Age-related changes in the composition, the molecular stoichiometry and the stability of proteoglycan aggregates extracted from human articular cartilage

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The heterogeneity of the components of proteoglycan aggregates, their stoichiometry within the aggregate and the aggregates' stability was investigated in normal human articular cartilage specimens (age-range newborn to 63 years). Proteoglycans were extracted from tissue by sequentially extracting them with PBS alone, PBS containing oligosaccharides of hyaluronan, and PBS containing solutions of increasing guanidinium chloride concentration (1 M, 2 M, 3 M and 4 M). A high proportion of each of the components of the proteoglycan aggregate, i.e. uronic acid, sulphated glycosaminoglycan, hyaluronan binding domain of aggrecan (G1-domain), link protein (LP) and hyaluronan, was extracted from immature cartilage by PBS alone and PBS containing oligosaccharides of hyaluronan. This was in marked contrast to adult cartilage, which required high concentrations of guanidinium chloride for the efficient extraction of these components. The molar ratios of total G1-domain:LP and the G1-domain associated with aggrecan:LP also differed markedly between immature and mature cartilage and between each of the

sequential extracts. The concentration of LP was less than that of the G1-domain in all extracts of cartilage from individuals over 13 years, but this was particularly noticeable in the 1 M guanidinium chloride extracts, and it was surmised that a deficiency in LP produces unstable aggregates *in situ*. The fragmentation of LP, which is known to occur with advancing age, did not influence the extractability of LP, and fragments were present in each of the sequential extracts. Therefore the generally accepted model of proteoglycan aggregation presented in the literature, which is mostly derived from analysis of immature animal cartilage, cannot be used to describe the structure and organization of aggregates in adult human articular cartilage, where a heterogeneous population of complexes exist that have varying degrees of stability.

Key words: aggrecan, competitive, heterogeneity, hyaluronan, link protein.

INTRODUCTION

The major space-filling molecule in the extracellular matrix of articular cartilage is the large aggregating proteoglycan, aggrecan. It is a complex macromolecule comprised of a central protein core to which numerous chondroitin sulphate and keratan sulphate (KS) glycosaminoglycan chains are covalently attached. The high negative charge of the glycosaminoglycan component renders the molecule osmotically active, thereby allowing the cartilage to deform reversibly under load.

The core protein of aggrecan contains three disulphide-bonded globular domains, designated G1 (the hyaluronan binding domain of aggrecan), G2 and G3 [1]. One molecule of aggrecan can contain up to 100 chondroitin sulphate chains, 30 KS chains and many O- and N-linked oligosaccharides [2]. The G3-domain is located at the C-terminus of the molecule and contains regions that share sequence similarity with C-type lectins, complement regulatory proteins (CRP) and members of the epidermal growth factor family [3]. The G3-domain is thought to be associated mainly with immature newly synthesized aggrecan, and the findings of Dudhia et al. [4] support this hypothesis. This domain binds other matrix components that may permit essential cross-links during extracellular matrix formation during development [5–7]. The CRP domain may also function by promoting glycosaminoglycan chain attachment to the core protein during

aggrecan synthesis [8], and both the G3-domain and chondroitin sulphate-rich core protein are required for effective secretion of aggrecan [9]. A requirement for a heat shock protein of 25 kDa ('HSP25') has also been implicated in G3-domain-mediated aggrecan secretion [10]. The G2-domain is located towards the N-terminus of the molecule, and shares some of the structural features of the G1-domain. However, it has no hyaluronan (HA) binding properties, despite sharing considerable sequence similarity with the G1-domain [11]. Although its exact function is still unknown, it appears to function by inhibiting aggrecan secretion [12]. It has been shown that both G1 and G2 work in concert with each other to inhibit the secretion of aggrecan, whilst G3 and the chondroitin sulphate-glycosylated core protein region promote secretion. Therefore, G2 acts as a secretion retardant, ensuring that only fully glycosylated aggrecan monomer is secreted [9].

The N-terminal G1-domain interacts with a decasaccharide length of HA and, by binding to the latter molecule, it facilitates the formation of multimolecular aggregates [13]. The G1-domain contains three regions important in binding to HA. Two tandem repeat sequences (designated B and B' or link modules) and the A region which has structural similarity with the immunoglobulin fold, are required to effect maximal binding to HA. The A region contains an O-linked carbohydrate side chain, which is important in fortifying the interaction between HA and the G1-domain

Abbreviations used: CRP, complement regulatory proteins; G1-domain, hyaluronan binding domain of aggrecan; GnHCl, guanidinium chloride; HA, hyaluronan; KS, keratan sulphate; LP, link protein; s-gag, sulphated glycosaminoglycan; UA, uronic acid.

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[13]. Interestingly, these carbohydrate side chains become elongated in preference to KS chains with age; whether this influences the G1–HA binding affinity and therefore modifies the aggrecan aggregate, is not known [14,15]. The G2 domain is unable to bind HA, despite having both B and B' components of the tandem repeat. It has been suggested that an extra N-linked oligosaccharide, which may exist in the B' region of G2, may disrupt the folding of this region and prevent binding [16]. Proteins of this family exist which, despite having only one tandem repeat region, are capable of binding HA [e.g. CD44 and TSG-6 (tumour-necrosis-factor-stimulated gene-6) [17]]. The reasons are as yet unclear why these only require one tandem repeat region, whilst aggrecan and versican require both B regions. In addition to inhibiting secretion, the G1-domain tandem repeats may also have a regulatory function in aggrecan biosynthesis, inhibiting glycosaminoglycan chain attachment to core protein. Each aggrecan/HA interaction is stabilized by the binding of one molecule of link protein (LP) [13,18]. The affinity of aggrecan G1 for HA is approx. $0.226 \mu\text{M}$, whilst the affinity of LP for HA is higher ($K_d = 0.089 \mu\text{M}$), thereby increasing the binding of the multimolecular complex [13]. The LP molecule is a 41–48 kDa glycoprotein that also shares sequence identity with the G1-domain of aggrecan, and the two interact via an immunoglobulin-like sequence [11,19].

The turnover of normal human articular cartilage results in extensive age-related changes in the structure of proteoglycan aggregates. As a consequence of C-terminal trimming and a reduction in the length of the chondroitin sulphate attachment region, aggrecan molecules lacking a G3-domain are present at higher concentrations in mature tissues [4]. Proteolytic cleavage also occurs between the G1- and G2-domains, liberating 'free' G1-domain that still has the ability to bind HA, and, as a consequence of this property, accumulates in the tissue during normal ageing [20,21]. The concentration of HA also increases with ageing, presumably to accommodate the accumulating G1-domain (intact and 'free') and the abundance of lower-molecular-mass aggrecan molecules [22]. However, there is an age-related decrease in the size of HA in adult cartilage that results in smaller aggregates.

LP also exhibits structural changes during normal ageing. Of the three isoforms, LP3 (41 kDa) is derived from the N-terminal cleavage of either LP1 (48 kDa) or LP2 (44 kDa) and is the major form of the molecule in adult cartilage [23]. In addition, fragments of LP have been identified in extracts of adult cartilage [22–26]. We have developed a strategy to investigate age-related changes in the *in situ* stoichiometry and hence stability of proteoglycan aggregates in human articular cartilage using differential extraction protocols. These have been devised by exploiting the specific interactions known to occur within the proteoglycan aggregate.

EXPERIMENTAL

Tissue and materials

Macroscopically normal human articular cartilage was obtained from the femoral condyles of an age-range of normal individuals (newborn to 63 years for the specimens that were put through the full analysis, but 58 only had G1 and LP analysis) following amputation for bone and cartilage tumours, not involving the knee joint, and in the case of the newborn specimen, from the femoral condyle at post-mortem within 12 h of death. Full thickness cartilage was stored at -70°C until it was processed.

Sepharose CL-2B, Sepharose CL-6B and Sephadex G50 and G100 were from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Acrylamide (ultrapure) and *N,N'*-methylenebisacrylamide

(ultrapure) were from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). PBS tablets and Dulbecco's modified Eagle's medium were from Flowgen Laboratories (Ashby-de-la-Zouch, Leics., U.K.). Chondroitinase ABC was from AMS Biotechnology (Abingdon, Oxfordshire, U.K.). Testicular hyaluronidase, papain, diphenylcarbamyl chloride-treated trypsin, anti-mouse IgG and anti-rabbit IgG antibodies conjugated to horseradish peroxidase were from Sigma–Aldrich. Enhanced chemiluminescence reagents (ECL[®]), HyperECL[®], X-ray film, Na¹²⁵I and Hybond N⁺ were from Amersham Biosciences. Immobilon PVDF transfer membrane was from Millipore (Watford, Herts., U.K.). 1,9-Dimethylmethylene Blue was from Sigma–Aldrich and the protein assay kit was from Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K.). This is a dye-binding assay based on the colour change (from 465 nm to 595 nm) of Coomassie Brilliant Blue G250 in response to various concentrations of protein. All other chemicals used were of analytical grade or equivalent. The rabbit polyclonal antisera, porcine anti-LP and human anti-G1-domain, were prepared as described by Dudhia et al. [4]. Mouse monoclonal antibodies 8-A-4, 3-B-3 and 2-B-6 were a gift from Professor Bruce Caterson (Connective Tissue Biology Laboratory, Department of Biosciences, Cardiff University, U.K.).

General procedures and measurements of effects on aggregation

Measurements of uronic acid (UA) were performed using an automated method [27]. Sulphated glycosaminoglycan (s-gag) concentrations were determined by a dye-binding assay as described by Ratcliffe et al. [28]. Protein concentrations were determined using the Bio-Rad protein assay. The galactosamine and glucosamine content of articular cartilage of different ages was determined after digesting the tissue with papain and hydrolysing the digest with 6 M HCl and separation on a Technicon II Autoanalyser [29].

Preparation of oligosaccharides of HA

Human umbilical cord HA (Sigma) at 1 mg/ml in 0.2 M sodium acetate, pH 5.0, was digested with testicular hyaluronidase (40 units/mg of HA) for 4 h at 37°C . The enzyme was precipitated by boiling the digest for 5 min and the supernatant was retained after centrifugation at $1500 g$ for 5 min. The pellet was washed in the digest buffer, re-centrifuged and the supernatant pooled with the original supernatant. The extent of HA fragmentation was determined by analysing $100 \mu\text{l}$ of the supernatant on a Sephadex G50 gel-filtration column ($120 \text{ cm} \times 1 \text{ cm}$) eluted with 0.2 M sodium acetate, pH 5.0. The ability of the oligosaccharides to interfere with aggregation was assessed as follows: porcine proteoglycan aggregates were prepared by mixing $100 \mu\text{l}$ porcine aggrecan (1.7 mg/ml in 0.5 M sodium acetate, pH 6.8) with $34 \mu\text{l}$ of HA ($100 \mu\text{g/ml}$) for 18 h at 4°C . Aggregates were also prepared by mixing the above with LP ($27 \mu\text{l}$ of a 0.67 mg/ml stock). The degree of aggregation in the presence of oligosaccharides of HA was assessed by analysing the elution profile of each mixture eluted from a column of Sepharose CL-2B.

Extraction of aggrecan from cartilage

Approx. 300 mg wet weight of full depth samples of normal human articular cartilage of various ages was cryosectioned at $20 \mu\text{m}$ in order to overcome any differences in the rates of extraction of the constituent macromolecules from the tissue before extracting it directly with 4 M guanidinium chloride (GnHCl), or treating it as follows.

Protocol A

Sections of cartilage were sequentially extracted with 10 vol. of the following extractants for 24 h periods at 4 °C in the presence of proteinase inhibitors: Extract 1 (E1), PBS alone, enables the extraction of freely extractable aggrecan from the tissue as well as any freely extractable aggregate; extract 2 (E2), PBS containing oligosaccharides of HA (1 mg of UA/100 mg of tissue), competitively extracts aggrecan that is non-LP-stabilized in the tissue [30] and extract 3 (E3), 4 M GnHCl containing 50 mM sodium acetate, pH 6.8, which extracts aggrecan complexed with HA and LP.

Protocol B

Extracts 1 and 2 were as for Protocol A, but E3 was replaced by four progressively increasing concentrations of GnHCl, i.e. 1 M, 2 M, 3 M and 4 M GnHCl (E3–E6), in order to investigate the presence of aggregates of intermediate stability, possibly arising from structural changes in LP and/or G1-domain, which could affect their ability to interact with HA. All extracts were dialysed extensively against 50 mM sodium acetate, pH 6.8, containing proteinase inhibitors, in order to promote proteoglycan aggregation. Tissue residues were digested with papain (2.8 units/ml of digest buffer) at 60 °C overnight.

Separation of oligosaccharides of HA from extracted proteoglycans

Freeze-dried extracts (50–75 µg of UA) were reconstituted in 4 M GnHCl and incubated at 4 °C for 18 h. Aggrecan was separated from the dissociated oligosaccharides of HA by filtration in a column (120 cm × 1 cm) of Sephadex G100. Aggrecan was excluded from the column as determined by immunolocalizing the G1-domain on dot blots of each fraction (results not shown).

Preparation of porcine LP

LP was prepared from a trypsin digest of porcine laryngeal aggregate according to the method of Bonnet et al. [31]. The G1-domain and LP were separated on a TSK SWG 3000 preparative column equilibrated with 4 M GnHCl. The concentration of purified LP was determined using its molar absorption coefficient.

Preparation of human G1-domain

The G1-domain was prepared from the articular cartilage of adult (> 60 years of age) femoral condyles by the method described by Bonnet et al. [31]. The concentration of G1-domain was determined using its molar absorption coefficient.

Radioimmunoassay of LP, G1-domain and KS

The iodination of tyrosine residues in porcine LP and human G1-domain was performed with Chloramine T as the oxidizing agent. Radioimmunoassays for LP (using monoclonal antibody 8-A-4), the G1-domain of aggrecan and KS were carried out essentially as described by Dudhia et al. [4].

Quantitation of 'free' G1-domain

Freeze-dried extracts containing 50–100 µg/ml UA were resuspended in 4 M GnHCl containing 50 mM sodium acetate, pH 6.8. Samples were fractionated on a Sepharose CL-6B column (12 cm × 0.5 cm) equilibrated with 2 M GnHCl and eluted at 2 ml/h. Radioimmunoassay for the G1-domain in the 0.5 ml fractions collected enabled a profile to be produced from which

the relative amounts of intact aggrecan core protein and 'free' G1-domain could be calculated.

ELISA-based assay for HA

Porcine G1-domain was prepared from pig laryngeal cartilage by the method of Bonnet et al. [31] and was biotinylated using the manufacturer's instructions. The G1-domain was bound to exogenous HA to protect the functional binding site during biotinylation. It was then dissociated from the HA by adding 4 M GnHCl and separated on a TSK SGW 4000 column, eluting with 4 M GnHCl. Extracts were mixed with an equal volume of 2 × PBS and digested with 5 µl of papain (1 unit of enzyme/ml of 0.4 M sodium acetate) containing 10 mM EDTA and 20 mM cysteine, pH 5.8, overnight at 60 °C, in order to liberate HA from proteoglycan aggregates. Prior to the assay, the papain activity was inhibited with 10 µl of 0.0416 mg/ml iodoacetic acid in H₂O, to prevent degradation of the ELISA reagents. HA was measured using an ELISA-based method based on a microtitre plate format described by Fosang et al. [32].

SDS/PAGE and Western-blotting

Freeze-dried extracts containing 6 µg of LP were dissolved in 10 µl of 0.5 M Tris/HCl, pH 6.8, containing 0.4% (w/v) SDS and proteinase inhibitors. Samples were digested with chondroitinase ABC (0.1 unit/mg of aggrecan) for 2 h at 37 °C. Buffer, containing 2% SDS and 2% 2-mercaptoethanol, was added to each sample and heated at 100 °C for 5 min. SDS/PAGE was carried out by the method of King and Laemmli [33] on 10–20% polyacrylamide gradient gels, using a 3% stacking gel. Proteins were transferred to Immobilon PVDF transfer membrane overnight in 25 mM Tris/HCl, pH 8.3, according to the method described by Towbin et al. [34]. After transfer, the membrane was blocked with 5% (w/v) low-fat milk solution in PBS, containing 0.1% (w/v) Tween 20. Primary (8-A-4) and secondary antisera were diluted in 2% (w/v) low-fat milk solution. The bound antibodies were localized with a peroxidase-conjugated second antibody and proteins were visualized using an ECL[®] kit.

Large pore agarose/polyacrylamide-gel electrophoresis and Western blots

Gels were prepared, equilibrated in 8 M urea and samples were applied as described by Bayliss et al. [35]. Glycosaminoglycan-containing molecules were visualized by staining the gels with Toluidine Blue, and aggrecan was localized on Western blots of these gels using the rabbit polyclonal anti-G1-domain, described previously, by the method of Bayliss et al. [35]. The epitope on KS chains was localized using the monoclonal antibody MZ-15. Similarly, the unsaturated Δdi6S epitope (which is a measure of chondroitin-6-sulphate) and the unsaturated Δdi4S epitope (which is a measure of chondroitin-4-sulphate), recognized by monoclonal antibodies 3-B-3 and 2-B-6 respectively after chondroitinase ABC digestion of aggrecan, were localized.

Preparation of extracts for rotary shadowing electron microscopy

The extracts were first separated by chromatography on columns of Sepharose CL-2B and the *V*₀ fractions were dialysed against 0.2 M ammonium hydrogen carbonate, pH 7.9, overnight at 4 °C, and prepared for electron microscopy by glycerol spraying on to freshly cleaved mica pieces, dried *in vacuo* for rotary shadowing as described previously [36]. The data from a large

number of electron micrographs were evaluated to determine the length of each aggrecan molecule and the proportion of molecules with a G3-domain.

RESULTS

Age-related changes in the concentration of the G1-domain and LP in 4 M GnHCl extracts of human articular cartilage

There was a significant increase in the molar concentrations of the G1-domain ($r = 0.92$) and in LP ($r = 0.72$) as a function of age (Figures 1a and 1b). However, only in the specimens from newborn and some of the younger cartilage specimens was the ratio of G1-domain:LP close to a value of 1:1 (Figure 1c). In specimens of cartilage from individuals between the ages of 5 and 25 years, the G1-domain:LP molar ratio increased to a maximum

Figure 1 The effect of age on the concentration of G1-domain (a), the concentration of LP (b) and the molar ratio of G1-domain:LP (c)

Assays were performed on extracts of human articular cartilage prepared in 4 M GnHCl. G1-domain and LP concentrations were assayed as described in the Experimental section.

Figure 2 Sepharose CL-6B gel chromatography of 4 M GnHCl extracts of cartilage from 5-year-old (a) and 39-year-old individuals (b)

Column fractions were monitored for sulphated glycosaminoglycan [□] and the G1-domain of aggrecan [◆]. The proportion of G1-domain present in peak II was determined by cutting and weighing the column profile.

value of 5–7.5:1, and remained at a value in excess of 1:1 in mature cartilage. The concentration of G1-domain and of LP was measured in the extracts of a number of specimens according to their gender. Applying a 95% confidence limit, no significant differences were observed between males and females when the 0–20 years (Group I), 21–40 years (Group II), 41–60 years (Group III) and 61 years and older (Group IV) age groups were compared: Group I, male = 25.81, female = 21.1, $P = 0.57$; Group II, male = 17.82, female = 25.9, $P = 0.89$; Group III, male = 41.4, female = 49.1, $P = 0.72$; Group IV, male = 25.1, female = 30.6, $P = 0.52$ (results not shown).

When cartilage extracts were analysed by chromatography on columns of Sepharose CL-6B, a higher concentration of 'free' G1-domain was observed in the extracts of adult cartilage (Figure 2). Furthermore, most of the extract was not associated with sulphated glycosaminoglycan (Figure 2), and SDS/PAGE showed that it consisted of two major proteins (molecular masses of 48 kDa and 68 kDa) that were immunolocalized on Western blots with the anti-human G1-domain polyclonal antiserum (results not shown). The ratio of 'free' G1-domain:LP and thus the ratio of aggrecan-associated G1-domain:LP was calculated for a number of samples (Table 1) and it was shown that even when the 'free' G1-domain was taken into account, there was an increase in the deficiency of LP in cartilage extracts during maturation of normal cartilage.

Table 1 The stoichiometry of reformed proteoglycan aggregates from extracts of human articular cartilage from individuals of various ages

4 M GnHCl extracts of cartilage were assayed for the G1-domain of aggrecan and LP using radioimmunoassay techniques and the molar ratio of these components was calculated. The proportion of G1-associated with aggrecan and that associated with 'free' G1-domain was determined by chromatography on Sepharose CL-6B columns, and the G1-domain associated with aggrecan:LP was calculated. ND, not determined.

Age (years)	LP (nmol/g wet wt.)	G1-domain (nmol/g wet wt.)	G1-domain:LP	% 'free' G1-domain	G1-domain associated aggrecan:LP
5	18.21	20.03	1.10	ND	1.10
11	26.56	57.15	2.15	21.1	1.69
19	21.73	79.38	3.65	22.50	2.83
26	35.62	115.15	3.23	18.50	2.63
44	31.20	161.17	5.16	36.30	3.29
49	21.30	134.19	6.30	60.20	3.79
55	78.63	208.87	2.66	34.00	1.75
70	70.31	208.40	2.96	35.50	1.91
85	81.04	225.86	2.78	37.90	1.73

Figure 3 Western blot of extracted cartilage specimens

Samples of normal human articular cartilage (5–65 years) were extracted with 4 M GnHCl and electrophoresed by SDS/PAGE in 10–20% polyacrylamide gradient gels. Proteins were transferred on to Immobilon membranes by Western blotting, and LP was localized by visualizing the binding of monoclonal antibody 8-A-4.

LP also undergoes fragmentation of its three major forms, LP1, LP2 and LP3, in adult cartilage, and this was observed by SDS/PAGE (Figure 3). On longer exposure (1 min), lower-molecular-mass fragments were observed in the extracts of both the immature and the mature cartilage.

Analysis of cartilage extracts

Proteoglycans were extracted from cartilage and the extracts were dialysed against 50 mM sodium acetate, pH 6.8. In order to eliminate the influence of the oligosaccharides of HA on the UA and HA analysis of tissue extracts, these low-molecular-mass fragments of HA were removed from the E2 extracts by dissociative Sephadex G100 chromatography. The oligosaccharides of HA, measured as UA, were well separated from aggrecan measured using the dye-binding technique and UA; the excluded peak of aggrecan also contained all of the G1-domain (results not shown). Subsequent analysis of aggrecan extracted as E2, was carried out using the excluded peak. The proportion of total proteoglycan present in the three sequential extracts, E1, E2 and E3–E6, was measured as the UA and s-gag (results not shown) contents of the extracts and papain digests of the tissue residues (Figure 4a). A large proportion of proteoglycan was extracted with the competitive extractant (E2) at all ages, indicating that link-stabilized aggregates were not present in this extract and a greater proportion was extracted from immature than from adult cartilage by extracts E1 and E2. Furthermore, at all ages, the dissociative extracts E3–E6 extracted the greatest

proportion of proteoglycan from the tissue, particularly from adult cartilage, where it reached 65% of the UA at 41 years of age.

(i) KS

KS was measured using an inhibition radioimmunoassay procedure which measures the KS epitope recognized by the monoclonal antibody MZ-15. The extractability of KS is shown in Figure 4(b). There was an age-related decrease in the ability of extract E1 to extract KS from the tissue. In contrast, KS extractability by E3–E6 was higher in the adult specimens. A large proportion of the KS epitope was also removed by E2 in specimens from all ages examined. When the ratio of KS:UA was calculated, it became clear that E2 had a high KS content when compared with the other extracts (Figure 4c). The increase in KS content with increasing age, measured using a mixture of antibody reaction and chemistry (UA), was also observed when cartilage aggregate preparations were analysed for their content of glucosamine and galactosamine (Figure 5). Electrophoresis in large-pore agarose gels (Figure 6) also confirmed that the E2 extracts were rich in KS. Although 3 µg of UA was loaded in each case, E2 had a higher Toluidine Blue reaction, consistent with it containing higher amounts of glycosaminoglycan (chondroitin sulphate and KS). An extensive analysis of these gels using a variety of polyclonal antisera and monoclonal antibodies showed that they could be used to analyse the composition of aggrecan in differential extracts (Table 2).

(ii) HA

The extractability of HA in the non-dissociative (E1) and competitive (E2) extracts was low in all tissue samples, but the proportion of this component also decreased with advancing age (Figure 7a). It should also be noted that HA was extracted with E2, particularly from immature cartilage, and it was assumed that removal of non-LP-stabilized aggrecan from proteoglycan aggregates by this extractant facilitated the extraction of HA. HA was readily extractable in specimens from all ages with the dissociative extract 4 M GnHCl (E3–E6). This was particularly true for adult tissue, where approx. 90% of total HA was extractable in tissue from individuals of 63 years of age.

(iii) G1-domain of aggrecan

The extractability of the G1-domain (intact aggrecan plus 'free' G1-domain of aggrecan) in each of the sequential extracts was

Figure 4 Effect of normal ageing of articular cartilage on extractability of UA (a) and KS (b)

The KS:UA ratio is shown in (c). E1–E6 denotes the extraction protocol used, as indicated in the key. Residue, papain digest of insoluble cartilage residue.

determined by inhibition radioimmunoassay (Figure 7b). The G1-domain was readily extracted by E1 and E2, particularly from immature cartilage, but this finding was less pronounced in adult tissue. Extract E1 also removed more G1-domain than E2, but this difference was more pronounced in adult cartilage. Furthermore, dissociating solutions of GnHCl removed the majority of G1-domain from mature articular cartilage.

(iv) LP

An inhibition radioimmunoassay was used to determine the proportion of LP present in each of the sequential extracts (Figure 7c). An age-related decrease was observed in the ability

Figure 5 The glucosamine and galactosamine content of full-depth normal human articular cartilage

The molar ratio was measured using an automated procedure on an amino acid analyser [28]. The Figure shows that the KS content increases with increasing age.

Figure 6 Large pore agarose/polyacrylamide-gel electrophoresis of cartilage extracted using Protocol B

Proteoglycans (arrows) were localized by staining the gel with Toluidine Blue. Cartilage was from individuals of various ages: (a) newborn, (b) 31 years and (c) 63 years. E, Extract 1–6, as indicated for each lane.

of LP to be non-dissociatively (E1) extracted from cartilage. A large proportion of LP was also extracted by E2 from immature cartilage and this also decreased with advancing age. A weaker dissociating solution was required to remove LP from immature cartilage than from adult cartilage, where 3 M or 4 M GnHCl was required.

The *in situ* molecular stoichiometry and stability of human articular cartilage aggregates

Aggrecan forms multimolecular aggregates in the extracellular matrix of cartilage. It is generally accepted that one molecule of aggrecan interacts with HA via its G1-domain and this arrangement is stabilized by its interaction with one molecule of LP. Therefore, any molar ratio of G1-domain:LP > 1 should indicate a relative deficiency in the LP content of extracted aggregates, thus rendering the interaction between aggrecan and HA less stable. The *in situ* stoichiometry of aggregates was therefore assessed by measuring the G1-domain:LP molar ratios

Table 2 Age-related changes in the composition of aggrecan fractionated by large pore, agarose/polyacrylamide gel electrophoresis

Epitopes for KS, chondroitin-6-sulphate, and chondroitin-4-sulphate were visualized using antibodies MZ-15, 3-B-3 and 2-B-6 respectively and enhanced chemiluminescence. The intensity of the signal was assessed as very strong (+++), strong (++), present (+) or absent (—). The presence (Y) or absence (N) of G1-domain immunoreactive (IR) bands is indicated.

Age (years)	Extract	IR bands	G1-Domain	MZ-15	3-B-3	2-B-6
Newborn	E1	Y	1	+	+	+++
		N	2	—	—	—
		N	3	+	—	—
	E2	Y	1	+	+	++
		N	2	+	+	++
		N	3	+	—	—
	E3	Y	1	+	+	+++
		Y	2	+	—	—
		N	3	+	—	—
	E4	Y	1	+	+	+++
		N	2	+	—	—
		N	3	+	—	—
	E5	Y	1	—	+	+++
		Y	2	+	+	++
		Y	3	+	+	++
	E6	Y	1	+	+	++
		Y	2	+	+	++
		Y	3	+	+	++
63	E1	Y	1	+++	+++	+
		Y	2	++	+++	+
		Y	3	+++	++	—
	E2	Y	1	+++	+++	+
		Y	2	++	+++	+
		Y	3	+++	++	—
	E3	Y	1	+++	+++	+
		Y	2	++	+++	+
		Y	3	+++	++	—
	E4	Y	1	+++	+++	+
		Y	2	++	+++	+
		Y	3	+++	+++	—
	E5	Y	1	+	+++	+
		Y	2	—	+++	+
		Y	3	+++	++	—
	E6	N	1	—	+	+
		Y	2	++	++	+
		Y	3	+++	+++	+

in each of the sequential extracts (Figure 7d). In immature cartilage, this was approx. 1 in all extracts. A substantial increase in the molar ratio of G1-domain:LP occurred in the non-dissociating (E1) and competitive (E2) extracts during maturation and subsequent ageing of cartilage and a similar age-related trend was observed for the dissociative extracts. An increase in the proportion of LP was demonstrated within the dissociative extracts, i.e. the G1-domain:LP molar ratio decreased as the concentration of GnHCl increased. However, this was always at a value > 1.

G3-domain of aggrecan

The G3 content and the length of the aggrecan molecule was determined for extracts from newborn, 5- and 41-year-old normal individuals, using rotary shadowing techniques. Table 3 shows that E1 and E2 contained the vast majority of G3 and that the size of the aggrecan molecule decreased dramatically as a consequence of normal ageing.

Further analysis of the G1-domain

The proteolytic modification of aggrecan during normal ageing results in the accumulation of the 'free' G1-domain still associated with HA in the tissue. The molar ratio of G1-domain:LP described above were therefore determined using values for the G1-domain associated with intact aggrecan and that present as 'free' G1-domain. 'Free' G1-domain was determined quantitatively in each extract by dissociative chromatography on a column of Sepharose CL-6B (Figure 8). An age-related increase was observed in the proportion of 'free' G1-domain present in the extracts (E1, E2 and E3–E6). The 'free' G1-domain in sequential extracts obtained indicated that proteolytic modification of the aggrecan core had occurred, even in cartilage from newborn, and that 'free' G1-domain could be extracted efficiently with the dissociating solutions 1 M (E3) and 2 M GnHCl (E4).

Proteoglycan stability was re-assessed as aggrecan-associated G1-domain:LP (Figure 9). In immature cartilage, the ratio approached 1 in all sequential extracts, indicating that sufficient LP was present to stabilize the interaction of intact aggrecan with HA. However, a deficiency in the LP content of all extracts was demonstrated for adult articular cartilage.

Analysis of LP structure

Although the competitive extract E2 was designed to remove non-LP-stabilized aggrecan from aggregates, LP was also extracted by this extractant. This could have resulted from an increase in the fragmentation of LP in the competitive extract, but electrophoresis of this extract did not show any differences in the structure of the LP molecule (Figure 10). LP was resolved into its three isoforms in every extract and fragments of the molecule were observed in all extracts at every age examined. LP fragments were also present in the dissociative extract of cartilage from newborns and were present in every extract from individuals above 21 years of age.

DISCUSSION

Significant changes occur in the structure and stoichiometry of the components of the proteoglycan aggregate during ageing of human articular cartilage. Proteolytic modification of the core protein of aggrecan results in the appearance of high concentrations of 'free' G1-domain in adult cartilage [21,37]. The LP molecule also exhibits age-related changes in structure [23], as does the HA in human articular cartilage [22]. These structural changes could prejudice the stabilizing power of LP in the formation of proteoglycan aggregate. In addition, our studies have shown that there is a progressive deficiency in the LP content of normal human articular cartilage during ageing. Therefore the traditional model governing proteoglycan aggregation, i.e. the interaction of one aggrecan molecule with HA which is stabilized by the binding of one LP molecule with both components [18], can only be strictly applied to immature human articular cartilage.

In addition to these age-related changes, the macromolecular organization of aggregates also changes through the depth of articular cartilage. For example, the superficial layer of canine articular cartilage contains approximately twice as much HA as the deep layers of cartilage [38] and there is also a deficiency of LP near the surface of the tissue and a higher concentration of non-link-stabilized aggregates in the pericellular matrix of immature porcine cartilage. In contrast, the proteoglycan aggregates in the inter-territorial matrix of the deep zone are mostly link-stabilized. As a consequence, the stoichiometry and

Figure 7 Effect of normal aging of articular cartilage on extractability of HA (a), G1-domain (b) and LP (c)

Data using Protocol B are shown in each case. The molar ratio of G1-domain:LP is shown in (d). E1–E6 denotes the extraction protocol used, as indicated in the key.

Table 3 Determination of the G3 content and the length of aggrecan molecules in extracts of normal articular cartilage

Proteoglycan aggregates were purified by gel chromatography on Sepharose CL-2B, and the G3 content and the length of the aggrecan molecules was determined by rotary shadowing electron microscopy [38].

Age	Extract	Aggrecan G3-domain (% of molecules)	Aggrecan length (nm)
Newborn	E1	26	195 ± 60
	E2	32	206 ± 50
5 years	E1	15	145 ± 74
	E2	20	151 ± 71
	E3	1–2	83 ± 54
	E4	1–2	66 ± 46
	E5	0	51 ± 42
41 years	E1	5	107 ± 82
	E2	6	74 ± 47
	E3	0	54 ± 48
	E4	0	65 ± 42
	E5	0	64 ± 42

stability of proteoglycan aggregates also vary throughout the depth of the tissue. These changes are accentuated in adult cartilage and may have a considerable effect on the properties of the tissue.

In order to investigate the possible *in situ* heterogeneity in the stoichiometry of proteoglycan aggregate components and in the stability of the aggregate complex, two differential extraction protocols were devised. A high proportion of each component was extracted from immature cartilage with the non-dissociative

extractant PBS. However, this amount decreased with age. Aggregate components extracted with PBS represent molecules that are freely extractable from the tissue and therefore they could be lost into the joint space *in vivo*. It has also been shown that a proportion of the total aggrecan molecules that are synthesized by human articular cartilage, have a low affinity for HA [39–42], and that only after a period of time do they mature to a high-affinity form and participate in aggregate formation like the endogenous molecules. The half-life of conversion is estimated as 12 h in immature lapine cartilage [41], but is greater than 24 h in mature human cartilage [42]. This increase in maturation time would enable the newly synthesized aggrecan to diffuse through the more densely packed adult tissue before it aggregated. A proportion of the freely extractable aggrecan in extract E1 may therefore arise from these sources. The requirement for activation of aggrecan would also explain why, in alginate cultures of chondrocytes, the proteoglycan in further removed matrix has a lower affinity for hyaluronic acid and LP and has had the opportunity to diffuse further, compared with proteoglycan in the cell-associated matrix [43].

The resident pool of aggrecan molecules within articular cartilage have a long half-life and are susceptible to proteolytic attack. This takes the form of C-terminal trimming, which removes the G3-domain [4], but proteolytic cleavage of the interglobular domain (between the aggrecan G1 and G2 domains) can also occur [44]. The findings presented in the present study (Table 3) support this hypothesis. Cleavage results in the generation of large chondroitin sulphate-bearing fragments that do not have the ability to interact with HA. Such fragments are rapidly lost from the tissue and may also constitute some of the freely extractable aggrecan within the PBS extract, although these form a polydisperse population and the larger fragments

Figure 8 Fractionation of extracts E1–E6 of cartilage from newborn (a) and 41-year-old (b) individuals on columns of Sepharose CL-6B

Column fractions were monitored for sulphated glycosaminoglycans (□) and for the G1-domain of aggrecan (◆). The proportion of G1-domain present in peak II was determined by cutting and weighing the column profile of each extract.

Figure 9 Aggrecan-associated G1-domain:LP ratio in extracts of cartilage from normal newborn and 41-year-old individuals

The concentration of the G1-domain that was associated with aggrecan (intact aggrecan) was measured by associative chromatography and expressed as a ratio of LP concentration. E1–E6 denotes the extraction protocol used, as indicated in the key.

appear to be retained by the tissue. Some of the cleaved G1-domain is lost from the tissue and some is retained, bound to HA, which is also susceptible to catabolic agents (M. T. Bayliss,

unpublished work). Therefore HA-free G1-LP complexes may also represent a proportion of the components of the aggregate in the PBS extract. In fact, LP has been shown to cross-link with aggrecan through the action of reducing sugars, and these advanced glycosylation end products accumulate with age [45].

A surprisingly large amount of LP was extracted with oligosaccharides of HA (E2) at all ages examined. The E2 extractant was designed to specifically extract aggrecan and HA that was not stabilized by LP. It is known that an intermediate complex of aggrecan and LP is released from the chondrocyte of the rat Swarm chondrosarcoma [46]. Moreover, a proportion of the newly synthesized aggrecan has a low affinity for HA, as described above. Aggrecan could therefore exist as a complex with LP prior to its diffusion through the matrix. A proportion of the LP present in extract E2 could therefore originate from this source. Similarly, LP has also been reported to undergo maturation [47], and it may exist as molecule with a low affinity for HA, but with a high affinity for the G1-domain of aggrecan. The rate of maturation of HA binding by LP could also be slower than it is for binding to the G1-domain. Therefore the interaction of aggrecan with such an LP molecule and its relatively low participation in aggregate formation could also enable the competitive extraction of LP by E2. Sztrolovics et al. [20] report that LP is lost from aggregate subsequent to the induction of aggrecanase activity and loss of aggrecan, without any apparent proteolytic degradation. This suggests that the proteolytic

Figure 10 SDS/PAGE on 10–20% gradient gels and transfer of proteins on to Immobilon membranes by Western-blotting

LP was localized by visualizing the binding of monoclonal antibody 8-A-4 to cartilage specimen extracts from normal newborn, 21-, 31-, 47-, and 63-year-old individuals. E, extract 1 to 6, as indicated for each lane.

mechanisms responsible, or the age-related accumulation of LP, are distinct from those induced by inflammatory mediators. The E2 extracts also contained large amounts of KS, and there were considerable differences in the sulphation patterns of aggrecan extracted by Protocol B (Table 1). This was taken as another sign of molecular heterogeneity within the aggrecan population.

The proteoglycan aggregates extracted with 4 M GnHCl (E3, Protocol A), constituted the largest proportion at all ages examined and was greater in mature cartilage. An age-related deficiency in LP was demonstrated in this pool, measured either as a ratio of total G1-domain:LP or as an increase in the ratio of aggrecan-associated G1-domain:LP. This suggested that there could be a progressive increase in the instability of these aggregates with advancing age. Protocol B was therefore used to explore this further. Substantial heterogeneity in the stoichiometry of aggregates, and hence in their ability to form stable aggregates, was demonstrated. The proportion of LP extracted increased as the concentration of GnHCl increased, and 1 M GnHCl extracted aggregates with a higher G1:LP ratio than did 4 M GnHCl. It therefore appears that aggregates with varying degrees of stability exist in adult human articular cartilage. Although the binding of aggrecan G1 to HA is random, the addition of LP results in clustering of aggrecan, indicating co-operativity [48]. This co-operativity may influence the stability of

aggregate. A population of G1/aggrecan may also bind to the cell surface via CD44/chondroitin sulphate interaction independently of HA [49], although the stability of this complex and the contribution this population makes to the extractants in our study is thought to be negligible.

The LP-stabilized pool of aggrecan molecules have a long half-life within the matrix and they are therefore susceptible to proteolytic attack as a result of growth and remodelling. LP appears to be the first molecule within the aggregate complex to be modified by proteinases and it probably protects the G1-domain of aggrecan from proteolytic degradation. Consequently, aggregates of varying stability could represent those that have already been modified *in situ*. For example, aggregates extracted with 1 M GnHCl, and which have the lowest LP concentration, may have been resident in the tissue for the longest period of time. However, additional experiments will have to be designed to test this hypothesis, but the studies of Maroudas et al. [21], showing that the 'free' G1-domain has a very long half-life compared with aggrecan-associated G1-domain, do support it.

LP is known to undergo fragmentation as it ages [23], and we therefore assumed that the concentration of these fragments would be higher in the lower stability extracts. However, although substantial structural modification of the LP was observed, this did not appear to be related to the degree of instability within the proteoglycan aggregate and fragmentation was observed at all ages and in all extracts of the cartilage examined. Our previous publications [4,35] have also shown that there is an age-related increase in the heterogeneity of the molecular mass of aggrecan molecules and that the proportion of them carrying the G3-domain decreases with age.

This study has demonstrated that the typical model governing proteoglycan aggregation cannot be applied as rigidly to mature human articular cartilage as it is to immature cartilage from other species. The long half-life of the molecules within the matrix allows the turnover and removal of specific components from aggregates, and this results in a substantial increase in structural heterogeneity. It is also likely that there is further heterogeneity within these molecular complexes that we have not yet detected. Furthermore, the location of each complex within specific compartments of the tissue is also not known, but extraction of full-depth histological cryo-sections, followed by the immunolocalization of aggregate components using polyclonal and monoclonal antibodies will hopefully clarify this.

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