The mechanism of aggrecan release from cartilage differs with tissue origin and the agent used to stimulate catabolism

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

*Joint Diseases Laboratory, Shriners Hospital for Children, McGill University, Montreal, Quebec, Canada H3G 1A6, †Genetics Unit, Shriners Hospital for Children, Department of Surgery, McGill University, Montreal, Quebec, Canada H3G 1A6, and ‡Department of Surgery, McGill University, Montreal, Quebec, Canada H3G 1A6

The mechanisms of aggrecan degradation in adult human articular, adult bovine nasal and fetal bovine epiphyseal cartilage in response to either interleukin-1 β (IL-1 β) or retinoic acid were compared using an explant culture system. Bovine nasal cartilage cultured with either IL-1 β or retinoic acid exhibited significant release of glycosaminoglycan (GAG). For both factors, aggrecan proteolysis occurred predominantly at the ‘aggrecanase’ site, with no evidence for the action of matrix metalloproteinases, and resulted in the appearance of the corresponding G1 fragment in tissue extracts and in culture media. In human cartilage, little effect of IL-1 β was seen, but abundant release of GAG occurred in the presence of retinoic acid, with evidence of aggrecanase action. Treatment of fetal epiphyseal cartilage with retinoic acid

resulted in significant GAG release, whereas treatment with IL-1 β did not. In the retinoic acid-treated tissue, however, no evidence for the cleavage of aggrecan in the interglobular region was apparent. Thus, in the fetal system, agents in addition to aggrecanase and matrix metalloproteinases appear to be active. Taken together, these data demonstrate that the pathways utilized for aggrecan catabolism may vary between different cartilages for a given stimulatory agent, and that, for a given tissue, different factors may elicit aggrecan release via different pathways.

Key words: aggrecanase, interleukin-1, matrix metalloproteinase, proteoglycan, retinoic acid.

INTRODUCTION

The large aggregating proteoglycan, aggrecan, is a major structural component of articular cartilage, on which it confers the ability to withstand compressive loads [1]. The domain structure of aggrecan has been established following the cloning of cDNAs from a number of species, including human [2] and bovine [3]. Beginning at the N-terminus, this molecule possesses two globular regions, termed G1 and G2, which are separated by an interglobular domain (IGD). The G1 domain is of great functional importance, since it mediates the binding of aggrecan to hyaluronate, an interaction which is stabilized by a small glycoprotein, link protein [4]. Following G1 and G2 is a long extended region bearing a large number of keratan and chondroitin sulphate glycosaminoglycan (GAG) chains, whose charge density provides the tissue with its osmotic properties. The chondroitin sulphate-containing region is divided into two parts, CS1 and CS2. A third globular domain, termed G3, is found at the C-terminus of the core protein.

After synthesis and secretion by the chondrocyte, aggrecan undergoes extensive proteolytic processing during its lifetime in the tissue [5]. While the aggrecan molecule is cleaved at numerous sites in its core protein, this process is of particular functional consequence in the IGD between G1 and G2. This region is potentially susceptible to proteolysis by many proteases, although only two major sites of cleavage have been identified *in situ* in articular cartilage using a variety of experimental approaches [6–8]. The first site, termed the MMP site, can be cleaved, *in vitro*, by a number of matrix metalloproteinases (MMPs) (MMP-1, -2, -3, -7, -8, -9 and -13). This site is located just C-terminal of the

final cysteine residue of the G1 domain, and involves the peptide bond between amino acid residues Asn-341 and Phe-342 of the mature core protein in the human. The second site, termed the aggrecanase site, is cleaved by members 4 and 5 of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family of metalloproteinases, also termed aggrecanases-1 and -2 respectively [9,10]. This site involves the bond between amino acids Glu-373 and Ala-374 of the mature core protein. Recent evidence indicates that trimming of the aggrecan core protein may occur at four other sites in the CS2 region close to the G3 domain before cleavage in the IGD takes place [11,12].

The degradation of aggrecan has been studied by a number of different experimental approaches. Characterization of the aggrecan cleavage products has been facilitated by the development of anti-neoepitope antibodies which are specific for degradation products resulting from specific protease action and which do not recognize the native uncleaved protein [13,14]. The presence of MMP- and aggrecanase-generated G1 fragments of aggrecan has been demonstrated in articular cartilage from both normal and arthritic individuals [15,16]. The degradation products increase with age and localize preferentially to cartilage regions exhibiting most damage. MMP-generated degradation products have likewise been demonstrated in cartilage of mice with type II collagen-induced arthritis [17]. In addition, synovial fluids from patients with osteoarthritis, rheumatoid arthritis and acute knee injuries contain aggrecan degradation products resulting from both aggrecanase and MMP action [18–20].

While significant advances have been made in characterizing the major sites of aggrecan cleavage, there is relatively little information on whether these pathways are adopted universally

Abbreviations used: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; AGG-C and AGG-N, C- and N-terminal neoepitopes ... NITEGE and ARGSV ... respectively generated on cleavage of aggrecan in the interglobular domain by aggrecanase; CS1 and CS2, first and second chondroitin sulphate attachment regions of aggrecan; G1, G2 and G3, the three globular domains in the aggrecan core protein (N- to C-terminal orientation); GAG, glycosaminoglycan; IGD, interglobular domain (between aggrecan G1 and G2 regions); IL-1 β , interleukin-1 β ; MMP, matrix metalloproteinase; MMP-C and MMP-N, C- and N-terminal neoepitopes ... DIPEN/S and FFGVG ... respectively generated on cleavage of aggrecan in the human/bovine IGD by MMPs; PBST, PBS containing 0.1% Tween 20.

¹ To whom correspondence should be addressed (e-mail jmort@shriners.mcgill.ca).

by all chondrocytes in response to a given degradative stimulus. To address this issue, an explant culture system was used to examine the roles of the MMP and aggrecanase pathways of aggrecan catabolism in cartilages that differ in both age and origin. The data obtained suggest that a variety of release mechanisms operate.

A preliminary version of this work was presented at the Orthopaedic Research Society, in Orlando, Florida, in March 2000.

MATERIAL AND METHODS

Cartilage explant cultures

Adult bovine nasal septum cartilage and fetal bovine femoral epiphyseal cartilage were obtained from a local abattoir. Adult human femoral 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 dissected free of any surrounding connective tissues and perichondrium, cut into pieces of approx. 5 mm³ and placed in 24-well culture plates (approx. 200 mg wet weight of tissue per well); for each tissue, six wells were set up. Tissue was then precultured for 24 h in Dulbecco's modified Eagle's medium buffered with 44 mM sodium bicarbonate and 25 mM Hepes and containing 10 mg/ml BSA. This was followed by a 6-day period in which the tissues in duplicate wells were cultured with the same medium supplemented with 5 ng/ml recombinant human interleukin-1 β (IL-1 β) (R&D, Minneapolis, MN, U.S.A.), 1 μ M all-*trans*-retinoic acid (Eastman Kodak, Rochester, NY, U.S.A.) or no additions. Since the weight of tissue differed slightly between wells, the amount of medium added throughout the cultures was proportional to the tissue weight (1 ml of medium/100 mg of tissue). At 2-day intervals during the 6-day period, the culture media were collected and fresh medium containing IL-1 β , retinoic acid or no factor was added. The tissue was then stored at -20 °C for later extraction and analysis. Media collected on days 2, 4 and 6 were pooled for later analysis. All culture experiments were performed three times for each cartilage type under study. Common trends were observed in each case, and representative data from one of the cultures are depicted in the Figures.

Preparation of tissue extracts

Tissue was 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 μ g/ml pepstatin A [21]. The extractions were performed with stirring in 6 ml Wheaton vials for 48 h at 4 °C. 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).

Enzymic deglycosylation of proteoglycan

Before subsequent immunoblot analysis, extracts and media (also dialysed against 10 mM sodium acetate, pH 6.0) were treated sequentially with keratanase II (Seikagaku) and chondroitinase ABC (ICN) [16]. Keratanase II was added at a final concentration of 0.01 unit/ml and the samples were incubated at 37 °C overnight. The buffer was then adjusted to 0.1 M sodium acetate and 0.1 M Tris/HCl, pH 7.3, and the samples were treated with chondroitinase ABC (0.1 unit/ml) for 4 h at 37 °C.

For subsequent analysis by SDS/PAGE, samples were mixed with an equal volume of loading buffer containing β -mercaptoethanol and heated at 100 °C for 5 min.

SDS/PAGE and immunoblotting

Samples were analysed on Tris/glycine SDS/10%-polyacrylamide gels, and following electrophoresis resolved proteins were transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON, U.S.A.). The membranes were then blocked overnight in 3% (w/v) BSA in Tris-buffered saline. Immuno-detection of the aggrecan degradation products was performed utilizing the antibodies described below and an alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibody (Promega). Colour was developed using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [16]. All washing of the blots was carried out in Tris-buffered saline containing 0.1% Tween 20.

To analyse high-molecular-mass aggrecan components, samples were run on Novex 4–12% gradient NuPage Bis-Tris gels (Invitrogen) and electroblotted as recommended by the manufacturer using the Novex transfer apparatus. Immuno-detection was as above, except that washing buffers contained 0.025% Tween 20.

Description and preparation of antibodies

Rabbit antibodies recognizing the C-terminal neopeptides (termed MMP-C and AGG-C) in G1-containing degradation products generated by aggrecan cleavage at the IGD MMP and aggrecanase sites respectively have been described previously [16]. Rabbit anti-neopeptide antibodies were also prepared to detect the N-terminal neopeptides (termed MMP-N and AGG-N) resulting from cleavage by MMP and aggrecanase respectively, using peptides based on the respective N-terminal amino acid sequences for immunization. The peptides FFGVGAKKGC (MMP-N) and ARGSVIGGC (AGG-N) were prepared on an ABI 430 solid-phase synthesizer using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry. The residues indicated in italics were added to create a spacer region and a C-terminal cysteine residue to permit conjugation to ovalbumin. In the case of the MMP-N neopeptide, a longer spacer region containing two lysine residues was included to increase the solubility of the peptide.

A general rabbit anti-(aggrecan G1) antibody was prepared by immunization with a mixture of peptide-ovalbumin conjugates (HDNSLSVSIPQPSGGC, RVLLGTSLTIPCYFIDPMHPVTT-APS, TEGRVVRNSAYQDKGGC and SSRYDAICYTG), whose sequences were derived from regions of the G1 domain chosen to minimize possible cross-reaction with the aggrecan G2 domain and the G1 domain of versican. Similarly, a rabbit anti-(aggrecan G2) antibody was prepared by immunization with a mixture of peptide-ovalbumin conjugates (CGGYRPGPTR-YSLTFEEA, IVSPRTPCVGD, VFFATRLEQFTGGC, EAL-EFCESHNATAT, TGQLYAAWSRGLDKGGC, PIVTPRPA-CGGDKPGV and TGLPDPLSRHHAFGGC), in this case chosen to minimize cross-reaction with the G1 domain. A mixture of ovalbumin conjugates of the peptides GWNKYQGHYR-HFPDR, TWVDAERRCREQQSHLSS, VEHARTGQKDKR-YGGC, CQPSGHWEEPRIT and CGGTTYKRRLQKRSSRH was used to prepare an antibody to the aggrecan G3 region. Italics represent linker regions as above. In all cases the human aggrecan sequence was used, but the resulting antisera were found to cross-react with the corresponding bovine antigens.

In order to study the aggrecanase cleavage neopeptides in the aggrecan CS2 domain [11] (termed AGG2 to AGG5), four

antibodies were raised using the following strategy. Peptides were prepared representing the C- and N-terminal regions of each cleavage site. In cases where the bovine sequence differed from that of human aggrecan, residues from both sequences were included at that cycle during the synthesis of the respective peptide. A mixture of two conjugates representing the C- and N-terminal neoepitope for each cleavage site was used for immunization. The following peptide pairs were used: AGG2, CGGTA(S/G)ELE and GRGTI(G/D)GGC; AGG3, CGGTFKEEE and GLGSVEGGC; AGG4, CGGAPTAQE and AGEGPSGGC; AGG5, CGGPT(I/V)SQE and LGQRPPGGC. Conjugation, immunization and characterization of the resulting antisera were carried out as described previously [13,22]. When peptide pairs had been used for immunization, serum samples were incubated with either peptide (1 mg of peptide/ml of serum) for 1 h at room temperature, and left at 4 °C overnight to block the corresponding antibody reactivity.

ELISA peptide inhibition assays

The selectivity of the anti-neoepitope antibodies was determined by ELISA as described previously [23]. Immulon-2 96-well plates were coated with the target peptide (2 ng/ml) in 10 mM sodium bicarbonate, pH 9.6, at 4 °C overnight. The wells were then washed with PBST (PBS containing 0.1 % Tween 20) and blocked with PBST containing 1 % (w/v) BSA. Mixtures of antibody and different potential competing peptides were then added, and the plate was incubated at 37 °C for 90 min. Following three washes with PBST, secondary antibody [alkaline phosphatase-conjugated goat anti-(rabbit IgG); Sigma, St. Louis, MO, U.S.A.] was added, followed by a further incubation at 37 °C for 90 min. The plate was then washed, followed by addition of the substrate, *p*-nitrophenyl phosphate, and colour development. Absorbance at 405 nm was read using a BioTek Instruments (Winooski, VT, U.S.A.) Elx808 reader. Peptide dilution curves were prepared, and peptide concentrations yielding 50 % inhibition of antibody binding (IC_{50}) were calculated.

GAG assay

The sulphated GAG content of tissue extracts was measured by the Dimethylmethylene Blue dye-binding colorimetric assay, modified for use in a microwell format [24,25]. Samples of extract or medium (10 or 20 μ l) were combined with assay reagent (46 mM Dimethylmethylene Blue/40 mM glycine/40 mM NaCl, pH 3.0) to a final volume of 100 μ l in a 96-well plate. The plates were read immediately for absorbance at 530 nm, as above.

RESULTS

Stimulation of GAG release by IL-1 β and retinoic acid

The catabolic effects of IL-1 β and retinoic acid were studied by quantifying the extent of GAG release from tissue maintained in culture (Figure 1). Culture of bovine nasal cartilage under control conditions resulted in the release of approx. 30 % of tissue GAG by day 6 of the culture period. In comparison, culture of the tissue in the presence of 5 ng/ml IL-1 β resulted in the release of approx. 80 % of the GAG in the tissue. Treatment of nasal cartilage with 1 μ M retinoic acid caused the release of approx. 60 % of the GAG.

Fetal bovine epiphyseal cartilage was analysed similarly. Control conditions caused GAG release of similar magnitude to that observed with nasal cartilage. In contrast, treatment with IL-1 β did not result in increased GAG release. However, culture in the presence of retinoic acid resulted in the release of 90 % of the tissue GAG. With human articular cartilage, spontaneous

Figure 1 Effects of IL-1 β and retinoic acid treatment on GAG release from cartilage explants maintained in culture

Bovine nasal cartilage (BNC), fetal bovine epiphyseal cartilage (FEC) and adult human articular cartilage (HAC) explants were cultured with 5 ng/ml IL-1 β (solid bars), 1 μ M retinoic acid (open bars) or no factor (hatched bars) for 6 days. Tissues were then extracted and the dialysed extracts were analysed for GAG content by the Dimethylmethylene Blue dye-binding assay. The levels of GAG released from the tissue samples are reported as a percentage of the GAG content in uncultured tissue from the same source.

Table 1 Specificity of anti-MMP-C and anti-AGG-C anti-neoepitope antibodies

The ability of variants of the MMP-C and AGG-C epitopes to compete with the immunizing peptide was assessed using a competitive ELISA.

Coating peptide	Competing peptide	IC_{50} (μ M)
CGGDIPEN	CGGDIPEN	0.02
	CGGDIPES	3.0
	GGDIPES	3.0
	GDIPENF	100.0
CGGNITEGE	GNITEGE	0.07
	GNITEGEA	18.0
	GDITEGE	1.4
	GNIYEGE	6.5
	GNIAEGE	1.0

release of GAG was higher than was observed with the bovine tissues, but following stimulation a release pattern similar to that of bovine epiphyseal cartilage was observed, namely a much greater increase in GAG release with retinoic acid than with IL-1 β .

Analysis of aggrecan degradation products

Release of GAG-containing fragments from cartilage is generally believed to be due to the action of proteases released from the chondrocytes. The action of MMPs and aggrecanases can be determined by analysis of neoepitopes present on the released fragments and on the G1-containing products retained in the tissue. Anti-neoepitope antibodies specific for the new N- and C-termini produced by the cleavage of aggrecan in the IGD have been described and characterized [16,23,26]. The selectivities of two of these antibodies, against MMP-C and AGG-C, were investigated further to determine their suitability for the present studies. First, substitution of the C-terminal asparagine residue with a serine in the MMP-C neoepitope was carried out to reproduce the sequence difference present in the human and bovine proteins (... VDIPEN and ... VDIPES respectively) [3].

Figure 2 Western blot analysis of AGG-N-containing aggrecan degradation products

Samples of culture medium (M) or tissue extract (T) from control (C), IL-1 β -treated (IL1) or retinoic acid-treated (RA) cartilage were separated by SDS/PAGE and immunoblotted using the anti-AGG-N serum. (A) Bovine nasal cartilage; (B) fetal bovine epiphyseal cartilage; (C) adult human articular cartilage. UC, untreated cartilage. Positions of molecular mass markers (shown in kDa) are indicated on the right.

This substitution was shown to decrease the affinity of the antibody by almost two orders of magnitude (Table 1), potentially limiting the usefulness of this reagent for analysis of the bovine system. Secondly, the possible problem with the use of the anti-AGG-C antibody in relation to modification of the ... NITEGE epitope by N- or O-linked glycosylation at the asparagine and/or threonine residues was studied. The effect of such modifications was mimicked by substitution of the threonine residue with either a tyrosine or an alanine, and substitution of the asparagine with an aspartic acid. In all cases reactivity was decreased by between one and two orders of magnitude, indicating that a lower level of epitope detection would be expected in the case of N- or O-linked modification. However, the results suggest that complete masking of the epitope does not occur.

Analysis of IGD N-terminal epitopes

Western blots of tissue extracts and culture media using the antibody against MMP-N (FFGVG...) showed no reactive material, indicating the absence of MMP activity able to cleave

Figure 3 Western blot analysis of AGG-C-containing aggrecan degradation products

Samples of culture medium (M) or tissue extract (T) from control (C), IL-1 β -treated (IL1) or retinoic acid-treated (RA) cartilage were separated by SDS/PAGE and immunoblotted using the anti-AGG-C serum. (A) Bovine nasal cartilage; (B) fetal bovine epiphyseal cartilage; (C) adult human articular cartilage. UC, untreated cartilage. Positions of molecular mass markers (shown in kDa) are indicated on the right.

within the aggrecan IGD in any of these systems. In contrast, the AGG-N (ARGSV ...) neoepitope was evident in the medium of bovine nasal cartilage cultures stimulated with either IL-1 β or retinoic acid (Figure 2A), indicating the action of aggrecanase in these cultures, as has been reported previously [8,27]. In the case of human articular cartilage, the AGG-N epitope was released into the culture medium on stimulation with retinoic acid, but not IL-1 β (Figure 2C). Surprisingly, no detectable AGG-N epitope was apparent (Figure 2B) with bovine fetal epiphyseal cartilage, even though GAG release was stimulated by the presence of retinoic acid (Figure 1).

Analysis of IGD C-terminal epitopes

With the bovine cartilages, no MMP-C (... DIPEN/S) epitope was detected under any culture conditions, consistent with the MMP-N data, although the lower sensitivity of this antibody with the bovine sequence may not allow detection of very low levels of this G1 product. As shown previously [16], endogenous MMP-C epitope was present in untreated adult human cartilage,

Figure 4 Western blot analysis of G1-containing aggrecan components

Samples of culture medium (M) or tissue extract (T) from control (C), IL-1 β -treated (IL1) or retinoic acid-treated (RA) cartilage were separated by SDS/PAGE and immunoblotted using the general anti-G1 serum. (A) Bovine nasal cartilage; (B) fetal bovine epiphyseal cartilage; (C) adult human articular cartilage. UC, untreated cartilage. Positions of molecular mass markers (shown in kDa) are indicated on the right.

Figure 5 Western blot analysis of aggrecanase-generated neoepitopes in the aggrecan CS2 region

Samples of culture medium from control (C), IL-1 β -treated (IL1) or retinoic acid-treated (RA) bovine nasal cartilage (BNC), fetal bovine epiphyseal cartilage (FEC) or adult human articular cartilage (HAC) were separated by SDS/PAGE and immunoblotted using the antiserum against the AGG4 cleavage site (upper panel) or that against the AGG5 cleavage site (lower panel). The amino acid sequence of the neoepitope being visualized is indicated on the right.

but no increase in abundance was apparent following culture or stimulation with IL-1 β or retinoic acid (results not shown). In contrast, AGG-C (... NITEGE)-containing material was seen in both the tissue extract and the medium of IL-1 β -treated and

retinoic acid-treated bovine nasal cartilage cultures (Figure 3A), consistent with the findings with the anti-AGG-N antibody. Also consistent with the AGG-N antibody data (Figure 2B), no AGG-C epitope was detected in the fetal bovine epiphyseal cartilage (Figure 3B). In the case of the human cartilage, AGG-C-containing G1 material was present in the uncultured tissue, as shown previously [16], but no release of this material was apparent in unstimulated cultures (Figure 3C). On stimulation with IL-1 β , a low level of AGG-C-containing material was detected in the culture medium. Stimulation with retinoic acid resulted in both an increase of AGG-C epitope in the tissue and release into the culture medium. In human cartilage, higher-molecular-mass anti-AGG-C reactive bands were observed with molecular sizes suggestive of oligomerization of the G1 domain.

Analysis of G1 regions

G1-containing material in the different culture systems was investigated by immunoblotting using a general anti-G1 antibody. As expected, bovine nasal cartilage extracts contained a diverse range of reactive material of sizes ranging from free G1 to intact aggrecan. On stimulation with IL-1 β or retinoic acid, the G1-containing material was reduced to an 80 kDa doublet, consistent with aggrecan cleavage at the aggrecanase site in the IGD. In addition, the G1 domain was released into the culture medium (Figure 4A). A dramatically different response was seen in the case of bovine fetal epiphyseal cartilage, whereby G1-containing material was detectable only at the very top of the gel in the case of tissue extracts, presumably representing intact aggrecan. Barely detectable levels of free G1 domain (with a molecular size of 80 kDa) were present in both the medium and the tissue extract of cartilage cultured in the presence of retinoic acid (Figure 4B). A diverse range of G1-containing material was present in the tissue extracts of adult human cartilage, but there was no increase in intensity of the various components in tissue cultured in the presence of IL-1 β . However, consistent with the release of AGG-C epitope, G1-containing material was released into the medium of cartilage cultured in the presence of retinoic acid (Figure 4C).

Detection of aggrecanase-generated CS2 neoepitopes

The lack of AGG-C and MMP-C neoepitopes and the weak general G1 staining in the fetal bovine samples cultured under stimulation with retinoic acid (Figures 3 and 4) suggested a lack of active aggrecanase or MMP production in this tissue. However, the majority of the GAG content of the tissue was lost following this treatment (Figure 1). The possibility that other aggrecanase cleavages were taking place was investigated by analysis of the culture medium for the aggrecanase neoepitopes in the CS2 region of the aggrecan core protein. Staining for two bands, representing the smallest G3-containing products generated by aggrecanase cleavage of aggrecan, was present in fetal bovine cartilage cultures stimulated with retinoic acid, as well as in adult human and bovine nasal cartilage cultures (Figure 5). This indicates that active aggrecanase is indeed present in the fetal bovine cartilage, and suggests that access to the aggrecanase cleavage site in the IGD is restricted in this tissue, as has been proposed from *in vitro* experiments [28].

Evidence for release of intact aggrecan from fetal bovine epiphyseal cartilage

Since it was possible that the standard SDS/10 %-PAGE system used to analyse aggrecan components would not allow pen-

Figure 6 Western blot analysis of G1-, G2- and G3-containing epitopes in aggrecan components released from fetal bovine epiphyseal cartilage

Samples of culture medium from control (C), IL-1 β -treated (IL1) or retinoic acid-treated (RA) fetal bovine epiphyseal cartilage were separated by SDS/PAGE on 4–12% NuPage Bis-Tris gradient gels and immunoblotted using anti-G1, anti-G2 or anti-G3 sera as indicated. A non-specific band at 155 kDa is observed in all samples with each of the antisera used. Positions of molecular mass markers (shown in kDa) are indicated on the left.

etration of the intact aggrecan molecule, even following GAG removal, samples were analysed using the NuPage 4–12% gradient gel system and transfer conditions recommended by the manufacturer (Figure 6). Under these conditions, a high-molecular-mass band reacting with anti-G1, anti-G2 and anti-G3 sera was observed in the medium of fetal bovine cartilage cultured in the presence of retinoic acid, indicating the release of intact aggrecan. In addition, slightly lower-molecular-mass bands reacting with anti-G1 and anti-G2 antibodies, consistent with cleavage in the CS2 region with corresponding loss of the G3 region, were observed. In contrast, no release of G3- or G1-containing material was found in the presence of IL-1 β .

DISCUSSION

The first clear point the present study makes is that the response of cartilage to stimulation by IL-1 β and retinoic acid differs depending on the type of cartilage being examined. As has been shown previously in short-term culture [26], no evidence for MMP activity was observed with any of the cartilages, even though it has been found in the past that proMMP expression is stimulated by IL-1 β [29]. However, as was shown in the case of collagen breakdown, it is possible that active MMPs capable of causing aggrecan cleavage may only be produced after a much more extended culture period [30]. At present it is not possible to ascribe the observed differences between the two bovine cartilages under study to either age or site variation, as both parameters varied. Previous work [31] has shown that articular cartilage from 1–2-week-old calves can respond to both IL-1 β and retinoic acid with GAG release and aggrecanase-dependent cleavage in the aggrecan IGD. This could be taken as evidence that the response to IL-1 β may be age-related and selective in the fetus, but site differences due to articular compared with epiphyseal or nasal sampling cannot be discounted. The work in calf articular cartilage also differed in the IL-1 β concentration being used (100 ng/ml in [31], compared with 5 ng/ml used in the present work), and it is possible that additional receptors were activated using the higher IL-1 β doses.

Bovine nasal cartilage behaved as expected, in that both IL-1 β and retinoic acid caused a high level of GAG release. The major aggrecan-degrading activity present in such cultures appears to be due to aggrecanases [9,10] belonging to the newly described ADAMTS family of metalloproteases. In addition to GAG release, however, high levels of aggrecan G1 domains were also

released from the tissue under stimulation by either agent. This release is difficult to explain on the basis of aggrecanase action alone, since aggrecanase-mediated proteolysis should not destabilize the interaction between the G1 domain and hyaluronate, suggesting that other mechanisms are acting in bovine nasal cartilage. Evidence for G1 domain release from other cartilages cultured in the presence of IL-1 β has been presented [31,32], but the mechanism behind this process is still unclear. It is also apparent that the free bovine aggrecan G1 region migrates as two components on SDS/PAGE, in contrast with that from human. These components are generated by the action of aggrecanase, as the same pattern can be produced *in vitro* upon treatment of purified bovine aggrecan with aggrecanase-1 or -2 (P. J. Roughley, R. J. White and J. S. Mort, unpublished work). The heterogeneity can be ascribed to differential oligosaccharide substitution of the bovine G1 region.

Human articular cartilage showed little response to IL-1 β in terms of GAG release, and there was no evidence for aggrecanase action. In contrast, retinoic acid appeared to stimulate aggrecanase activity, with the concomitant release of GAG and production of the corresponding G1 region. As with bovine nasal cartilage, release of the aggrecan G1 domain into the culture medium was observed. In contrast with the simple pattern seen for the bovine G1 region, however, several higher-molecular-mass bands reactive with the anti-AGG-C antibody were seen in human cartilage, in addition to that expected for the aggrecanase-generated G1 domain. The spacing of these bands suggests that they represent oligomers of the G1 domain. Since the gels were run under reducing conditions and a diverse array of other proteins were present in the samples, these bands are unlikely to result from intermolecular disulphide bond formation. Using a different antibody, a similar result has been described for human cartilage by Yasumoto et al. in a preliminary report [33]. It was proposed that these bands represent the products of natural chemical cross-linking of the G1 components, as has been described by Bank et al. [34]. The relatively long lifetime of aggrecan molecules in adult human cartilage [35], and the close association between G1 regions in the native proteoglycan aggregates, could lead to the formation and accumulation of such cross-linked species.

Fetal bovine epiphyseal cartilage appeared to respond to stimulatory agents in a similar way as adult human articular cartilage, in that only retinoic acid induced release of GAG-containing products into the culture medium in major excess of that in unstimulated cultures. However, no evidence for the production of either MMP- or aggrecanase-generated G1 domains was observed. In principle, the lack of AGG-C-containing material could be attributed, at least in part, to decreased reactivity of the anti-AGG-C serum with G1 domains containing carbohydrate modification on the asparagine or threonine residues of the ... NITEGE epitope, as indicated by the decrease in reactivity in ELISA assays following residue substitution at these positions. Indeed, our own preliminary studies of bovine aggrecan degradation by recombinant aggrecanase demonstrated that the AGG-C neopeptide is partially masked with respect to immunodetection in both the fetal epiphyseal and adult nasal cartilage used in this work. This explains the relatively low immunoreactivity observed in the nasal cartilage experiment (Figure 3A), whereas the anti-G1 antibody indicated much more abundant product (Figure 4A). With the fetal cartilage, the lack of extensive G1 domain generation was, however, confirmed by the low abundance of staining of 80 kDa material with the general anti-G1 antibody.

Evidence for the C-terminal trimming of aggrecan under various conditions was provided by the analysis with antibodies

specific for neopeptides generated by the action of aggrecanase in the CS2 region. However, such cleavages can only provide a partial explanation for the almost complete release of the tissue GAG content from the fetal bovine cartilage in response to retinoic acid. If it can be assumed that all of the chondroitin sulphate substitution sites are occupied by chains of the same size, according to the sequence of bovine aggrecan [3], cleavage at the first aggrecanase site in the CS2 region would result in the release of only 50% of the total chondroitin sulphate. Thus it seems that, in fetal cartilage, there is an additional mechanism for releasing high-molecular-mass aggrecan components without IGD cleavage. The absence of IGD cleavage itself may be due to protection of the substrate by glycosylation near the aggrecanase cleavage site [28]. The demonstration of cleavage in the CS2 region, particularly in the fetal bovine system where no free G1 is generated, would predict the generation of large aggrecan fragments bearing G1 domains. In agreement with this hypothesis, such fragments were observed when an appropriate gel system was used to analyse aggrecan products released from fetal bovine cartilage. In addition, intact aggrecan, as demonstrated by the presence of G1, G2 and G3 domains, was observed in these media. Thus it appears that aggrecan release can occur in the absence of core protein cleavage.

Several possible mechanisms could result in the release of intact aggrecan, or of the G1 domain following aggrecan cleavage by aggrecanases. First, the interaction between the G1 domain and hyaluronate could become destabilized either by proteolysis or by chemical modification, for example by free radical attack. Alternatively, modification of link protein could also destabilize the proteoglycan aggregate. Evidence for the generation of free radicals in cartilage has been presented [36,37], and some modifications of adult human link protein are compatible with free radical action [38]. Hyaluronate depolymerization could also result in the loss of bound aggrecan or its G1-containing degradation products from the tissue. Partial hyaluronate depolymerization may explain why hyaluronate size decreases with age in human cartilage [39]. Two mechanisms for hyaluronate cleavage are possible, i.e. free radical attack and glycosidase action, and indirect evidence supports both routes. Depolymerization of hyaluronate by reactive oxygen species is well established [40], indicating that this is a possible mechanism. While evidence for hyaluronidase expression has been demonstrated in chondrocytes [41], the very low pH optimum of the well characterized forms of these enzymes argues against their action in the extracellular environment. However, cDNAs for new hyaluronidases have been cloned [42], and it remains possible that one or more of these enzymes could function outside the cell.

We thank Elisa de Miguel for peptide synthesis and Guylaine Bedard for preparing the figures. This work was supported by the Arthritis Society of Canada and the Shriners of North America.

REFERENCES

- Hardingham, T. E. and Fosang, A. J. (1992) Proteoglycans: many forms and many functions. *FASEB J.* **6**, 861–870
- Doege, K. J., Sasaki, M., Kimura, T. and Yamada, Y. (1991) Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, aggrecan. Human-specific repeats, and additional alternatively spliced forms. *J. Biol. Chem.* **266**, 894–902
- Hering, T. M., Kollar, J. and Huynh, T. D. (1997) Complete coding sequence of bovine aggrecan: comparative structural analysis. *Arch. Biochem. Biophys.* **345**, 259–270
- Neame, P. J. and Barry, F. P. (1993) The link proteins. *Experientia* **49**, 393–402
- Roughley, P. J. and Mort, J. S. (1986) Ageing and the aggregating proteoglycans of human articular cartilage. *Clin. Sci.* **71**, 337–344
- Flannery, C. R., Lark, M. W. and Sandy, J. D. (1992) Identification of a stromelysin cleavage site within the interglobular domain of human aggrecan. Evidence for proteolysis at this site *in vivo* in human articular cartilage. *J. Biol. Chem.* **267**, 1008–1014
- Sandy, J. D., Neame, P. J., Boynton, R. E. and Flannery, C. R. (1991) Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. *J. Biol. Chem.* **266**, 8683–8685
- Hughes, C. E., Caterson, B., Fosang, A. J., Roughley, P. J. and Mort, J. S. (1995) Monoclonal antibodies that specifically recognize neopeptide sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: application to catabolism *in situ* and *in vitro*. *Biochem. J.* **305**, 799–804
- Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbaszade, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R. et al. (1999) Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. *Science* **284**, 1664–1666
- Abbaszade, I., Liu, R. Q., Yang, F., Rosenfeld, S. A., Ross, O. H., Link, J. R., Ellis, D. M., Tortorella, M. D., Pratta, M. A., Hollis, J. M. et al. (1999) Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family. *J. Biol. Chem.* **274**, 23443–23450
- Tortorella, M. D., Pratta, M., Liu, R. Q., Austin, J., Ross, O. H., Abbaszade, I., Burn, T. and Arner, E. (2000) Sites of aggrecan cleavage by recombinant human aggrecanase-1 (ADAMTS-4). *J. Biol. Chem.* **275**, 18566–18573
- Sandy, J. D., Thompson, V., Doege, K. and Verscharen, C. (2000) The intermediates of aggrecanase-dependent cleavage of aggrecan in rat chondrosarcoma cells treated with interleukin-1. *Biochem. J.* **351**, 161–166
- Mort, J. S. and Buttle, D. J. (1999) The use of cleavage site specific antibodies to delineate protein processing and breakdown pathways. *J. Clin. Pathol. Mol. Pathol.* **52**, 11–18
- Hughes, C. E., Caterson, B., White, R. J., Roughley, P. J. and Mort, J. S. (1992) Monoclonal antibodies recognizing protease-generated neopeptides from cartilage proteoglycan degradation: application to studies of human link protein cleaved by stromelysin. *J. Biol. Chem.* **267**, 16011–16014
- Lark, M. W., Bayne, E. K., Flanagan, J., Harper, C. F., Hoerrner, L. A., Hutchinson, N. I., Singer, I. I., Donatelli, S. A., Weidner, J. R., Williams, H. R. et al. (1997) Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. *J. Clin. Invest.* **100**, 93–106
- Sztrolovics, R., Alini, M., Roughley, P. J. and Mort, J. S. (1997) Aggrecan degradation in human intervertebral disc and articular cartilage. *Biochem. J.* **326**, 235–241
- Singer, I. I., Kawka, D. W., Bayne, E. K., Donatelli, S. A., Weidner, J. R., Williams, H. R., Ayala, J. M., Mumford, R. A., Lark, M. W. and Glant, T. T. et al. (1995) VDIPEN, a metalloproteinase-generated neopeptide, is induced and immunolocalized in articular cartilage during inflammatory arthritis. *J. Clin. Invest.* **95**, 2178–2186
- Lohmander, L. S., Hoerrner, L. A., Dahlberg, L., Roos, H., Björnsson, S. and Lark, M. W. (1993) Stromelysin, tissue inhibitor of metalloproteinases and proteoglycan fragments in human knee joint after injury. *J. Rheumatol.* **20**, 1362–1368
- Lohmander, L. S., Hoerrner, L. A. and Lark, M. W. (1993) Metalloproteinases, tissue inhibitor, and proteoglycan fragments in knee synovial fluid in human osteoarthritis. *Arthritis Rheum.* **36**, 181–189
- Lohmander, L. S., Neame, P. J. and Sandy, J. D. (1993) The structure of aggrecan fragments in human synovial fluid: evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury and osteoarthritis. *Arthritis Rheum.* **36**, 1214–1222
- Roughley, P. J. and White, R. J. (1980) Age-related changes in the structure of the proteoglycan subunits from human articular cartilage. *J. Biol. Chem.* **255**, 217–224
- Mach, L., Schwihla, H., Stüwe, K., Rowan, A. D., Mort, J. S. and Glössl, J. (1993) Activation of procathepsin B in human hepatoma cells: the conversion to the mature enzyme relies on the action of cathepsin B itself. *Biochem. J.* **293**, 437–442
- Lee, E. R., Lamplugh, L., Leblond, C. P., Mordier, S., Magny, M.-C. and Mort, J. S. (1998) Immunolocalization of the cleavage of the aggrecan core protein at the Asn³⁴¹-Phe³⁴² bond, as an indicator of the location of the metalloproteinases active in the lysis of the rat growth plate. *Anat. Rec.* **252**, 117–132
- Farndale, R. W., Sayers, C. A. and Barrett, A. J. (1982) A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. *Connect. Tissue Res.* **9**, 247–248
- Hollander, A. P., Atkins, R. M., Eastwood, D. M., Dieppe, P. A. and Elson, C. J. (1991) Human cartilage is degraded by rheumatoid arthritis synovial fluid but not by recombinant cytokines *in vitro*. *Clin. Exp. Immunol.* **83**, 52–57
- Little, C. B., Flannery, C. R., Hughes, C. E., Mort, J. S., Roughley, P. J., Dent, C. and Caterson, B. (1999) Aggrecanase versus matrix metalloproteinases in the catabolism of the interglobular domain of articular cartilage aggrecan *in vitro*. *Biochem. J.* **334**, 61–68
- Ilic, M. Z., Handley, C. J., Robinson, H. C. and Mok, M. T. (1992) Mechanism of catabolism of aggrecan by articular cartilage. *Arch. Biochem. Biophys.* **294**, 115–122

- 28 Pratta, M. A., Tortorella, M. D. and Arner, E. C. (2000) Age-related changes in aggrecan glycosylation affect cleavage by aggrecanase. *J. Biol. Chem.* **275**, 39096–39102
- 29 Nguyen, Q., Mort, J. S. and Roughley, P. J. (1992) Preferential mRNA expression of prostromelysin relative to procollagenase and in situ localization in human articular cartilage. *J. Clin. Invest.* **89**, 1189–1197
- 30 Cawston, T. E., Ellis, A. J., Humm, G., Lean, E., Ward, D. and Curry, V. (1995) Interleukin-1 and oncostatin M in combination promote the release of collagen fragments from bovine nasal cartilage. *Biochem. Biophys. Res. Commun.* **215**, 377–385
- 31 Bonassar, L. J., Sandy, J. D., Lark, M. W., Plaas, A. H., Frank, E. H. and Grodzinsky, A. J. (1997) Inhibition of cartilage degradation and changes in physical properties induced by IL-1 β and retinoic acid using matrix metalloproteinase inhibitors. *Arch. Biochem. Biophys.* **344**, 404–412
- 32 Ratcliffe, A., Tyler, J. A. and Hardingham, T. E. (1986) Articular cartilage cultured with interleukin 1. Increased release of link protein, hyaluronic-binding region and other proteoglycan fragments. *Biochem. J.* **238**, 571–580
- 33 Yasumoto, T., Bayliss, M. T., Bird, J. T. E. and Mason, R. M. (1999) Species and age-related differences in the turnover of aggrecan and link protein. *Trans. Orthop. Res. Soc.* **45**, 82
- 34 Bank, R. A., Bayliss, M. T., Lefebvre, F. P., Maroudas, A. and TeKoppele, J. M. (1998) Ageing and zonal variation in post-translational modification of collagen in normal human articular cartilage. The age-related increase in non-enzymatic glycation affects biomechanical properties of cartilage. *Biochem. J.* **330**, 345–351
- 35 Maroudas, A., Bayliss, M. T., Uchitel-Kaushansky, N., Schneiderman, R. and Gilav, E. (1998) Aggrecan turnover in human articular cartilage: use of aspartic acid racemization as a marker of molecular age. *Arch. Biochem. Biophys.* **350**, 61–71
- 36 Tikku, M. L., Yan, Y. P. and Chen, K. Y. (1998) Hydroxyl radical formation in chondrocytes and cartilage as detected by electron paramagnetic resonance spectroscopy using spin trapping reagents. *Free Radical Res.* **29**, 177–187
- 37 Tikku, M. L., Shah, R. and Allison, G. T. (2000) Evidence linking chondrocyte lipid peroxidation to cartilage matrix protein degradation. Possible role in cartilage aging and the pathogenesis of osteoarthritis. *J. Biol. Chem.* **275**, 20069–20076
- 38 Roberts, C. R., Mort, J. S. and Roughley, P. J. (1987) Treatment of cartilage proteoglycan aggregate with hydrogen peroxide: relationship between observed degradation products and those which occur naturally during aging. *Biochem. J.* **247**, 349–357
- 39 Holmes, M. W. A., Bayliss, M. T. and Muir, H. (1988) Hyaluronic acid in human articular cartilage. Age-related changes in content and size. *Biochem. J.* **250**, 435–441
- 40 McCord, J. M. (1974) Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. *Science* **185**, 529–531
- 41 Flannery, C. R., Little, C. B., Hughes, C. E. and Caterson, B. (1998) Expression and activity of articular cartilage hyaluronidases. *Biochem. Biophys. Res. Commun.* **251**, 824–829
- 42 Csóka, A. B., Scherer, S. W. and Stern, R. (1999) Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31. *Genomics* **60**, 356–361

Received 20 April 2001/16 October 2001; accepted 13 December 2001