Analysis of aggrecan in human knee cartilage and synovial fluid indicates that aggrecanase (ADAMTS) activity is responsible for the catabolic turnover and loss of whole aggrecan whereas other protease activity is required for C-terminal processing *in vivo*

John D. SANDY¹ and Christie VERSCHAREN

Institute for Research in Skeletal Development and Paediatric Orthopaedics, Shriners Hospital for Children, Department of Pharmacology and Therapeutics, University of South Florida, Tampa, FL 33612-9499, U.S.A.

Studies of aggrecan proteolysis in human joints have implicated both the aggrecanase [ADAMTS, a disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif] and matrix metalloproteinase (MMP) families. We have analysed the aggrecan core protein species present *in vivo* in both articular cartilage and synovial fluids from normal, acutely injured and osteoarthritic joints. Normal cartilage contains at least seven major G1 domain (the N-terminal globular domain of aggrecan)-bearing species, of which three (full-length core, G1-NITEGE³⁷³ and G1-VDIPEN³⁴¹) have been identified. The C-terminals of the others are unknown but digestion of fetal human aggrecan with MMP-3 and crude aggrecanase suggests that they are products of MMP-like activity *in vivo*. Normal synovial fluids contain at least 10 species, of which nine result from ADAMTS-dependent cleavage, and this cleavage occurs at all of the five known aggrecanase sites. Aggrecan fragments in the cartilage and synovial fluids of acutely injured joints are generally similar

to normal, but all contain a markedly increased ratio of G1-NITEGE to G1-VDIPEN. Aggrecan from the cartilage of late-stage osteoarthritis patients is remarkably similar to normal, whereas the synovial fluid aggrecan is more fragmented than that from normal or injured knees. The analyses suggest that the role of the ADAMTS and these MMP-like activities in human cartilage are distinctly different. Excessive ADAMTS activity *in vivo* is destructive to cartilage matrix, since the bulk of the glycosaminoglycan (GAG)-bearing products are released from the tissue into the synovial fluid following cleavage of the Glu³⁷³–Ala³⁷⁴ bond. In contrast, the MMP-like activity appears to be essentially non-destructive, since much of the GAG-bearing product is retained in the tissue following cleavages that are in the more C-terminal regions of the molecule.

Key words: glycosaminoglycan, osteoarthritis, proteinase.

INTRODUCTION

The core-protein heterogeneity of aggrecan in human articular cartilages has been investigated using a range of methods, including amino acid analysis [1], protein sequencing [2,3], aspartate racemization [4], electron microscopy [5], buoyant-density centrifugation [6], composite gel electrophoresis [7], size-exclusion chromatography [8], immunoreactivity [9] and Western blotting [10–12]. Taken together, these studies have suggested that heterogeneity can be most readily explained by the presence of some intact full-length aggrecan together with a series of products generated by varying degrees of proteolytic truncation from the C-terminal end of the core protein. These truncations result in the formation of distinct and electrophoretically separable species of aggrecan core, all of which lack the G3 globular domain (the C-terminal globular domain of aggrecan) and appear to be retained in the tissue through the hyaluronan-binding properties of the G1 domain (the N-terminal globular domain of aggrecan).

While the presence of many of these species in aggrecan-containing tissues has been known for many years, their exact proteolytic origin remains unsolved. In this regard, recent studies of these natural products by protein sequencing and neopeptide

antisera [2,12–15] have shown that some of the quantitatively minor species are generated by the action of metalloproteinases of the matrix metalloproteinase (MMP) and ADAMTS [which stands for ‘a disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif’] families. However, the precise C-termini and the identity of the processing protease(s) for the bulk of the aggrecan are unclear at present.

Despite the lack of precise structural data there is a general consensus regarding the approximate C-terminal location of the major species in mammalian cartilages. First, maybe 30–50 % of the total aggrecan core protein, measured as moles of G1 domain, is present as the full-length species (residues 1–2396 in the human) which migrates at an estimated 350 kDa on SDS/PAGE [7]. A second major form is the so-called keratan sulphate (KS)-rich aggrecan [16], which migrates at about 250 kDa on SDS/PAGE and which has been suggested to terminate [3] in the region near the border of the chondroitin sulphate (CS)-1 and CS-2 domains. A third major form is the so-called ‘double-globe’ aggrecan [11,17], which migrates at about 160 kDa on SDS/PAGE, is composed of the G1 and G2 (the second N-terminal globular domain of aggrecan) domains only and appears to terminate between the G2 and KS-rich domains. A fourth major form is a group of molecules that migrate at about

Abbreviations used: ADAMTS, a disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif; MMP, matrix metalloproteinase; G1 domain, the N-terminal globular domain of aggrecan; G2 domain, the second N-terminal globular domain of aggrecan; G3 domain, the C-terminal globular domain of aggrecan; KS, keratan sulphate; CS, chondroitin sulphate; OA, osteoarthritis; AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; GAG, glycosaminoglycan; DE52, diaminoethylcellulose anion exchanger; ACL, anterior cruciate ligament.

¹ To whom correspondence should be addressed (e-mail jsandy@shctampa.usf.edu).

100 kDa on SDS/PAGE and terminate within the C-terminal region of the interglobular domain (IGD) and which may be the products of metalloproteinase cleavage in this region [18]. The lowest-molecular-mass group migrates at about 55–65 kDa on SDS/PAGE and is composed of G1 domains with the known C-termini, G1-NITEGE³⁷³ [12,19] and G1-VDIPEN³⁴¹ [2].

Quantitatively important information on the turnover of aggrecan in human joints can be obtained by analysis of synovial fluids. Thus it can be estimated that the fluid present in a normal knee joint at any one time probably accounts for about 0.1 % of the total aggrecan present in all the joint tissues. If, by extrapolation from rabbit studies, the half-life of the human synovial fluid aggrecan is 8 h [20], then a significant amount (perhaps 5 % of the total) would pass through the fluid compartment in a month. This value is probably considerably higher in joints following injury or in progressive osteoarthritis (OA). We previously analysed the fragments in normal and diseased human synovial fluids by N-terminal sequencing of species isolated by CsCl gradient separation and gel-permeation chromatography [21,22]. This showed that the major high-buoyant-density fragments were generated by cleavage of tissue aggrecan at the Glu³⁷³–Ala³⁷⁴ site, which is now known to be due to the action of one or more of the aggrecanases, ADAMTS-1, -4 and -5 [23–26]. However, since these analyses were done on the high-buoyant-density fragments alone and did not include an analysis of G1 domain fragments and other low-buoyant-density species [27,28], the earlier studies were incomplete. Indeed, it has been shown [28] that a proportion of the low-buoyant-density species in human synovial fluids is generated by cleavage by MMP-like activity at the Asn³⁴¹–Phe³⁴² site. To address this issue and to provide further information on the proteolytic origin of the species present in human articular cartilages and synovial fluids, we have now analysed aggrecan fragments from cartilage and fluids which have been prepared by an ion-exchange method that isolates both high- and low-buoyant-density species in high yield.

EXPERIMENTAL

Materials

The ECL detection system was from Amersham (Arlington Heights, IL, U.S.A.) and was used according to the manufacturer's instructions. Goat anti-mouse IgG (peroxidase-conjugated) was from Sigma. Goat anti-rabbit IgG and goat anti-mouse IgM (peroxidase-conjugated) were from Boehringer. The production, source, characterization and use of the antisera has been described in the following papers: anti-NITEGE and anti-VDIPEN [29,30]; anti-TFKEEE, anti-APTAQE and anti-PTVSQE [12]; anti-TASELE and anti-G3 (LEC-7) [31]. Affinity-purified antisera NITEGE and VDIPEN were gifts from Dr John Mort (Shriners Hospital for Children, Montreal, Canada), and anti-G2 antiserum and AF-28 were gifts from Dr Amanda Fosang (University of Melbourne, Parkville, Australia). Antiserum ARGSVI was raised in rabbits against the synthetic peptide ARGSVILTVK at Research Genetics (Huntsville, AL, U.S.A.) by the multi-antigen presentation (MAP) protocol. Antiserum LGQRPP (produced in rabbits by injection of the keyhole-limpet-haemocyanin-conjugated peptide CGGLG-QRPP) and human recombinant MMP-3 were from Merck (Rahway, NJ, U.S.A.). Antibody 3-B-3, chondroitinase ABC (protease-free), keratanase II and endo- β -galactosidase were from Seikagaku (Falmouth, MA, U.S.A.). Crude aggrecanase was prepared from the medium of cartilage explanted from the metacarpal-phalangeal joints of 32-week-old pigs. Explants (about 25 mg wet weight/ml of medium) were maintained for

2 days in serum-free Dulbecco's modified Eagle's medium and then treated in this medium with human recombinant interleukin-1 β (10 ng/ml). The medium collected over the next 4 days was concentrated 15-fold on Microcon 10000 Da molecular-mass cut-off filtration units for use in digestions.

Proteinase digestion of aggrecan

Portions of fetal human aggrecan (final concentration 330 nM) were partially digested with recombinant human MMP-3 or 10 μ l of crude aggrecanase (kindly provided by Dr Jennifer Westling, Shriners Hospital for Children, Tampa, FL, U.S.A.) for 24 h at 37 °C in 50 mM Tris/HCl, pH 8.5/10 mM CaCl₂/100 mM NaCl. Digestion was terminated by addition of 10 mM EDTA, the aggrecan was deglycosylated, and portions (about 10 μ g of glycosaminoglycan, GAG) were taken for Western-blot analysis.

Source and description of articular cartilages and synovial fluids

Normal and acute-injury human synovial fluids were a generous gift of Dr Stefan Lohmander (Lund University, Lund, Sweden). Undiluted fluid was aspirated from normal volunteers and from joint-injured patients: patient ages, type of injury and interval between injury and aspiration were as follows; 38 years, anterior cruciate ligament (ACL) rupture and meniscus tear, 277 weeks; 39 years, blunt trauma, 2 days; 36 years, meniscal tear, 1 week; 53 years, joint distortion, 1 week. Cartilage from the femoral notch was collected from tissue donors with no evidence of joint disease, and from joint-injured patients at the time of notchplasty (widening of the femoral notch) for ACL reconstruction (Dr Fred Nelson, Department of Orthopaedics, San Diego Naval Base, San Diego, CA, U.S.A.): patient ages, type of injury and interval between injury and surgery were as follows: 36 years, ACL rupture and medial meniscal tear, 8 weeks; 40 years, ACL rupture and lateral meniscal tear, 11 weeks. Fluids and cartilage from patients undergoing joint-replacement surgery were obtained with patient consent from Dr Bill Kilgore (Morton Plant Hospital, Clearwater, FL, U.S.A.). Normal human tibial cartilage was from Dr Ada Cole (Rush Medical Center, Chicago, IL, U.S.A.).

Isolation of aggrecan fragments from articular cartilages

The cartilage (typically 5–50 mg wet weight) was rinsed in cold PBS, chopped finely and extracted by rocking at 4 °C (15 μ l of extractant per mg wet weight) for 48 h in 4 M guanidine hydrochloride/10 mM Mes/50 mM sodium acetate/5 mM EDTA/0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF)/5 mM iodoacetic acid/0.3 M aminohexanoic acid/15 mM benzamidine/1 μ g/ml pepstatin, pH 6.8. A clear extract was obtained after centrifugation to pellet the tissue (which was digested with papain) and the extract was desalted on Sephadex G-50 (4 ml bed volume) in 7 M urea/50 mM Tris/acetate, pH 8.0. The yield of GAG from the tissue was greater than 90 % in all samples. The GAG-rich void-volume fractions from Sephadex G-50 were combined and applied to a column (0.5 ml bed volume) of DE52 cellulose (diaminoethylcellulose anion exchanger) in 5 ml Poly-Prep columns equilibrated in 7 M urea/50 mM Tris/acetate, pH 8.0. After sample loading and collection of unbound material (flow-through), the column was eluted as follows: (i) 2 ml of 7 M urea/50 mM Tris/acetate, pH 8.0, which was added to the flow-through, and (ii) 1.5 ml of 0.8 M NaCl/7 M urea/50 mM Tris/acetate, pH 8.0. For some

Figure 1 Schematic diagram of the aggrecan core species described in this study

The figure illustrates the structures of the human peptides described in this paper. Some peptides (1, 4–10, 12, 13, *e*) have been identified in this study by double immunoreactivity and migration behaviour on SDS/PAGE. Others (peptides 2 and 3) were not detected in extracts of human cartilages, but have been identified previously in rat chondrosarcoma [31]. Peptide 11 has not been identified in any extracts or in the products of ADAMTS digestions of aggrecan. Peptides *a–d* and *f–i* are only characterized by single immunoreactivity and migration behaviour, so that the likely site of the unknown C- or N-terminus is given by a residue range. The N-terminus of species *i* is in parentheses, because it has not been determined here but is suggested from previous work [25]. The scheme for ADAMTS-generated human aggrecan peptides used throughout this text (peptides 1–13) is identical with that described for the rat [31]. Residue numbering is for human aggrecan [43] and assumes that the natural N-terminus (residue 1) is Val²⁰. Filled circle, G1 domain; checked circle, G2 domain; stippled circle, G3 domain.

samples, the column was eluted sequentially with 1.5 ml of buffer containing 0.1 M NaCl and 0.2 M NaCl before the 0.8 M NaCl elution. In these cases all salt-eluted fractions were combined for the analyses shown. The recovery in the 0.8 M NaCl eluate (or combined eluates) of guanidine-extracted GAG was always greater than 85% if more than 100 µg of GAG was fractionated. High-buoyant-density human fetal and mature aggrecan (the D1 fraction, > 1.5 g/ml) was also isolated by CsCl gradient centrifugation. Associative Sepharose CL-2B analysis has been described previously [32].

Isolation of aggrecan fragments from synovial fluids

Human synovial fluid (typically 2.0 ml containing about 100 µg of GAG) was digested with 2 turbidity-reducing units of *Streptomyces* hyaluronidase by incubation at 60 °C for 3 h after addition of 0.4 ml of 0.5 M ammonium acetate/20 mM EDTA/0.5 mM AEBSE, pH 6.0. The digest was dried to remove the ammonium acetate, and taken up in about 2 ml of 7 M urea/50 mM Tris/acetate, pH 8.0, for DE52 chromatography as described in detail above. The yield of GAG from the DE52 column was greater than 90% when 100 µg or more GAG was applied.

Deglycosylation and Western-blot analysis of fragments

The flow-through and NaCl eluates from DE52 chromatography were dialysed exhaustively against distilled water, and the retentates were dried and dissolved in 50 mM sodium acetate/50 mM Tris/10 mM EDTA, pH 7.6. Protease-free chondroitinase ABC (25 m-units/100 µg of GAG) was added, followed by incubation at 37 °C for 1–2 h. Samples were then adjusted to pH 6.0 with acetic acid, and 0.5 m-units (per 100 µg of GAG) of endo-β-galactosidase and 0.5 m-units (per 100 µg of GAG) of keratanase II were added followed by incubation at 37 °C for 2–4 h. Samples were then dried and dissolved in gel-loading buffer for SDS/PAGE. Quantification and determination of the relative abundance of proteins by densitometric analysis of Western blots was done as described in [33]. CS was assayed by reactivity with dimethyl-Methylene Blue as described in [34].

RESULTS

A schematic representation of the aggrecan core species described here

Figure 1 provides a schematic diagram of all the aggrecan species described in the text and characterized on the basis of electro-

Figure 2 The major aggrecan core species present in normal human articular cartilages and synovial fluids

Aggrecan fragments were isolated in high yield from normal weight-bearing tibial cartilage and from normal human knee synovial fluids. Portions (about 10 μ g of GAG) were deglycosylated and analysed by Western blotting with anti-G1 antiserum. The age (in years) of donors is shown. Also shown (left-hand lanes) is an analysis of fetal human aggrecan before and after digestion with aggrecanase and MMP-3. The species labelled *a–e*, 1 and 6 are shown in Figure 1.

Figure 3 Identification of G1-KEEE¹⁷¹⁴ and G1-SELE¹⁵⁴⁵ in normal mature human articular cartilages

High-buoyant-density mature human aggrecan (fraction D1) from the 29-year-old normal donor (shown in Figure 2) was deglycosylated and analysed by SDS/PAGE (4% gel). The blots were probed with anti-G1 (lane 1), anti-KEEE (lane 2) and anti-SELE (lane 3). Band 4 (G1-KEEE¹⁷¹⁴) and band 5 (G1-SELE¹⁵⁴⁵) migrate between bands 1 and *a*. See text for explanation.

phoretic migration and Western-blot analysis (see details below). Those species identified by numbers (1–13) follow the numbering scheme developed in a recent paper [31] on the aggrecanase-mediated degradation of aggrecan in a rat chondrosarcoma cell system. These represent full-length aggrecan (species 1) and both experimentally verified (species 2–10, 12 and 13) and hypothetical (species 11) products, which are generated by ADAMTS-dependent cleavage alone at one or more of the five known aggrecanase sites. On the other hand, the species identified by letters (*a–i*) have not been identified in terms of a specific C-terminal sequence (except for species *e*, which is G1-VDIPEN³⁴¹), but have been characterized only on the basis of migration behaviour and immunoreactivity with one or two antisera.

The aggrecan core species present in normal human articular cartilages

The isolation protocol used in this work was developed to ensure that all human aggrecan core fragments (whether CS/KS-substituted, KS-substituted or non-GAG substituted) are recovered in high yield from the guanidine hydrochloride extracts. To check this, portions of MMP-3-digested or ADAMTS-4-digested fetal and mature human aggrecan were fractionated on Sephadex G-50 and DE52 (see the Experimental section) and the percentage recovery of all species was determined by GAG analysis and Western blotting with multiple antisera. This established that more than 85 % of all species were recovered in the 0.8 M NaCl eluate from DE52, the product that was used for all the Western-blot analyses shown here.

The profile of G1-bearing species in normal human knee articular cartilages from individuals aged 16–71 years was remarkably similar at all ages (Figure 2). It consists of seven major products (1, *a*, *b*, *c*, *d*, 6 and *e*), although, as suggested previously [10], core-protein heterogeneity within some of these populations is likely. More samples would need to be analysed to determine if the variation in relative abundance of the different bands exhibits significant age-related change. The seven bands (labelled on the left of Figure 2) migrated at about 350 kDa (full-length aggrecan, species 1), 250 kDa (species *a*), 160 kDa (species *b*), 110 kDa (species *c*), 90 kDa (species *d*), 65 kDa (species 6, G1-NITEGE) and 50 kDa (species *e*, G1-VDIPEN). This supports previous data [11] that mature human articular cartilage contains as many as 12 separable G1-bearing aggrecan core species. In this regard it is clear that bands *a* and *b* are rather broad and the region of the gel between bands *a* and *b* contains a significant amount of heterogeneous immunoreactive material. It seems likely that with higher-resolution methods, this region of the gel (from about 300 kDa to 160 kDa) would resolve into multiple species.

Band 1 was identified as full-length aggrecan, since it was the only tissue species that also reacted with the anti-G3 antiserum (results not shown). The high-molecular-mass (> 150 kDa) core species described here as 1, *a* and *b* were heavily substituted with sulphated GAGs in cartilage, since they were also found in the D1 fraction (> 1.5 g/ml) on CsCl gradient centrifugation of guanidine hydrochloride extracts of human cartilages of all post-natal ages (see Figure 3, see the left lane for example). The identification of species 6 (G1-NITEGE³⁷³) and *e* (G1-VDIPEN³⁴¹) was made on the basis of their size and reactivity with their respective neopeptide antisera (results not shown), much as described previously for human cartilage extracts [12]. Since species *a–d* appeared to represent at least 50 % of the G1-aggrecan in human articular cartilages (Figure 2) we were interested to determine the precise C-termini for these species. Initially we examined the possibility that they might represent species such as 2–5 (see Figure 1), which are the known products of ADAMTS-dependent cleavage of aggrecan at the four CS-attachment region sites [12]. For this purpose we probed blots of both DE52-purified and CsCl-gradient-purified human aggrecan with antisera specific to these C-terminal sequences and which have been shown to react with their cognate epitopes in rat chondrosarcoma extracts [31]. No reactivity was observed with anti-ISQE¹⁹¹⁹ or anti-TAQ¹⁸¹⁹, suggesting that species 2 and 3 (Figure 1) do not accumulate in human articular cartilages. On the other hand, both anti-SELE¹⁵⁴⁵ and anti-KEEE¹⁷¹⁴ detected high-molecular-mass immunoreactive species in these samples, suggesting the presence of species 4 and 5 (Figure 1). Surprisingly, however, the migration position of these species (see Figure 3) did not correspond to either of the major species labelled as *a* and *b*. Instead, the species that reacted with these two antisera consistently migrated in the region of the gel between the full-length species 1 and band *a*. In other studies involving higher loading of Western blots and Sepharose CL-2B chromatography (results not shown), we found that species 4 and 5 did, as expected, also react with the anti-G1 antiserum and form aggregates with hyaluronan. The apparent absence of G1 immunoreactivity in this region of the gel (Figure 3) can be explained by the high sensitivity of the neopeptide antisera relative to the anti-G1 antiserum. In summary, the data show that mature human cartilage extracts contain detectable levels of aggrecan products generated by ADAMTS cleavage at Glu¹⁷¹⁴ and Glu¹⁵⁴⁵; however, together they represent a very minor proportion (apparently less than 2 %) of the C-terminally truncated aggrecan present. In a previous study (J. D. Sandy, R. A. Mumford, P. J. Roughley and M. W. Lark, unpublished work) we showed that these species are also present in these minor proportions in human cartilages from fetal, 2 month-, 1 year-, 5 year- and 15 year-old individuals.

While the structure of species *a* remains unknown, its migration behaviour (at about 250 kDa) suggests that it is considerably smaller than G1-SELE¹⁵⁴⁵, and it therefore appears to represent the product of cleavage within the C-terminal region of the CS-1 domain at about residues 1200–1300 (see Figure 1). The C-terminus of species *b* (Figures 2 and 3) is also unknown, but its migration behaviour at about 160 kDa, and its strong reactivity with an anti-G2 antiserum (result not shown), suggests that it is the naturally occurring form of the G1–G2 double-globe species [17] and that it therefore probably has a C-terminus at about residues 650–750, which is the region between the G2 domain and the KS-rich region (see Figure 1). The C-termini of species *c* and *d* (Figure 2) are also unknown, but their migration behaviours and immunoreactivities are consistent with them being aggrecan G1 domains with distinct C-termini, which are within the interglobular domain between Ala³⁷⁴ (the ADAMTS site) and the G2 domain, which initiates at Cys⁴⁸¹ (see Figure 1).

While the C-termini of these naturally occurring species *a–d* (Figure 2) have not yet been identified, it is significant that species with the same migration behaviour can be generated on partial digestion of human fetal aggrecan (largely band 1) with MMP-3 (Figure 2, left-hand lanes). As expected, in the same digest a good yield of the expected MMP product, species *e*, was obtained. In contrast, digestion of human fetal aggrecan with a crude aggrecanase preparation (see the Experimental section) generated little or no product that co-migrated with the natural species *a–e*, but, as expected, a high yield of products with the migration behaviour of species 4–6. For these reasons, we will use the term ‘MMP-like’ to describe the unknown proteinase(s) that generate species *a–d* in human cartilages.

The aggrecan core species present in normal human synovial fluids

The predominant G1-bearing aggrecan fragment present in normal synovial fluids from individuals ranging from 14 to 39 years (Figure 2) was species 6 (G1-NITEGE), with smaller amounts of species *b–e*. The high abundance of G1-NITEGE was particularly marked in the 14–21 age group, where it represented more than 90 % of the immunoreactive material. There was a clear age-related change, with G1-VDIPEN³⁴¹ and species corresponding approximately in size to bands *b–d* being more abundant in the fluids from older individuals. While the precise nature of the species in the 100–250 kDa size range is unclear, the 65 kDa and 50 kDa species have previously been identified in fluids as G1-NITEGE and G1-VDIPEN respectively [12]. When the fluid samples shown in Figure 2 were analysed instead with the G3 antiserum, a different set of fragments (G1-negative and G3-positive) was detected in the fluids (Figure 4). Image overlay of the patterns obtained with the G1 and G3 antisera showed that, appropriately, only the full-length aggrecan (species 1) reacted with both. Up to seven G3-reactive species (labelled as 1, 10, 9, 8, 7, *g* and *h* in Figure 4) were detected in some samples (see Figure 1 for a schematic diagram of the species), and two of these (*g* and *h*) appeared as doublet bands. In keeping with the notation of G1-bearing species, the readily identifiable G3-bearing fragments have been given numbers (see [31] for more description), whereas the partially identified species (*g* and *h*) have been given letters. Species 8 and 9 together appear to represent the majority of the G3-bearing fragments present in most samples, and these are identified here as the ADAMTS-generated peptides with N-termini at Ala¹⁸²⁰ and Gly¹⁷¹⁵ respectively. This identification, while not definitive, is based on the finding [12,31] that the equivalent fragments have been shown by N-terminal sequencing in rat chondrosarcoma aggrecan to represent the ADAMTS-generated fragments with N-terminals at Ala¹⁵⁶⁴ and Gly¹⁴⁵⁹ (rat residue numbers). This is also supported by a new finding (Figure 5) that the human fragment 7 (a doublet) specifically reacted with a neopeptide antiserum (anti-LGQRPP) to the ADAMTS-generated N-terminus at Leu¹⁹²⁰ (see Figure 1). Fragment 10 has not been unambiguously identified, but from the immunoreactivity and mobility of the peptide and by analogy with the rat aggrecan fragment analysis [31], it almost certainly represents the ADAMTS-generated G3-bearing peptide 10, with an N-terminal at Gly¹⁵⁴⁰ (Figure 1). A variably abundant and previously undescribed G3-bearing fragment (peptide *g*, a doublet) was also present in normal human fluids (Figure 4); from the mobility of this peptide it appears to represent a fragment generated by cleavage, by an unknown proteinase, within the ‘gap’ region (residues Ser²⁰⁶⁹–Val²¹⁴⁷) between the most C-terminal CS sub-

Figure 4 Further characterization of aggrecan fragments in normal human synovial fluids

Portions (10 μ g of GAG) of the deglycosylated fragments from normal synovial fluids shown in Figure 2 were run for Western-blot analysis with the anti-G3 antiserum (lanes 1–6, from left to right) and antibody 3-B-3 (lanes 7 and 8). The age (in years) of donors is shown above each lane and the species labelling refers to the structures shown in Figure 1. Antibody 3-B-3 detects an epitope closely related to the unsaturated disaccharide (Δ -di6S) which is generated as a linkage region stub by chondroitinase ABC treatment of whole aggrecan [41].

Figure 5 Aggrecan fragments in human synovial fluids: identification of peptide 7

High-buoyant-density (D1) aggrecan fragments were isolated by CsCl gradient centrifugation of synovial fluids collected from mature patients after knee joint trauma. Portions (10 μ g of GAG) were deglycosylated and run for Western blotting with the anti-G3 (lanes 1 and 2, from the left) and anti-LGQRPP (lanes 3 and 4) antisera.

stitution site and the globular G3 domain (see Figure 1). Species *h* is likely to be derived from species *g* by further N-terminal proteolysis.

The samples from 17 and 33 year-olds were also run and probed with antibody 3-B-3 (Figure 4, right-hand lanes). This illustrates that peptides 1, 8, 9 and 10, as expected, appear to carry the 3-B-3 (+) epitope on the core protein after

chondroitinase ABC digestion. Interestingly, peptides 7, *g* and *h* from the extreme C-terminal region of the molecule (see Figure 1) appeared to be poorly reactive with 3-B-3, suggesting that the epitope is not abundant in this region after chondroitinase ABC digestion. Also consistent with the structures and migration positions proposed for peptides 4, 5, 12 and 13 was the finding that this region of the gel was strongly 3-B-3 (+) positive and at the same time G3-negative (Figure 4, see all six anti-G3 lanes). These data do not, however, exclude the possibility that this region of the gel includes 3-B-3-positive bands other than peptides 4, 5, 12 and 13.

Since normal fluids contained abundant ADAMTS-generated G1-NITEGE and also ADAMTS-generated G3-domain fragments (peptides 8 and 9), it seemed likely that they would also contain core fragments from the central regions of the molecule; indeed this was evident on Western-blot analysis of the same samples with anti-KEEE, anti-SELE and anti-ARGSV (Figure 6). With anti-KEEE a single or double band of reactivity at about 300 kDa was typically seen, and this is consistent with the presence of peptides 4 and 12 (Figure 1). The KEEE-reactive species at about 70 kDa in the 33 year-old sample (species *i*) is probably the small intervening fragment generated by cleavage of peptide 4 to peptide 5 (see Figure 1), a product which has been observed in ADAMTS-4 digests of aggrecan in solution [25]. With anti-SELE, a doublet migrating slightly faster than the KEEE-reactive doublet was observed, consistent with the presence of peptides 5 and 13. The presence of peptides 12 and 13 in some samples was confirmed by the appropriate-sized doublet of reactivity with the anti-ARGSV antiserum (33 and 39 year samples). The anti-ARGSV also showed 'false' reactivity with full-length aggrecan in some samples (33 years), suggesting that under some conditions this antiserum can detect the peptide ARGSVILTVK in the intact molecule. Anti-ARGSV also detected an abundant and discrete product of about 100 kDa in

Figure 6 Aggrecan fragments in normal human synovial fluids

Portions (10 μ g of GAG) of the deglycosylated fragments from normal synovial fluids shown in Figure 2 were run for Western-blot analysis with anti-KEEE, anti-SELE and anti-ARGS. The age (in years) of donors is shown above each lane, and the species labelling refers to the structures shown in Figure 1.

Figure 7 Aggrecan in human femoral notch cartilage and knee synovial fluids following acute joint injury

Aggrecan fragments were isolated in high yield from normal and post-injury notch cartilage and from post-injury synovial fluids. Portions (10 μ g of GAG) were deglycosylated and run for Western-blot analysis with anti-G1 antiserum. The age (in years) of donors is shown above each lane and the species labelling refers to the structures shown in Figure 1. All patient descriptions are given in the Experimental section.

the 19 year normal sample and in several late-stage OA samples (results not shown). This species has not been further studied, but is represented schematically as fragment *f* in Figure 1. The

reactivity at about 70 kDa with anti-ARGSV in all samples (Figure 6) appears to be non-specific, since it was also observed in blank lanes (results not shown).

Figure 8 Further characterization of the aggrecan fragments in human synovial fluids following acute joint injury

Portions (10 μ g of GAG) of the deglycosylated fragments from the post-injury synovial fluids shown in Figure 7 were run for Western-blot analysis with anti-G3, anti-ARGS, anti-KEEE and anti-SELE. The age (in years) of donors is shown above each lane, and the species labelling refers to the structures shown in Figure 1. All patient descriptions are given in the Experimental section.

In summary, the analysis of normal cartilage and fluids supports the view that while the tissue contains abundant aggrecan, which is C-terminally truncated by MMP-like activity, the aggrecanase(s) represent the major proteolytic activity responsible for the degradative processing of aggrecan, which leads to its release into the synovial fluid. Consistent with this conclusion was the finding that we could not detect species in these fluid samples with antibody AF-28, which reacts with the N-terminal sequence FFGVG generated by MMP activity [28].

The aggrecan core species present in human cartilage and synovial fluids following knee joint injury

To examine aggrecan turnover in human joints in response to joint injury, we have analysed samples from two groups of mature patients (Figure 7). In the first group (Figure 7, left-hand lanes), we analysed cartilage obtained from the low-weight-bearing margin of the intercondylar notch of the distal femur. Tissue was obtained from cadavers with no evidence of joint disease and from the identical site obtained at notchplasty during ACL reconstructive surgery. The aggrecan core species were isolated and the Western-blot analysis with anti-G1 for two typical samples from each group is shown. The analysis of normal notch cartilage gave data that were, as expected, very similar to results obtained with normal tibial cartilage samples from weight-bearing areas (Figure 2), with evidence for all seven of the core species described previously. However, the same analysis of notch cartilage from post-injury patients (8 and 11 weeks post ACL rupture) was distinctly different, with evidence for the depletion of bands *a*, *d* and particularly G1-VDIPEN (band *e*) with a concomitant increase in band *b* and particularly G1-NITEGE (band 6).

In the second group (Figure 7, right-hand lanes) we analysed the fluid taken from joint-injured patients. In these patients (36–53 years) the G1-bearing fragment profiles were remarkably similar in each patient, and consisted predominantly of species 6 (G1-NITEGE) and a very small and variable amount of G1-VDIPEN together with some band-*c*-like material. This profile

was therefore very similar to that seen with normal synovial fluids taken from individuals of 14–21 years (Figure 2). Analysis of these fluids with anti-G3, anti-ARGS, anti-KEEE and anti-SELE (Figure 8) supported the view that the composition of acute injury fluids was most similar to normal fluids taken from the younger patients in the 14–39 age group (see Figures 4 and 6). The bands at about 70 kDa in the anti-ARGS, anti-KEEE and anti-SELE blots were consistently seen in blank lanes (results not shown) and therefore appear to be non-specific.

In summary, the data obtained with cartilage and fluids after joint injury suggest that it is accompanied by an acceleration of the normal pathways of aggrecan turnover by ADAMTS activity. Most strikingly, a rapid accumulation of G1-NITEGE was seen in the notch cartilage at 8–11 weeks after injury, and the fluids were G1-NITEGE-rich when taken as early as 2 days and as late as 277 weeks after joint injury.

The aggrecan core species present in human cartilage and synovial fluids at joint-replacement surgery for advanced OA

The aggrecan present in the cartilage and synovial fluid taken from patients at joint-replacement surgery for end-stage OA was also analysed. The tissue profiles with anti-G1 (Figure 9, left-hand lanes) were remarkably similar to those obtained with aggrecan from normal cartilage in this age range (see Figure 2), again consisting of the seven well-recognized core species. Unlike the tissue from acutely injured joints, the late-stage OA tissue had a higher and therefore more 'normal' content of G1-VDIPEN relative to G1-NITEGE. The G1-positive species in the synovial fluids from this patient group (Figure 9, right-hand lanes) were similar to those found in the mature normal fluids, again with a 'normal' content of G1-VDIPEN relative to G1-NITEGE. In contrast, the fluid from these patients exhibited abnormal and variable profiles when analysed with anti-G3, anti-ARGS, anti-KEEE and anti-SELE (Figure 10). Particularly striking was the absence of the 'ladder' of G3-reactive species 7–10, which is a consistent feature of normal and injury fluids. Instead these

Figure 9 Aggrecan fragments in human articular cartilage and synovial fluids taken at joint-replacement surgery

Aggrecan fragments were isolated in high yield from pooled articular cartilages and from synovial fluids obtained at knee-joint arthroplasty for late-stage OA. Portions (10 μ g of GAG) were deglycosylated and run for Western-blot analysis with anti-G1. The patient age in years is given above each lane, and the species labelling refers to the structures shown in Figure 1.

Figure 10 Further characterization of the aggrecan fragments in human synovial fluids at joint-replacement surgery

Portions (10 μ g of GAG) of the deglycosylated fragments from the fluids shown in Figure 9 were run for Western analysis with anti-G3, anti-ARGS, anti-KEEE and anti-SELE. The age (in years) of donors is shown above each lane, and the species labelling refers to the structures shown in Figure 1.

samples were highly enriched in the low-molecular-mass G3-positive doublet, designated species *g* (see Figure 1). The anti-ARGSV probe showed an unusual triplet of species in the region of species 12 and 13; however, there was less evidence for the KEEE-reactive species 4 and 12 but apparently high concentrations of the SELE-positive species 13. In summary, the data obtained with cartilage and fluid from end-stage OA joints has features that distinguish it from both normal and joint-injured patients. Whereas the tissue is more similar to the normal state than to post-injury, the fluid is unlike either of the other groups. Instead it is characterized by evidence of more extensive proteolysis of the CS-bearing fragments by proteinases that do not appear to be active in normal or acutely injured knees.

DISCUSSION

The data presented provide several new and important insights into the pathways of aggrecan proteolysis in human knee joint cartilage *in vivo*. First, analysis of aggrecan from normal, injured and late-stage OA mature cartilages (Figures 2, 3, 7 and 9) has shown that there are at least 7 major core species in human cartilages and that five of these, which appear to represent more than 50% of the total core protein present, are not the product of ADAMTS-dependent cleavage at the established 'aggrecanase' sites. Instead, these species (described as bands *a–e* in Figure 2) have C-termini that can either be generated by MMPs (species *e*, G1-VDIPEN³⁴¹) or which appear to be in regions of the core protein (residues 1200–1300, 653–755 and 374–480) with known MMP-sensitive cleavage sites. For example, species *a* might have a C-terminal sequence of GVED¹²⁷⁵, since it is known that MMP-3 treatment of cartilage releases fragments with an N-terminal sequence of ISGL [35], which would be generated by cleavage at Asp¹²⁷⁵–Ile¹²⁷⁶ or at other Asp/Glu–Ile bonds in this region of human aggrecan. In the same way, species *b* might have a C-terminus of CFRG⁶⁵⁶, since MMP-1 is known to cleave aggrecan at the Gly⁶⁵⁶–Ile⁶⁵⁷ bond (C. R. Flannery and J. D. Sandy, unpublished work). Also, species *c* and *d* might have C-termini of FTSED⁴⁴¹ and TVKP³⁸⁴, respectively, since these are known MMP-dependent cleavage sites in the interglobular domain [18,36]. In support of this idea is the finding (Figure 2) that MMP-3 digestion of fetal human aggrecan generates products that co-migrate with species *a–e*. In this regard it is also interesting that the equivalent species are also abundant in extracts of human, bovine and porcine epiphyseal growth cartilage (J. D. Sandy and C. Verscharen, unpublished work), suggesting that they are formed during cartilage growth [29] and may therefore be species that represent the 'so-called' long-lived pool of tissue aggrecan [4,27]. Definitive identification of these species will, however, require isolation and sequencing of the natural C-terminal peptides.

Secondly, the only major tissue aggrecan species that is an end-product of glutamyl endoproteinase (ADAMTS)-dependent cleavage is G1-NITEGE³⁷³. Two other related products (G1-KEEE¹⁷¹⁴ and G1-SELE¹⁵⁴⁵) are present, but only in trace amounts. The accumulation of G1-NITEGE³⁷³, in the virtual absence of G1-KEEE¹⁷¹⁴ and G1-SELE¹⁵⁴⁵, suggests that the G1-NITEGE represents a relatively stable or 'terminal' product, whereas the other species may be short-lived intermediates in the pathway of proteolysis which leads to the abundant matrix forms represented by species *a–d*.

Thirdly, in contrast with the cartilage, the normal synovial fluids (Figures 2, 4 and 6) are composed primarily of fragments

generated by ADAMTS activity. Thus the 3-B-3-positive species (Figure 4), which almost certainly represent the bulk of the CS-bearing fragments, co-migrate with the ADAMTS-generated species 4, 5, 8–10, 12 and 13. This finding is consistent with our earlier conclusion [21,22] that the quantitatively major aggrecan fragments present in fluids are generated by 'aggrecanase'. The only clear exception to this is the appearance of G1-VDIPEN³⁴¹ in normal fluids from older individuals (Figure 2) and in fluids from joint-replacement patients (Figure 9). This result is clearly consistent with the finding that small interglobular domain fragments generated by cleavage of the Asn³⁴¹–Phe³⁴² bond [28,37] are also present in human fluids. Therefore, our suggestion from cell-culture studies that cleavage at the Asn³⁴¹–Phe³⁴² bond is always secondary to cleavage at the Glu³⁷³–Ala³⁷⁴ bond in chondrocyte-mediated aggrecanolysis [38] also appears to pertain to cartilage explants [15] and human cartilage *in vivo*.

Fourthly, analysis of fluids from joint-injured patients (Figures 7 and 8) gave patterns that were very similar to normal fluids in most respects. Therefore the marked increase in the concentration and content of aggrecan fragments following acute knee injury [39] appears to result from an acceleration of the normal ADAMTS-dependent pathway of aggrecan degradation. Indeed the finding that the vast majority of G1-reactive material in injury was represented by the G1-NITEGE fragment is consistent with a post-injury return to the pathway expressed in young individuals (Figure 2). An exception to this rule was that the anti-G3 blot of acute injury fluid (Figure 9) showed the expected ladder of fragments 7–10, but little or no evidence for the small doublet, fragment *g*, seen in most normal fluids (Figure 6).

Fifthly, analysis of fluid taken at joint replacement (Figures 9 and 10) showed some marked differences from normal and acute injury samples, which suggested that in late-stage OA more extensive cleavage of the aggrecan occurs. Thus the pattern of G1-reactive fragments was enriched in the G1-VDIPEN³⁴¹ product and the anti-G3 blot showed a relative paucity of the large G3 fragments, 7–10, and a marked abundance of the small fragment *g*. Analysis with the ARGS, KEEE and SELE antisera also suggested that more extensive proteolysis of the large fragments (12 and 13) had occurred in these joints.

Sixthly, the marked differences in fluid composition between normal (Figures 1 and 4) and late-stage OA (Figures 9 and 10) samples were not accompanied by a detectable difference in tissue composition (Figures 2 and 9). Analysis of three samples of normal aged and end-stage OA cartilage aggrecan showed that the general pattern and relative abundance of high-buoyant-density species (1, 4, 5, *a* and *b*) was very similar in all samples. This suggests that the aggrecan core protein composition of the tissue does not change markedly during progression of the disease, which is in contrast with the situation in human aging where the G1-VDIPEN and G1-NITEGE fragments appear to represent an increasing proportion of the G1-bearing species in the tissue [40]. On the other hand, the tissue composition appears to undergo a marked change following injury, a change that is not evident in end-stage disease (Figure 7). This is seen as a marked increase in the ratio of G1-NITEGE to G1-VDIPEN; thus, densitometric analysis shows that this ratio is about 2.5 in normal cartilage, but increases to about 12 in the early post-injury period, a change that presumably reflects a period of rapid matrix remodelling that must involve the preferential addition of G1-NITEGE to the matrix as a result of enhanced aggrecanase activity.

Finally, the essentially normal profile of aggrecan core structure in articular cartilage at end-stage OA is in clear contrast with the effect of this disease on the CS composition [41,42], which undergoes a marked change in the non-reducing terminal isomer

ratio towards an 'adolescent' phenotype. When taken together, these results suggest that the changes in aggrecan turnover which accompany joint injury, and which are associated with progression to end-stage OA, are most readily explained as an acceleration of the normal ADAMTS-dependent proteolytic pathways. However, these extracellular events occur at the same time as an activation of altered pathways of intracellular CS synthesis (A. H. Plaas, M. E. Adams and J. D. Sandy, unpublished work). It therefore appears that the response of the mature articular chondrocyte to joint injury, which commonly leads to osteoarthritic lesions, involves an activation of both anabolic and catabolic pathways, and that a full understanding of OA and its progressive nature will require elucidation of the cellular mechanisms responsible for both of these changes.

This work was funded by grants to J. D. S. from the Shriners of North America and the Arthritis Foundation. The expert technical assistance of Vivian Thompson, Charlene MacPherson and Troy Howell is gratefully acknowledged. Special thanks are due to Dr Stefan Lohmander and colleagues for the supply of human synovial fluids, to Dr Fred Nelson and colleagues for supply of notchplasty cartilage and fluids and to Dr Bill Kilgore for supply of late-stage OA cartilage and fluids.

REFERENCES

- Bayliss, M. T. and Ali, S. Y. (1978) Age-related changes in the composition and structure of human articular-cartilage proteoglycans. *Biochem. J.* **176**, 683–693
- 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
- Ilic, M. Z., Robinson, H. C. and Handley, C. J. (1998) Characterization of aggrecan retained and lost from the extracellular matrix of articular cartilage. Involvement of carboxyl-terminal processing in the catabolism of aggrecan. *J. Biol. Chem.* **273**, 17451–17458
- 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
- Buckwalter, J. A., Roughley, P. J. and Rosenberg, L. C. (1994) Age-related changes in cartilage proteoglycans: quantitative electron microscopic studies. *Microsc. Res. Tech.* **28**, 398–408
- Roughley, P. J. and White, R. J. (1983) The use of caesium sulphate density gradient centrifugation to analyse proteoglycans from human articular cartilages of different ages. *Biochim. Biophys. Acta* **759**, 58–66
- Dudhia, J., Davidson, C. M., Wells, T. M., Vynios, D. H., Hardingham, T. E. and Bayliss, M. T. (1996) Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage. *Biochem. J.* **313**, 933–940
- Webber, C., Glant, T. T., Roughley, P. J. and Poole, A. R. (1987) The identification and characterization of two populations of aggregating proteoglycans of high buoyant density isolated from post-natal human articular cartilages of different ages. *Biochem. J.* **248**, 735–740
- Glant, T. T., Mikecz, K., Roughley, P. J., Buzas, E. and Poole, A. R. (1986) Age-related changes in protein-related epitopes of human articular-cartilage proteoglycans. *Biochem. J.* **236**, 71–75
- Vilim, V. and Fosang, A. J. (1993) Characterization of proteoglycans isolated from associative extracts of human articular cartilage. *Biochem. J.* **293**, 165–172
- Vilim, V. and Fosang, A. J. (1994) Proteoglycans isolated from dissociative extracts of differently aged human articular cartilage: characterization of naturally occurring hyaluronan-binding fragments of aggrecan. *Biochem. J.* **304**, 887–894
- Sandy, J. D., Plaas, A. H. and Koob, T. J. (1995) Pathways of aggrecan processing in joint tissues. Implications for disease mechanism and monitoring. *Acta Orthop. Scand. Suppl.* **266**, 26–32
- 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
- Mort, J. S. and Buttle, D. J. (1999) The use of cleavage site specific antibodies to delineate protein processing and breakdown pathways. *Mol. Pathol.* **52**, 11–18
- 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 aggrecan *in vitro*. *Biochem. J.* **344**, 61–68
- Heinegard, D., Wieslander, J., Sheehan, J., Paulsson, M. and Sommarin, Y. (1985) Separation and characterization of two populations of aggregating proteoglycans from cartilage. *Biochem. J.* **225**, 95–106
- Fosang, A. J. and Hardingham, T. E. (1989) Isolation of the N-terminal globular protein domains from cartilage proteoglycans. Identification of G2 domain and its lack of interaction with hyaluronate and link protein. *Biochem. J.* **261**, 801–809
- Fosang, A. J., Last, K., Knauper, V., Neame, P. J., Murphy, G., Hardingham, T. E., Tschesche, H. and Hamilton, J. A. (1993) Fibroblast and neutrophil collagenases cleave at two sites in the cartilage aggrecan interglobular domain. *Biochem. J.* **295**, 273–276
- Cole, A. A. and Kuettner, K. E. (1995) MMP-8 (neutrophil collagenase) mRNA and aggrecanase cleavage products are present in normal and osteoarthritic human articular cartilage. *Acta Orthop. Scand. Suppl.* **266**, 98–102
- Williams, J. M., Downey, C. and Thonar, E. J. (1988) Increase in levels of serum keratan sulfate following cartilage proteoglycan degradation in the rabbit knee joint. *Arthritis Rheum.* **31**, 557–560
- Sandy, J. D., Flannery, C. R., Neame, P. J. and Lohmander, L. S. (1992) The structure of aggrecan fragments in human synovial fluid. Evidence for the involvement in osteoarthritis of a novel proteinase which cleaves the Glu 373-Ala 374 bond of the interglobular domain. *J. Clin. Invest.* **89**, 1512–1516
- 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
- Sandy, J. D., Westling, J., Kenagy, R. D., Iruela-Arispe, M. L., Verscharen, C., Rodrigue-Mazaneque, J. C., Zimmerman, D., Lemire, J. M., Fischer, J. W., Wight, T. N. and Clowes, A. W. (2001) Versican V1 proteolysis in human aorta *in vivo* occurs at the Glu441-Ala442 bond, a site which is cleaved by recombinant ADAMTS-1 and ADAMTS-4. *J. Biol. Chem.* **276**, 13372–13378
- 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
- Kuno, K., Okada, Y., Kawashima, H., Nakamura, H., Miyasaka, M., Ohno, H. and Matsushima, K. (2000) ADAMTS-1 cleaves a cartilage proteoglycan, aggrecan. *FEBS Lett.* **478**, 241–245
- Bayliss, M. T. and Roughley, P. J. (1985) The properties of proteoglycan prepared from human articular cartilage by using associative caesium chloride gradients of high and low starting densities. *Biochem. J.* **232**, 111–117
- Fosang, A. J., Last, K. and Maciewicz, R. A. (1996) Aggrecan is degraded by matrix metalloproteinases in human arthritis. Evidence that matrix metalloproteinase and aggrecanase activities can be independent. *J. Clin. Invest.* **98**, 2292–2299
- 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
- Mort, J. S., Magny, M. C. and Lee, E. R. (1998) Cathepsin B: an alternative protease for the generation of an aggrecan 'metalloproteinase' cleavage neopeptide. *Biochem. J.* **335**, 491–494
- 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
- Sandy, J. D. and Plaas, A. H. (1989) Studies on the hyaluronate binding properties of newly synthesized proteoglycans purified from articular chondrocyte cultures. *Arch. Biochem. Biophys.* **271**, 300–314
- Sandy, J. D., Gamett, D., Thompson, V. and Verscharen, C. (1998) Chondrocyte-mediated catabolism of aggrecan: aggrecanase-dependent cleavage induced by interleukin-1 or retinoic acid can be inhibited by glucosamine. *Biochem. J.* **335**, 59–66
- Farndale, R. W., Buttle, D. J. and Barrett, A. J. (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim. Biophys. Acta* **883**, 173–177
- Bonassar, L. J., Frank, E. H., Murray, J. C., Paguio, C. G., Moore, V. L., Lark, M. W., Sandy, J. D., Wu, J. J., Eyre, D. R. and Grodzinsky, A. J. (1995) Changes in cartilage composition and physical properties due to stromelysin degradation. *Arthritis Rheum.* **38**, 173–183
- Fosang, A. J., Last, K., Knauper, V., Murphy, G. and Neame, P. J. (1996) Degradation of cartilage aggrecan by collagenase-3 (MMP-13). *FEBS Lett.* **380**, 17–20

- 37 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
- 38 Lark, M. W., Gordy, J. T., Weidner, J. R., Ayala, J., Kimura, J. H., Williams, H. R., Mumford, R. A., Flannery, C. R., Carlson, S. S., Iwata, M. et al. (1995) Cell-mediated catabolism of aggrecan. Evidence that cleavage at the “aggrecanase” site (Glu373-Ala374) is a primary event in proteolysis of the interglobular domain. *J. Biol. Chem.* **270**, 2550–2556
- 39 Dahlberg, L., Friden, T., Roos, H., Lark, M. W. and Lohmander, L. S. (1994) A longitudinal study of cartilage matrix metabolism in patients with cruciate ligament rupture – synovial fluid concentrations of aggrecan fragments, stromelysin-1 and tissue inhibitor of metalloproteinase-1. *Br. J. Rheumatol.* **33**, 1107–1111
- 40 Sztrvolovics, 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
- 41 Plaas, A. H., West, L. A., Wong-Palms, S. and Nelson, F. R. (1998) Glycosaminoglycan sulfation in human osteoarthritis. Disease-related alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J. Biol. Chem.* **273**, 12642–12649
- 42 Brown, M. P., West, L. A., Merritt, K. A. and Plaas, A. H. (1998) Changes in sulfation patterns of chondroitin sulfate in equine articular cartilage and synovial fluid in response to aging and osteoarthritis. *Am. J. Vet. Res.* **59**, 786–791
- 43 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

Received 9 November 2000/30 May 2001; accepted 9 July 2001