Characterization of proteoglycans isolated from associative extracts of human articular cartilage

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Approx. 10% of the total proteoglycan content of normal young human articular cartilage was extracted under associative conditions with Dulbecco's PBS. Proteoglycans isolated from the extract by Q-Sepharose chromatography were separated by gel chromatography and characterized by gradient-gel SDS/PAGE and immunoblotting. Three species of small proteoglycans, two main populations of aggrecan and a population of its smaller fragments were identified. The major populations of aggrecan contained chondroitin sulphate chains, all or part of the N-terminal G1 and G2 domains and, therefore, intact keratan sulphate domains. The larger population was estimated by gradient SDS/PAGE to have a molecular mass of approx. 600 kDa or greater. The second population had an apparent molecular mass of approx. 300–600 kDa. Core proteins derived from these populations of proteoglycans separated on SDS/PAGE into several clusters of bands in the range from 120 to approx. 360 kDa. The extract further contained smaller fragments which lacked chondroitin sulphate but reacted with antibodies against keratan sulphate, and against epitopes present in the G2 domain of aggrecan. The presence of the G2 domain in a broad range of populations of decreasing size indicated extensive cleavage of the aggrecan core protein within its chondroitin sulphate domain. These findings suggest that fragmentation of aggrecan probably occurs *in vivo* in normal articular cartilage of young individuals. Associative extracts also contained decorin, biglycan and fibromodulin. These were resolved from aggrecan by gel chromatography and identified by immunodetection.

INTRODUCTION

The large aggregating proteoglycan, aggrecan, is a major structural component of cartilage and represents 5–10% of tissue wet weight [1]. cDNA cloning of the human aggrecan core protein [2] has revealed two globular domains (G1 and G2) comprising the N-terminus, a G3 globular domain at the C-terminus, and a long extended glycosaminoglycan-rich domain between G2 and G3 which can be further divided into a keratan sulphate domain and two chondroitin sulphate domains (CS-1 and CS-2) [1–3]. Analyses of aggrecan populations in human cartilage have shown that several major large species occur [4–7]. A small G1 fragment that still exhibits an ability to bind to hyaluronan represents a major degradation product of aggrecan that accumulates in the tissue with age [8].

The small proteoglycans containing leucine-rich core proteins represent another family of proteoglycans present in cartilage. These proteoglycans comprise decorin and biglycan, which carry chondroitin sulphate/dermatan sulphate chains, and fibromodulin which carries keratan sulphate chains [1,3]. Biglycan and decorin have previously been identified in fetal [9] and juvenile [10] human articular cartilage, but only trace amounts of [9], or no [11], biglycan have been found in adult cartilage.

Our knowledge of the mechanism involved in aggrecan degradation in the cartilage matrix derives mainly from experiments *in vitro*. Explanted cultures of articular cartilage release into the medium aggrecan fragments that lack the G1 domain [12–14], indicating that proteolytic attack within the interglobular domain between G1 and G2 is a primary event in turnover. A major metalloproteinase cleavage site within this domain has been identified at the Asn^{341}–Phe^{342} bond [15,16], and it has recently been shown that cleavage at this site occurs *in vivo* in human articular cartilage [17]. However, the predominant cleavage within the interglobular domain appears to occur *in vivo* at a site that is different from the major metalloproteinase cleavage site, between Glu^{373} and Ala^{374} [18,19]. The identity of the proteinase(s) responsible for this action remains unknown.

Although the separation of G1 from the rest of the molecule has been identified as a major event enabling aggrecan to diffuse out of the tissue, the possibility that proteolytic processing may occur within the glycosaminoglycan-rich domain has been paid less attention. Stromelysin and leucocyte elastase are able to cleave aggrecan within its chondroitin sulphate domain *in vitro* [20–22], and evidence *in vivo* for cleavage in this region in human cartilage has recently been provided [19]. Processing of this nature may yield fragments comprising an intact keratan sulphate domain with all, or part, of the N-terminal globular domains, and these may correspond to keratan sulphate-rich populations of aggrecan that have previously been identified [7,23].

It is thought that the cleaved products of aggrecan that are no longer held in the cartilage as part of aggregates diffuse into the synovial fluid, and indeed proteoglycan fragments of various sizes have been found in pathological human synovial fluids [24–27]. Our previous experiments have demonstrated that a small but significant proportion (~ 10%) of total sulphated glycosaminoglycans present in human articular cartilage can be extracted under associative conditions with PBS [28]. We consider that this PBS-extractable population may represent proteoglycans that are released into the synovial fluid under normal conditions of tissue turnover. The aim of the present study was to characterize all PBS-extractable proteoglycans of human articular cartilage in order that the results may provide a basis

Abbreviations used: G1, first globular domain of aggrecan (N-terminal); G2, second globular domain of aggrecan; CAPAGE, composite agarose/polyacrylamide gel electrophoresis; mAb, monoclonal antibody.

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for comparing the proteoglycan degredation products released from the cartilage under conditions of normal turnover with those released into synovial fluid during arthritic disease.

EXPERIMENTAL

Tissues and materials

Human articular cartilage was obtained from femoral heads at the time of autopsy within 12 h of death. The cartilage appeared macroscopically normal. Tissue was stored at −60 °C until processing.

Q-Sepharose and Sephacryl S-400 were from Pharmacia LKB Biotechnology (Sweden), and nitrocellulose membranes from Micro Filtration Systems (Dublin, CA, U.S.A.). Agarose type HSC used to prepare composite gels was purchased from PS Park Scientific (U.K.). Chondroitinase ABC and endo-β-D-galactosidase (keratanase; *Pseudomonas* sp.) were obtained from Sigma (St. Louis, MO, U.S.A.). Pig anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were from USOL (Praha, Czech Republic). Stains-all dye was from BDH Chemicals (Poole, Dorset, U.K.). All other chemicals used were of analytical grade. Bovine nasal cartilage proteoglycan monomer purified by density-gradient centrifugation under associative and dissociative conditions (A_1D_1 fraction) was a gift from Dr. Radka Poláková, Institute of Rheumatology, Praha.

Extraction and isolation of proteoglycans

A 10 g portion of full-depth cartilage slices was sectioned at 20 μm and extracted with Dulbecco's PBS (without Ca^{2+} and Mg^{2+}), pH 7.4 (hereafter, just PBS), in the presence of proteinase inhibitors as described previously [28]. The concentration of sulphated glycosaminoglycans in the extract was estimated by the 1,9-Dimethylmethylene Blue assay [28,29] with chondroitin sulphate as a standard. The extract was chromatographed on Q-Sepharose in the presence of 7 M urea [28], and column fractions were assayed by dye-binding assay for sulphated glycosaminoglycan content. Samples of every fourth fraction were dialysed against distilled water and analysed by SDS/PAGE and SDS/composite agarose/polyacrylamide-gel electrophoresis (SDS/CAPAGE). Proteoglycans were separated into two pools on Q-Sepharose, and these were designated pools I and II. Pools were dialysed against distilled water, freeze-dried and weighed.

Gel chromatography on Sephacryl S-400

Chromatography on a Sephacryl S-400 column (1.1 cm × 195 cm) was done in 4 M guanidine HCl/50 mM Tris/HCl, pH 8.5 [30], at a flow rate of 4 ml/h. The eluate was monitored at 280 nm, and 4 ml fractions were collected and assayed for dye-binding activity. Fractions were pooled, dialysed and freeze-dried. The pools of fractions that were analysed further were designated by capital letters.

Gel electrophoresis

Samples were dissolved in reducing buffer containing 2% SDS and 5% mercaptoethanol, and heated at 100 °C for 5 min. SDS/PAGE on 1.3–20%-polyacrylamide gradient gels was performed essentially as described [31], except that the stacking gel was made from composite polyacrylamide/agarose gel. Because calibration curves constructed for extremely loose gradient gels are not linear [31], the molecular masses determined from the extrapolated part of the calibration curve are likely to be underestimated and should be considered approximate.

SDS was removed from the gels before staining by washing with 4–5 changes of 40% methanol/8% acetic acid over 4 h. Staining of proteoglycans with the cationic carbocyanin dye Stains-all was as described by Goldberg et al. [32]. Standard glycosaminoglycans (generously provided by Dr. M. B. Mathews and Dr. J. A. Cifonelli, University of Chicago, U.S.A.) tested with Stains-all dye showed that, under the conditions used, chondroitin sulphate stained dark blue and keratan sulphate stained red.

Gels for CAPAGE were prepared essentially as described [4], except that the running buffer contained 0.1% SDS instead of urea as the dissociating agent. The gels were stained with Toluidine Blue.

Antibodies and immunoblotting

Electrophoretically separated samples were transferred on to nitrocellulose membranes either in Tris/glycine buffer, pH 8.3, containing 20% methanol [33] for 3 h (core protein samples) or in 25 mM phosphate buffer, pH 6.5 [34], at 20 V overnight (native proteoglycans). After the transfer, molecular-mass standards were revealed with Ponceau S and the membranes were blocked with 5% (w/v) skim milk in 20 mM Tris-buffered saline, pH 7.5 (TBS), at 4 °C overnight. Antibodies were diluted in 2% skim milk in TBS. The peroxidase reaction was developed with 4-chloro-1-naphthol as a substrate.

The antibodies used in this study were as follows. (1) Monoclonal antibodies (mAbs) recognizing different epitopes present on aggrecan (kindly given by Dr. Bruce Caterson, University of North Carolina, Chapel Hill, NC, U.S.A.). The anti-keratan sulphate antibody 5-D-4 recognizes polysulphated carbohydrate epitopes that have been extensively characterized [35–37]. The 3-B-3 and 2-B-6 antibodies recognize neo-epitopes at the non-reducing end of oligosaccharide 'stubs' generated by chondroitinase ABC digestion of chondroitin 6-sulphate and chondroitin 4-sulphate respectively [38], and mAb 1-C-6 recognizes a peptide epitope present on the G1 domain [39,40]. (2) Rabbit polyclonal antibodies raised against synthetic peptides. The anti-G2 antiserum recognizes a peptide sequence in the B′ loop of the G2 domain of aggrecan. When tested with pig antigens under reducing conditions, this antiserum recognized G2 exclusively and did not cross-react with G1 or link protein. LF-15 and LF-30 antibodies recognize peptides derived from core proteins of biglycan and decorin, respectively [41], and were gifts from Dr. Larry W. Fisher, Bone Research Branch, NIDR, NIH, Bethesda, MA, U.S.A. (3) Rabbit polyclonal antibodies to human fibromodulin, a gift from Dr. Anna H. K. Plaas, Shriners Hospital for Crippled Children, Tampa, FL, U.S.A. Before incubating with the first antibody, immunoblots probed with anti-G2 or 1-C-6 antibodies were reduced by treating the blocked nitrocellulose membrane with 10 mM dithiothreitol at 37 °C for 2 h; immunoblots of native proteoglycans prepared for probing with 5-D-4, 3-B-3, 2-B-6, LF-15 or LF-30 antibodies were treated with 0.05 unit/ml chondroitinase ABC for 2 h at 37 °C.

Enzymic treatments

Enzyme digestions were done at 37 °C for 4 h. Samples (160 μg/40 μl) were digested with 0.02 unit of chondroitinase ABC and/or 0.1 unit of keratanase. Samples digested simultaneously with both enzymes or with chondroitinase ABC only were dissolved in 0.1 M Tris/acetate buffer, pH 7.3. Samples digested with keratanase only were dissolved in 50 mM Tris/acetate buffer, pH 7.4. The buffers contained BSA (0.5 mg/ml) and the following proteinase inhibitors: 10 mM EDTA, 10 mM

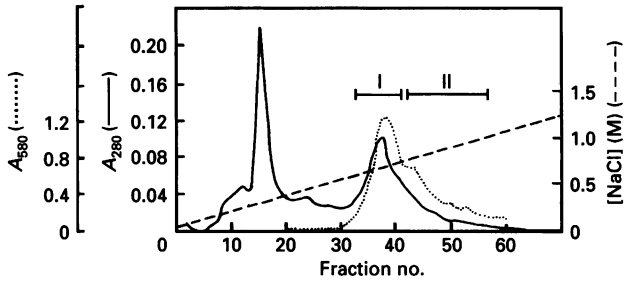

Figure 1 Chromatography of PBS extract of human articular cartilage on Q-Sepharose in 7 M urea

The eluate was monitored for protein (A_{280}) and assayed for sulphated glycosaminoglycans (A_{580}). Fractions were pooled as indicated by bars; pools are designated by Roman numerals.

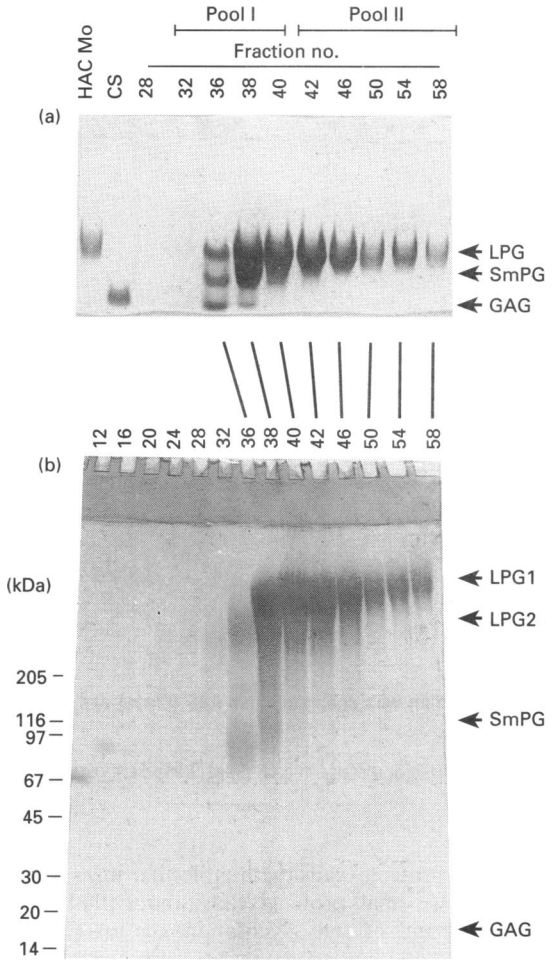

Figure 2 Electrophoretic analysis of fractions separated by Q-Sepharose chromatography

(**a**) CAPAGE, stained with Toluidine Blue; (**b**) SDS/PAGE in a 1.3–20% gradient gel, stained with Alcian Blue and Coomassie Blue. HAC Mo, standard of proteoglycan monomer (A_1D_1 fraction) from human articular cartilage; CS, chondroitin sulphate; LPG, large proteoglycans; SmPG, small proteoglycans; GAG, glycosaminoglycans.

N-ethylmaleimide, 5 mM phenylmethanesulphonyl fluoride and 5 μg/ml pepstatin. The digests were stopped by adding SDS/PAGE sample buffer and boiling for 5 min.

Table 1 Amounts of material recovered after freeze-drying from pools that were separated by Q-Sepharose and Sephacryl S-400 chromatography

Proteoglycans isolated by Q-Sepharose chromatography (mg dry wt./g wet wt. of cartilage)		Mass of proteoglycan recovered after Sephacryl S-400 chromatography (mg dry wt./g wet wt. of cartilage)	
Pool I	5.3	Pool A	0.5
		B	0.45
		C	0.43
		D	0.39
		E	0.45
		F	0.29
		G	0.3
		H	0.22
		J	1.06
Pool II	5.1	Pool O	1.05
		P	0.85
		R	0.7
		S	0.4
		T	0.1

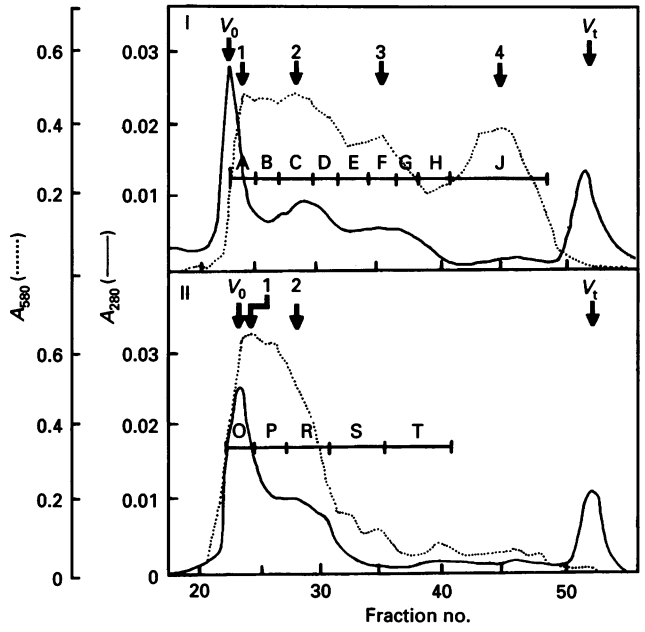

Figure 3 Chromatography of Q-Sepharose-separated pools I and II on Sephacryl S-400

The eluate was monitored for protein (A_{280}) and assayed for sulphated glycosaminoglycans (A_{580})/ V_0, V_t and individual peaks are indicated by arrows. Fractions were pooled as indicated by bars; pools are designated by capital letters.

RESULTS

Isolation and fractionation of proteoglycans

Articular cartilage of two young adults, aged 18 and 22 years, was pooled and extracted with PBS. The content of sulphated glycosaminoglycans in the extract was estimated by the dye-binding assay as equivalent to 4.8 mg of chondroitin sulphate/g wet wt. of cartilage. Proteoglycans were isolated from the extract by chromatography on Q-Sepharose and the fractions were

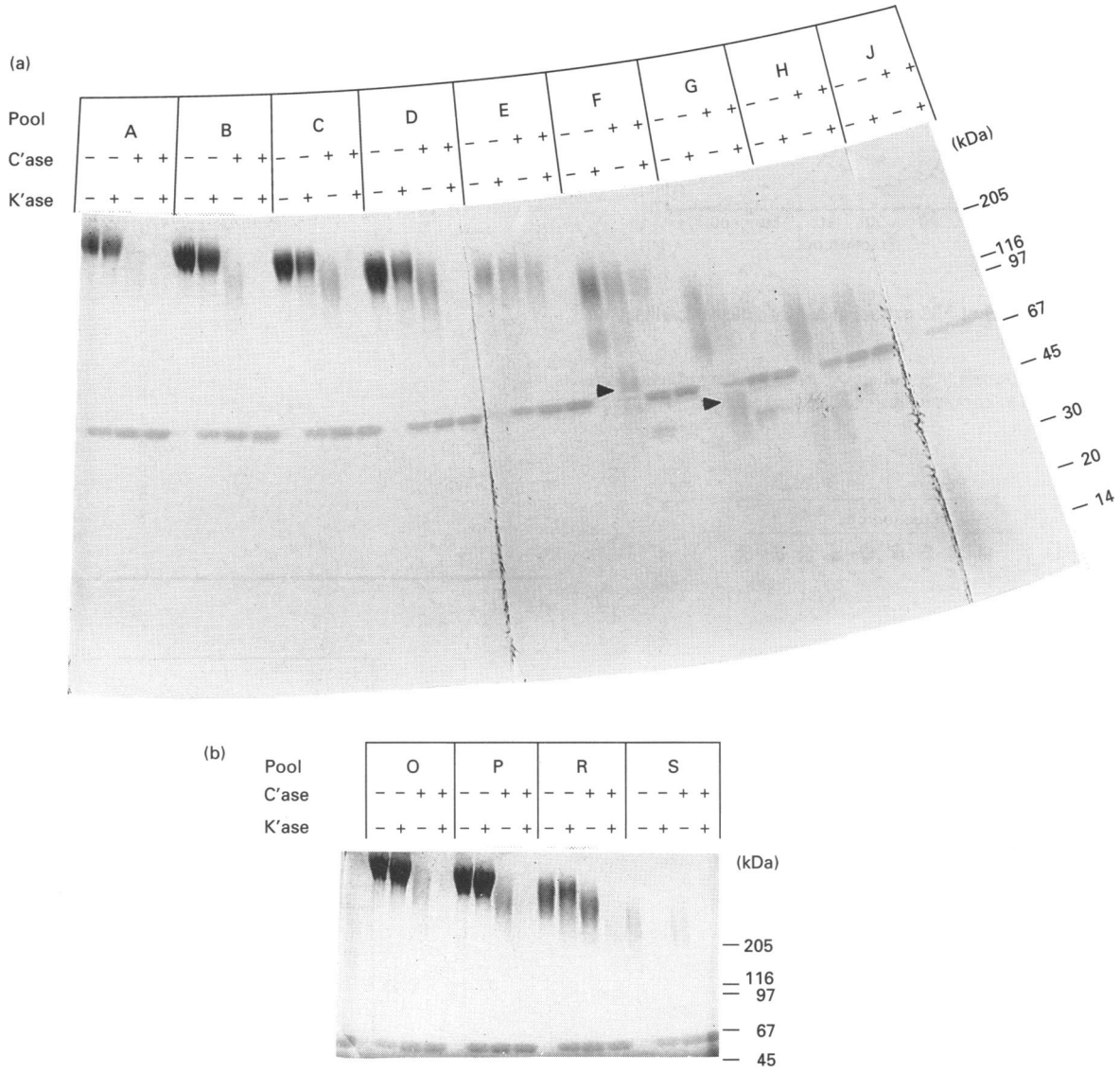

Figure 4 SDS/PAGE of proteoglycans of Sephacryl S-400-separated pools before and after digestion with chondroitinase ABC (C'ase) and/or keratanase (K'ase)

Gradient gels (1.3–20%) were stained with Stains-all dye and Coomassie Blue. Arrowheads indicate new proteoglycan bands that appeared in pools F and G after keratanase treatment.

pooled as indicated by bars in Figure 1. The yield of proteoglycans recovered in each of pools I and II after dialysis and freeze-drying is given in Table 1. Total PBS-extractable proteoglycan represented approx. 1% of the cartilage wet weight (10.4 mg/g of cartilage), which is equivalent to approx. 10% of the total tissue proteoglycan, assuming that adult cartilage contains about 10% proteoglycan [1].

Pool I was eluted from the Q-Sepharose column with 0.5–0.75 M NaCl, and pool II with 0.75–1.0 M NaCl. Samples of column fractions were analysed by gel electrophoresis. CAPAGE, which resolves large proteoglycans from small proteoglycans and glycosaminoglycans [28], showed that large proteoglycans were present in both pools I and II, whereas small proteoglycans and free glycosaminoglycans were eluted in pool I, but were not present in pool II (Figure 2a). SDS/PAGE revealed the presence of several different populations of proteoglycans. These included two broad, partially overlapping, bands corresponding to two species of large proteoglycans (with molecular mass in excess of approx. 300 kDa); small proteoglycans (about 100 kDa); and a fast-migrating band of free glycosaminoglycans (10–25 kDa). The two species of large proteoglycan were partially resolved from one another on the column, and Figure 2(b) shows that the species designated LPG2 is the predominant proteoglycan in fraction 36, and is present up to fraction 46. The species designated LPG1 is present in fractions 38–58. The two populations of large proteoglycan were also distinguishable from each other by their staining with Stains-all dye. LPG1 stained blue/violet, whereas LPG2 stained red/orange, suggesting that they probably differed in their ratio of keratan sulphate to chondroitin sulphate.

Pools I and II were further fractioned by gel chromatography on Sephacryl S-400 (Figure 3). Pool I was separated into three peaks containing protein and sulphated glycosaminoglycans (Figure 3; peaks 1, 2 and 3) and one peak of sulphated

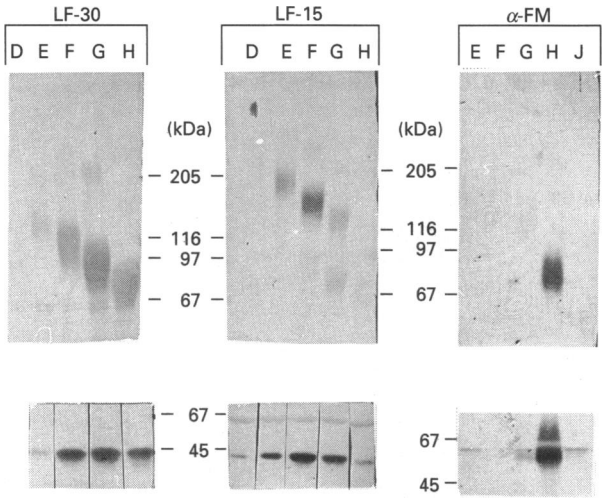

Figure 5. Immunolocalization of small proteoglycans

Native (upper panels) and enzyme-digested (lower panels) proteoglycans of Sephacryl S-400-separated pools were electrophoresed in SDS/1.3–20% gradient gels, transferred on to nitrocellulose, and detected with antibodies as indicated. Core proteins detected with anti-biglycan (LF-15) and anti-decorin (LF-30) antibodies were digested with chondroitinase ABC. Core proteins detected with anti-fibromodulin antibodies (α-FM) were digested with keratanase. The antibody LF-15 was raised against BSA-conjugated peptide and reacts also with BSA present in the chondroitinase ABC buffer.

glycosaminoglycans (peak 4). Pool II was eluted as two overlapping peaks containing both protein and sulphated glycosaminoglycans (Figure 3; peaks 1 and 2). As demonstrated below, the main components of the peaks were: peak 1, LPG1; peak 2, LPG2; peak 3, small proteoglycans; peak 4, free glycosaminoglycans. Collected fractions were pooled as indicated by the bars, and pools were designated by capital letters (Figure 3; A–J and O–T). The pools were dialysed, freeze-dried and weighed (Table 1). Total recoveries after gel chromatography were approx. 77% for proteoglycans of pool I and approx. 61% for proteoglycans of pool II.

Samples of Sephacryl S-400-separated pools were analysed by SDS/PAGE (Figure 4; lane 1 of each set of four represents native, undigested sample). Peak 1 (pools A and O) eluted at the void volume of the column represented the LPG1 population, and it migrated as a single broad blue-stained band of apparent molecular mass estimated to be in excess of at least 600 kDa. Peak 2 (pools C, D and R) represented the LPG2 population, and it migrated as a violet/orange band with decreasing molecular mass from about 600 kDa to approx. 300 kDa. Proteoglycans eluted in the trailing edge of peak 2 (pool S) migrated on SDS/PAGE with molecular mass decreasing to almost 200 kDa, and the molecular mass of proteoglycans present in pools E–H decreased continuously from more than 200 kDa to less than 100 kDa. SDS/PAGE of proteoglycans present in pools F and G revealed the presence of at least two species of small proteoglycan.

Small proteoglycans

Small proteoglycans present in pools E–H were identified by immunolocalization experiments with specific antibodies before and after chondroitinase ABC or keratanase treatment (Figure 5). The antibody LF-15 (anti-biglycan) recognized a proteoglycan of molecular mass 140–200 kDa that was present in the pools D–G, and the antibody LF-30 (anti-decorin) recognized a proteoglycan of molecular mass 70–120 kDa that was present in pools E–H. Decorin and biglycan core proteins were also identified by immunolocalization after treatment with chondroitinase ABC, and each migrated on SDS/PAGE with an identical molecular mass of 45 kDa. Since the protein cores liberated by chondroitinase ABC treatment were the same size, the polydispersity of the native proteoglycans was due to variations in the length of their chondroitin sulphate chain(s). A faster-migrating band of molecular mass 70–90 kDa recognized by the LF-15 antibody (pools F and G) might represent biglycan protein core with only one glycosaminoglycan chain attached. The antibody to human fibromodulin (anti-FM) recognized a 70–90 kDa proteoglycan that was present exclusively in pool H and stained red with Stains-all dye. The protein core of fibromodulin was identified by immunolocalization after treatment with keratanase and migrated on SDS/PAGE with molecular mass of approx. 60 kDa. Keratan sulphate chains present on fibromodulin also reacted strongly with the 5-D-4 antibody (Figures 6 and 7).

Enzymic deglycosylation

Samples of each of pools A–J and O–S were digested with chondroitinase ABC and/or keratanase and subjected to SDS/PAGE in 1.3–20% gradient gels (Figure 4). Whereas native proteoglycans stained either blue or violet with Stains-all dye, all keratanase-treated samples stained blue and all chondroitinase ABC-treated samples stained red. The mobilities of the large proteoglycans (pools A–D and O–P) in gradient gels increased after chondroitinase ABC treatment, but did not change after keratanase treatment, indicating that chondroitin sulphate, rather than keratan sulphate, contributed significantly to the size of the whole molecule.

As was demonstrated above, native proteoglycans of pools F and G separated on gradient SDS/PAGE into two bands, representing decorin and biglycan respectively. After keratanase treatment, bands representing biglycan and decorin remained at the same position, and a third, faster-migrating, blue band appeared (see arrowheads in Figure 4) with apparent molecular mass decreasing from about 70 kDa (F) to less than 50 kDa (G). After these pools were digested with chondroitinase ABC, the band of native decorin completely disappeared and the violet band with the mobility of biglycan changed in colour to red. No other new band appeared, except for a doublet of decorin/biglycan protein cores that stained with Coomassie Blue (45 kDa). This indicated the presence of another proteoglycan containing a significant proportion of keratan sulphate and co-migrating with biglycan on SDS/PAGE. The other glycosaminoglycan present in this proteoglycan may be chondroitin sulphate with a proportional content of 3-B-3 and 2-B-6 epitopes too low to detect on immunoblots, as was the case for the decorin and biglycan. This proteoglycan might represent another type of aggrecan fragment containing predominantly the keratan sulphate domain.

Populations of aggrecan

Samples of the Sephacryl S-400-separated pools were analysed by immunoblotting with antibodies specific for different structural domains within the protein core of aggrecan. These were mAb 1-C-6 (G1 domain), anti-G2 antiserum (G2 domain), mAb 5-D-4 (keratan sulphate chains) and mAbs 3-B-3 and 2-B-6 (chondroitin sulphate chains). The mAbs 5-D-4, 3-B-3 and 2-B-6 recognize different carbohydrate epitopes on glycosaminoglycan chains (see the Experimental section), and these are

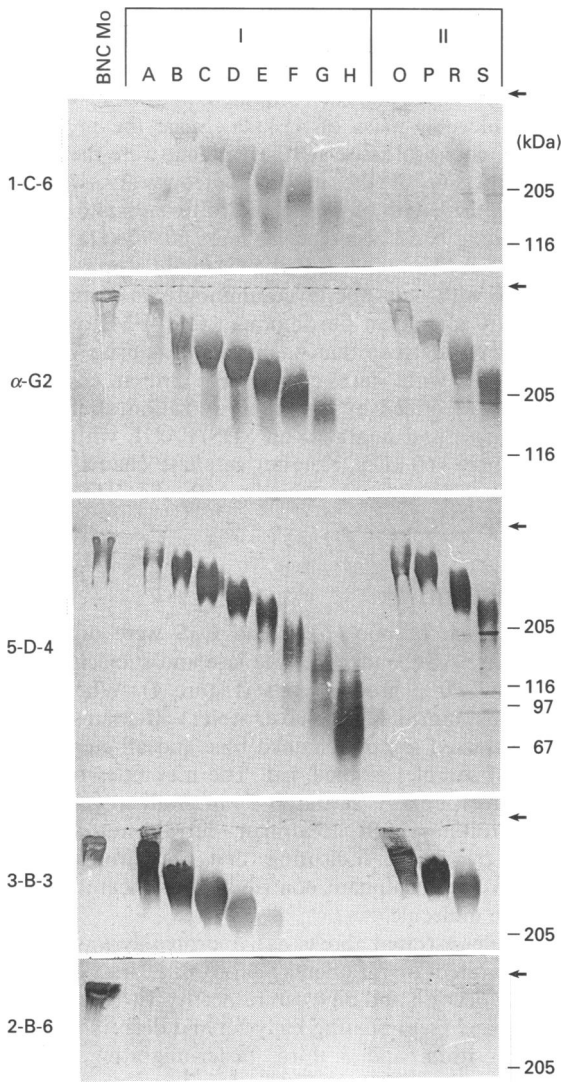

Figure 6. Immunolocalization of Sephacryl S-400-separated pools with antibodies specific for the structural domains of aggrecan

Proteoglycans separated by SDS/PAGE in 1.3–20% gradient gels were transferred on to nitrocellulose and detected with antibodies as indicated. Arrows indicate the start of the separating gel. BNC Mo, standard of proteoglycan monomer (A_1D_1 fraction) from bovine nasal cartilage..

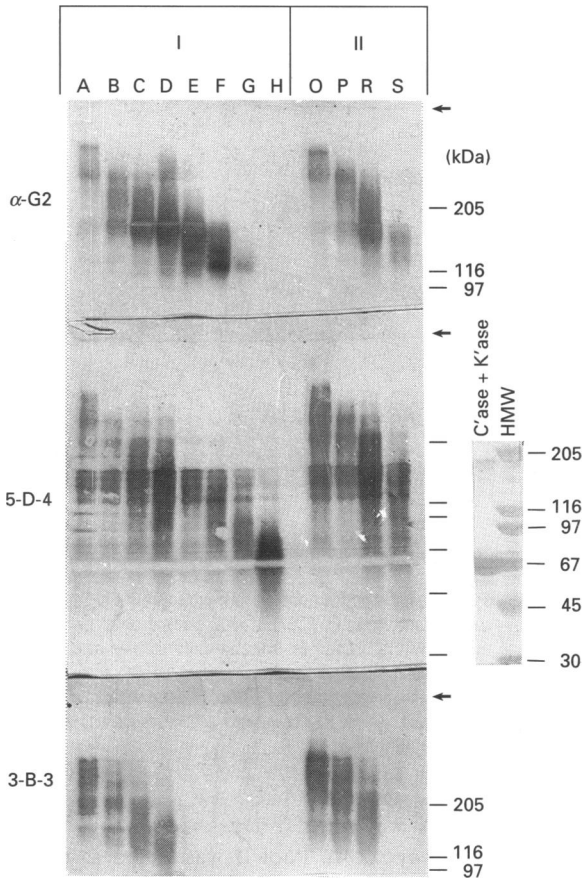

Figure 7. Immunolocalization of core proteins from proteoglycans separated on Sephacryl S-400

Core proteins separated by SDS/PAGE in 1.3–20% gradient gels were transferred on to nitrocellulose and detected with antibodies as indicated. Arrows indicate the start of the separating gel. Two separate lanes (right side of the middle immunoblot) were stained with Ponceau S to show the position of protein bands in keratanase and chondroitinase ABC (K'ase + C'ase). The enzymes were overloaded. HMW, high-molecular-mass standards.

distributed among different protein domains on the core protein. However, keratan sulphate chains are located not only in the keratan sulphate domain but also interspersed among chondroitin sulphate chains in the chondroitin sulphate domain and within the N-terminal globular and interglobular domains [16].

The 1-C-6 epitope, as well as the epitope specific for the G2 domain, were present on proteoglycans in pools A–G and O–S, confirming that the fragments with molecular mass decreasing to approx. 200 kDa were derived from aggrecan. All populations of aggrecan in pools A–G and O–S reacted strongly with the 5-D-4 antibody. The signal given by mAb 2-B-6 was very weak or not present at all, confirming previously published data [9,11] that indicated a very low abundance of chondroitin 4-sulphate in human aggrecan. There was strong reactivity with mAb 3-B-3 in pools A–D and O–R, confirming that the populations of aggrecan contain chondroitin 6-sulphate. Smaller fragments of aggrecan (pools E–G and S) carried keratan sulphate and the G2 domain, but did not contain detectable amounts of chondroitin sulphate. The concomitant loss of 3-B-3 reactivity and the decreasing molecular mass indicate that there is significant proteolytic processing occurring C-terminally to the keratan sulphate domain.

Immunoblotting was used to localize aggrecan core-protein fragments after their enzymic deglycosylation (Figure 7). The core proteins spanned a broad range of molecular masses from 120 to approx. 360 kDa and separated into several clusters. All core proteins that reacted positively with the anti-G2 antiserum (pools A–G and O–S) also did so with the 5-D-4 antibody. mAb 3-B-3 recognized core proteins in the same size range as those recognized by anti-G2 antiserum and mAb 5-D-4, but, in agreement with the results obtained with the undigested samples, only in pools that contained large populations of aggrecan (pools A–D and O–R). At least three core protein bands exceeding approx. 280 kDa were present exclusively in pools A–B and O–P. A cluster of four conspicuous bands present on the immunoblots with 5-D-4 (in the range 140–180 kDa) is an artefact related to the presence of deglycosylating enzymes in the samples. The same effect was caused by BSA, the presence of which also

caused interruptions in the broad bands of proteoglycan core proteins on immunoblots (Figure 7).

DISCUSSION

Three overlapping populations of aggrecan and three species of small proteoglycans were extracted from human articular cartilage under very mild associative conditions, and thus present the possibility that all these proteoglycans may be found in normal synovial fluid. It is quite likely that the proteoglycans obtained in associative extracts of finely sectioned tissue may be the same as those that are lost from the tissue *in vivo*.

The presence of small proteoglycans in the associative extract was unexpected, at least for decorin and fibromodulin, as assays *in vitro* have shown that both decorin and fibromodulin bind to collagen types I and II [42,43]. The present findings suggest that for a proportion of molecules *in vivo* this binding may be weak, or that a proportion of decorin and fibromodulin is not bound to collagen. Biglycan, on the other hand, does not bind to collagen, and its possible function in articular cartilage is unknown [3]. The unambiguous identification of the small proteoglycans in the extract was possible only by the use of specific antibodies, since chromatographic and electrophoretic separation techniques usually result in their overlap with each other, or with cleavage products of aggrecan.

Two main large populations of aggrecan were identified in the extract. Both populations comprised the G2 domain and keratan sulphate domain, but the proportion of chondroitin sulphate decreased with decreasing size of the whole molecule. Hence the smaller population most probably represents a population of truncated molecules of aggrecan derived from the larger population by removing a part of the chondroitin sulphate domain. Accordingly, several different-sized core proteins are found in each population. The mobilities of these proteoglycans on gradient SDS/PAGE, as well as their colours after staining with the Stains-all dye, corresponded to two populations of aggrecan that are routinely isolated from the A_1D_1 fraction of human articular cartilage extracts (V. Vilím, unpublished work). Similar populations isolated from cartilage of various species have been designated in the literature as chondroitin sulphate-rich (the larger population) and keratan sulphate-rich (the smaller population) [7,44–47]. The third population present in the associative extract represented aggrecan fragments with molecular mass less then approx. 300 kDa, which contain the G2 domain and keratan sulphate chains, but are lacking chondroitin sulphate chains.

The immunolocalization experiments do not allow a quantitative estimate of the relative proportion of G1 or G2 domains. The interglobular domain between G1 and G2 is highly susceptible to proteolytic attack; therefore it is likely that aggrecan populations positive for both 1-C-6 and anti-G2 epitopes represent a mixed population of molecules either lacking or containing the G1 domain. Although there is a copy of the 1-C-6 epitope in both the G1 and G2 domains, previous studies using pig aggrecan have shown that this epitope is masked in G2 by substitution with keratan sulphate [40]. Since G1–G2 fragments isolated from human aggrecan contain significantly more keratan sulphate than those of pig (A. J. Fosang, unpublished work), it is reasonable to predict that the 1-C-6 epitope in human G2 is also masked by keratan sulphate.

The pattern of aggrecan fragments described in the present study is similar to that observed in cultures of explanted bovine articular cartilage [13,48]. The predominant core proteins released into the culture medium represented two large proteins with intact N-termini and a number of fragments, ranging from less than 100 kDa to approx. 250 kDa. Two similar species of aggrecan fragments, both produced by cleavage within the interglobular domain, and comprising the G2 domain, keratan sulphate domains, and variable lengths of the chondroitin sulphate domain, have recently been purified from human synovial fluids [19].

In summary, our results demonstrate heterogeneity and polydispersity of proteoglycans found in associative extracts of normal human articular cartilage. The data indicate that, in addition to cleavage within the interglobular domain of aggrecan, there are other proteolytic cleavage sites present along the core protein and several of these are located in the chondroitin sulphate domain. Identification of aggrecan fragments in associative extracts of normal cartilage suggests that cleavage at these sites occurs *in vivo* under normal conditions of aggrecan turnover.

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