SDS/PAGE and immunoblotting

Samples were analysed on SDS/10 %-polyacrylamide gels. Following electrophoresis, resolved proteins were transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON, U.S.A.). The membranes were then blocked overnight with 3 % (w/v) BSA in Tris-buffered saline. The mouse monoclonal antibody 8A4 used to detect link protein has been described previously [16], and was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, U.S.A.). Bound antibody was visualized by incubation with an alkaline phosphatase-conjugated goat anti-(mouse IgG) antibody (Promega) and colour development using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [17]. In the case of column and gradient fractions, ECL® (Amersham) detection was used.

RESULTS

Release of link protein from cartilage explants in culture

In the accompanying paper [9a] it was shown that culture of bovine nasal, fetal bovine epiphyseal or human articular cartilage in the presence of retinoic acid resulted in dramatic release of GAG into the culture medium. Evidence for aggrecan cleavage in the IGD was observed for bovine nasal and human articular cartilage. In addition, retinoic acid promoted the release of the aggrecan G1 domain from bovine nasal cartilage and human articular cartilage. However, little evidence for cleavage of aggrecan in the IGD was seen in the case of fetal bovine epiphyseal cartilage, suggesting that intact proteoglycan, or aggrecan cleaved only in the chondroitin sulphate attachment region, was being lost from this tissue. To determine whether aggrecan components were being released in an aggregate form, the link protein content of the culture media was evaluated. In agreement with the high level of G1 release from bovine nasal cartilage in the presence of retinoic acid, release of link protein into the medium was observed (Figure 1A). Link protein was also released from bovine epiphyseal cartilage (Figure 1B), and to a lesser degree from human articular cartilage (Figure 1C). While retinoic acid stimulated GAG release from all three cartilage types, IL-1β was only effective in the case of bovine nasal cartilage, and liberation of link protein followed the same pattern (Figure 1A).

The bovine nasal cartilage, which was taken from adult animals, and adult human cartilage showed faster-migrating link protein bands, which, as reported by us previously [18,19], indicate that cleavage of the molecule had occurred *in situ* with aging. However, no additional proteolysis of link protein was observed following stimulation of the cultures with retinoic acid or IL-1β.

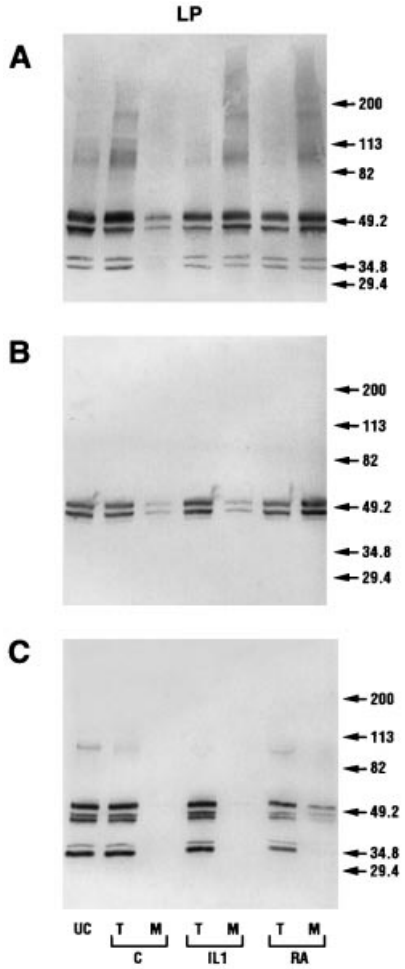

Figure 1 Release of link protein from cartilage cultures

Samples of culture medium (M) or tissue extract (T) from control (C), IL-1β-treated (IL-1) or retinoic acid-treated (RA) cartilage were separated by SDS/PAGE and immunoblotted using the monoclonal antibody 8A4 to detect link protein (LP). (**A**) Bovine nasal cartilage; (**B**) fetal bovine epiphyseal cartilage; (**C**) adult human articular cartilage. UC, untreated cartilage. In each case, sample loading represents material from the same amount of tissue. Positions of molecular mass markers (shown in kDa) are indicated on the right.

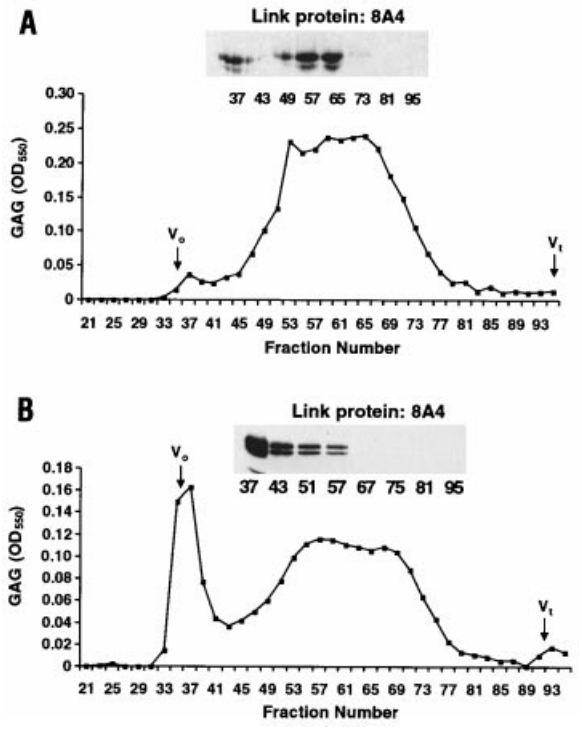

Figure 2 Size-exclusion analysis of GAG and link protein in cartilage culture media

Pooled culture medium (days 2–6) was analysed by Sepharose CL-2B chromatography, and the GAG content of the fractions was determined by measurement of absorbance (OD) at 550 nm. Selected fractions were analysed for link protein by immunoblotting (inset). V_o and V_t indicate the void volume and total column volume respectively. (**A**) Adult bovine nasal cartilage cultured in the presence of IL-1β; (**B**) fetal bovine epiphyseal cartilage cultured in the presence of retinoic acid.

Analysis of link protein function in bovine nasal cartilage cultures

Release of link protein from bovine nasal cartilage could be due either to its modification, rendering it unable to interact with aggrecan and hyaluronate, or to its release as part of a complex with these components. To determine the state of the released link protein, medium from bovine nasal cartilage cultured in the presence of IL-1β was analysed by Sepharose CL-2B chromatography under associative conditions (Figure 2A). The vast majority of the GAG-containing material was eluted in the included volume, indicating that most of the aggrecan was not released as an intact multimolecular aggregate. Western blotting analysis of the column fractions showed that a small portion of the link protein eluted with a minor void volume peak, but the majority was found to co-elute with the major GAG-containing peak, indicating that it is not present as a monomeric molecule, but is involved in interaction with the aggrecan fragments or another tissue component, or is undergoing association. In addition, G1 domains produced by proteolytic cleavage of aggrecan in the IGD co-eluted with the link protein (results not shown) and must therefore also be interacting with another tissue component.

To characterize the interactions of link protein further, the peak fractions containing both GAG and link protein (Figure 2A) were pooled and analysed further by associative caesium chloride density-gradient centrifugation (Figure 3). All of the link protein in this preparation migrated to the bottom of

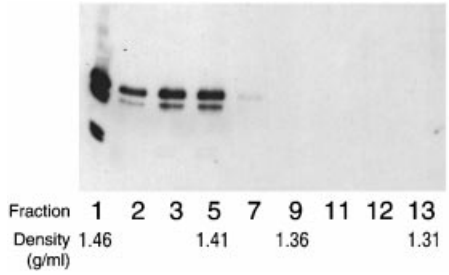

Figure 3 Density-gradient centrifugation analysis of link protein released from IL-1β-treated bovine nasal cartilage

Fractions 50–70 from the Sepharose CL-2B chromatographic analysis of IL-1β-treated bovine nasal cartilage (Figure 2A) were analysed, under associative conditions, by caesium chloride density-gradient centrifugation. The link protein content of the gradient fractions was determined by immunoblotting.

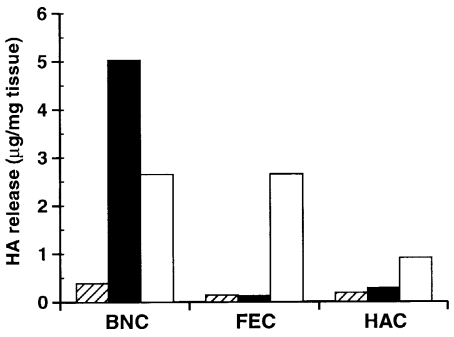

Figure 4 Hyaluronate release from cartilage cultures

Hyaluronate (HA) was quantified in pooled medium (days 2–6) from cultures of bovine nasal cartilage (BNC), fetal bovine epiphyseal cartilage (FEC) and adult human articular cartilage (HAC). Explants were cultured with 5 ng/ml IL-1β (solid bars), 1 μM retinoic acid (open bars) or no additions (hatched bars). Media samples were reduced and alkylated, and hyaluronate was assayed using the competitive ELISA method.

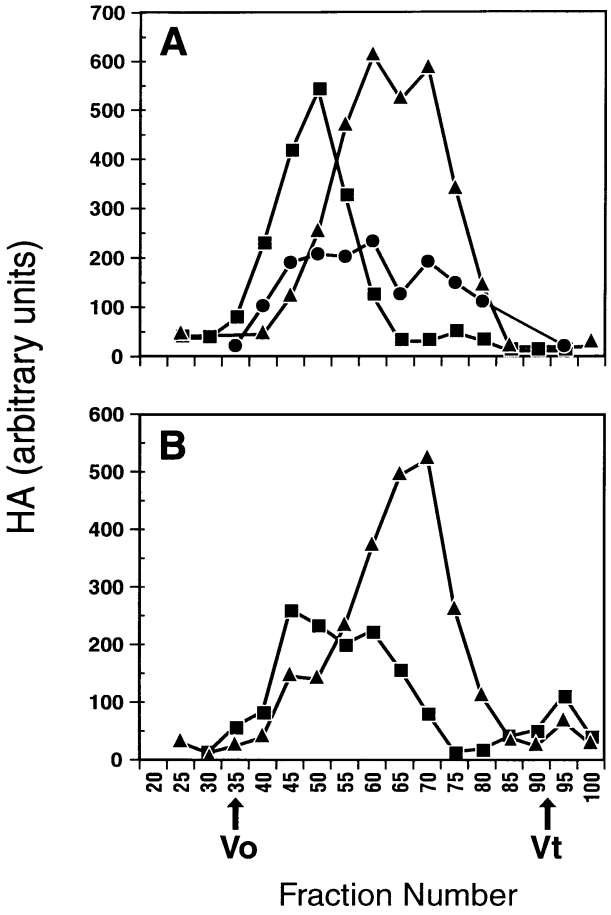

Figure 5 Hyaluronate size estimation

Culture medium and uncultured cartilage extract samples were reduced and alkylated, analysed by Sepharose CL-2B chromatography, and the fractions were assayed for hyaluronate (HA) content. ■, Uncultured cartilage extract; ▲, medium from retinoic acid-treated explant cultures; ●, medium from IL-1β-treated explant cultures. Vo and Vt indicate the void volume and total column volume respectively. (**A**) Bovine nasal cartilage; (**B**) fetal bovine epiphyseal cartilage.

the gradient, indicating that it formed a strong association with a tissue component of high density and was not merely undergoing self-association. This high-density component was not aggrecan, as the use of a higher starting density for centrifugation resulted in migration of link protein to the top of the gradient, while aggrecan remained at the bottom (results not shown). It is likely, therefore, that the link protein interacts with hyaluronate in these preparations.

Analysis of link protein in fetal bovine epiphyseal cartilage cultures

The previous analysis [9a] indicated that, although aggrecan was released from fetal bovine epiphyseal cartilage stimulated with retinoic acid, essentially no cleavage occurred in the IGD. Associative Sepharose CL-2B chromatography of culture medium showed that a large portion of the GAG-containing material eluted at the void volume, suggesting that relatively intact aggregates of aggrecan were released from the tissue under these conditions (Figure 2B). The majority of the link protein eluted with this void volume peak, while a smaller amount co-migrated with the included peak of GAG-containing material.

Measurement of hyaluronate release

The apparent release of stable proteoglycan aggregates from fetal bovine epiphyseal cartilage suggested that, in addition to aggrecan and link protein, hyaluronate is also released from cartilage under the appropriate stimulus. Using an inhibition ELISA method, hyaluronate content was measured in medium samples (Figure 4). Hyaluronate release was seen to mirror the release of GAG reported previously and also that of link protein, being essentially complete by day 8 of culture (results not shown). In bovine nasal cartilage, both IL-1β and retinoic acid stimulated hyaluronate release dramatically, with IL-1β being more effective than retinoic acid; these two agents induced the release of approx. 35% and 15% of tissue hyaluronate content respectively. However, with fetal bovine epiphyseal and adult human cartilage, only retinoic acid caused an increase in the hyaluronate content of the culture medium, inducing the release of approx. 25% and 5% of tissue hyaluronate respectively.

Size estimation of released hyaluronate

The sizes of the hyaluronate released into the culture medium relative to those of the material that could be extracted from the tissue with 4 M guanidinium chloride were compared by Sepharose CL-2B chromatography following reduction and alkylation of the samples. With bovine nasal cartilage, the endogenous hyaluronate eluted as a symmetrical peak close to the void volume, while the hyaluronate in the medium from retinoic acid-stimulated tissue eluted substantially later as a disperse peak (Figure 5A). This indicates that much of the hyaluronate was smaller in size than that present in cartilage, and suggests that partial depolymerization had occurred. The pattern was similar for the medium from IL-1β-stimulated cartilage, except that even more polydispersity was apparent.

The endogenous hyaluronate from fetal bovine epiphyseal cartilage eluted at a similar position, but as a slightly broader peak, compared with that extracted from bovine nasal cartilage (Figure 5B). Again the elution of the material released from tissue cultured in the presence of retinoic acid was much retarded, indicative of a smaller size. Hyaluronate released from retinoic acid-stimulated human articular cartilage showed a similar late elution profile (results not shown), suggesting that degradation of hyaluronate occurs as a consequence of stimulation of different cartilage types by retinoic acid or IL-1β.

DISCUSSION

The present study demonstrates that, for bovine and human cartilage cultured in the presence of catabolic stimulators, release of intact link protein and hyaluronate occurs in addition to that of aggrecan fragments. In the case of fetal epiphyseal cartilage, link protein release occurred in conjunction with release of aggrecan that had not undergone cleavage in the IGD, suggesting that a mechanism other than proteolysis may be responsible for the release of components of the proteoglycan aggregates in this tissue. In addition, in the other culture systems where aggrecan degradation was observed within the IGD, isolated G1 region was released from the tissue, also supporting the presence of a non-proteolytic mechanism for release. Furthermore, the released hyaluronate was much reduced in size relative to that which could be extracted from the original tissue, indicating that it had undergone partial depolymerization. These results are compatible with the release of aggrecan, link protein and hyaluronate fragments as complexes, but the mechanism underlying hyaluronate cleavage within the cartilage matrix is not clear at the present time. Depolymerization by reactive oxygen species known to be produced by chondrocytes is one possibility [20], as is cleavage by hyaluronidases, several of which are expressed in chondrocytes [21]. Hyaluronate cleavage could also provide an explanation for previous findings obtained using pig cartilage, where link protein and hyaluronate release had been reported [22,23]. The demonstration of hyaluronate fragmentation and release suggests that this process is also under the control of catabolic stimulators and contributes to the loss of components of the cartilage matrix.

While hyaluronate depolymerization appears to be a plausible explanation for the present data, one must consider the possibility of other mechanisms being responsible for the increase in hyaluronate release. This is particularly true for the action of IL-1β, which is known to stimulate hyaluronate production by chondrocytes [13], and it could therefore be argued that a burst of synthetic activity could potentially be responsible for the increased hyaluronate release induced by this cytokine. While some participation of this mechanism cannot categorically be discounted, there are several lines of evidence that suggest that it is not a major contributor. First, it is unlikely that new synthesis could account for the magnitude of hyaluronate release observed. Secondly, there is considerable evidence that most newly synthesized hyaluronate undergoes internalization and intracellular degradation rather than extracellular degradation and release into the culture medium [24–26]. In addition, it is not clear how the release of newly synthesized hyaluronate could account for the release of matrix-bound link protein, which in the absence of proteolytic modification should remain stably bound to hyaluronate in the tissue. The results of the present work show clearly that the released link protein is all bound to hyaluronate, and that much of the hyaluronate is of a decreased size compared with that in the tissue, indicating partial cleavage. In fact, for fetal bovine cartilage, approx. 25% of the released hyaluronate was smaller than any of the material present in the unstimulated tissue. It could be argued that such depolymerization of hyaluronate could have occurred in the culture medium following release. However, any degradative agent able to depolymerize hyaluronate in the medium would have to be generated by the chondrocytes as a consequence of the action of IL-1β, and it is difficult to explain why such agents would not act preferentially at their site of origin within the matrix.

A second mechanism to account for aggrecan release, but in this case without the requirement for hyaluronate cleavage, can be proposed, whereby 'mini-aggregates' composed of aggrecan

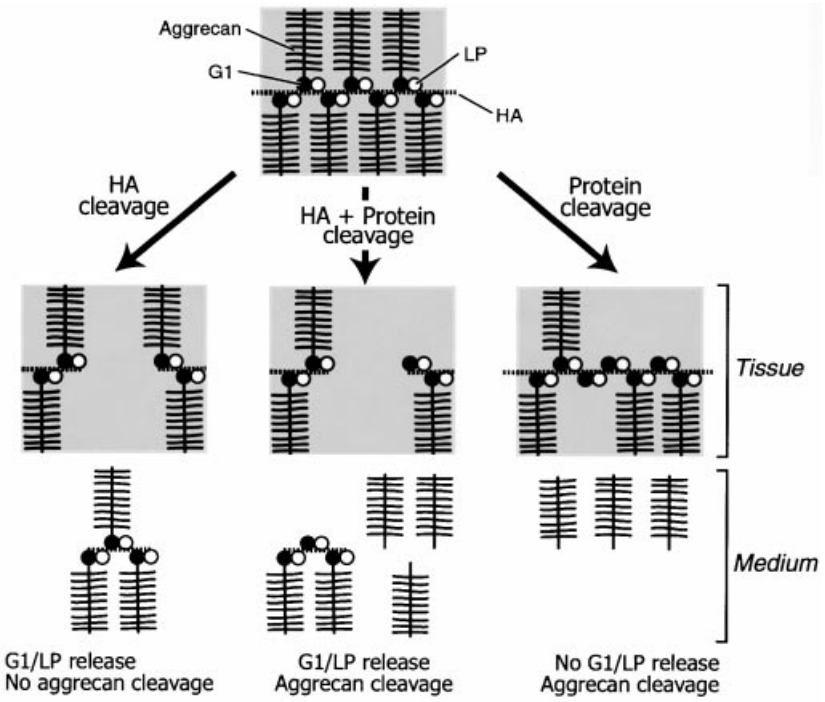

Figure 6 Representation of mechanisms resulting in the release of hyaluronate (HA), link protein (LP) and aggrecan from cartilage

The figure depicts the consequences of independent mechanisms for the degradation of proteoglycan aggregates – hyaluronate cleavage and protein cleavage – and the consequences of their combined action. For simplicity, aggrecan G2 and G3 regions are not indicated, and it is assumed that intact proteoglycan aggregates are unable to diffuse from the tissue without catabolic processing.

and link protein complexed with small hyaluronate fragments pre-exist in the matrix. Such complexes may be retained in the tissue, either due to their size or through lectin-like interactions between the G3 domain and other polyvalent components of the extracellular matrix such as tenascin, as has been proposed in brain for the aggrecan homologues neurocan and brevican [27]. Upon aggrecan proteolysis, these mini-aggregates could be lost from the tissue due to either their decreased size or elimination of G3-mediated interactions. Such a mechanism could account for the release of G1-containing components following cleavage by aggrecanase in the IGD or CS2 regions, but would not allow for the release of intact aggrecan, as was observed with fetal bovine cartilage cultured in the presence of retinoic acid.

A model can be proposed to account for the results presented (Figure 6). In the majority of studies of the effects of stimulatory agents on cartilage metabolism, only release of GAG is monitored, in large part due to the use of the simple and reproducible colorimetric assay developed by Farndale et al. [15]. The molecular nature of the released GAG is not, however, determined by this method. The data from the present study suggest that two independent mechanisms exist for the release of aggrecan from cartilage, and that the composition of the released material may be quite different depending on the tissue under study and the agents used to stimulate cartilage breakdown. Firstly, as has been well established, proteolytic cleavage of aggrecan at any point along the core protein prior to the G3 domain will result in the production of diffusible, GAG-containing products, although the closer to the G1 region the cleavage occurs, the higher the relative GAG yield will be. While aggrecan is an excellent substrate for many proteases *in vitro* [5,28], aggrecanases and matrix metalloproteinases fulfil this role predominantly *in vivo* [17]. The second mechanism for GAG release is fragmentation of hyaluronate. This allows the release of small complexes of link protein and intact or partially degraded aggrecan. It is likely that both of these degradative pathways are functioning simultaneously, but that they are controlled by different mechanisms. It is also possible that hyaluronate fragmentation could be facilitated by proteolysis of aggrecan, with aggrecan removal facilitating enzyme access or eliminating radical scavenging.

In the present study fetal bovine epiphyseal cartilage cultured in the presence of retinoic acid represents an example of aggrecan release where the second pathway is predominant. Two possible explanations can be proposed to explain this deficiency in proteolysis. First, retinoic acid may not increase the expression of the appropriate proteolytic enzymes (aggrecanases) in this particular tissue. However, examination of aggrecan cleavage neoepitopes in the CS2 domain demonstrated that active aggrecanase is being produced in fetal bovine epiphyseal cartilage under retinoic acid stimulation [9a]. Alternatively, the increased oligosaccharide content of the IGD of fetal bovine aggrecan [29] may render this region resistant to proteolytic cleavage due to the close proximity of the oligosaccharide chains to the aggrecanase cleavage site. Such a resistance of fetal bovine aggrecan to cleavage by aggrecanase in the IGD has been reported *in vitro* [30]. Aggrecan IGD neoepitope analysis is commonly used as an indicator of aggrecan degradation in cartilage [31–34], and the present study suggests that this may not be of universal application for all cartilage systems. A similar situation has been reported with bovine fetal rib growth plate cartilage upon treatment with retinoic acid, with both proteolytic and non-proteolytic pathways being suggested [35]. The non-proteolytic pathway was postulated to be the direct diffusion of partially degraded proteoglycan aggregates, although no information on whether depolymerization of hyaluronate may be occurring was presented. Hence it is possible that hyaluronate depolymerization may be a major factor in immature cartilages.

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