Age-Related Changes in the Composition and Structure of Human Articular-Cartilage Proteoglycans

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1. Analysis of the purified proteoglycans extracted from normal human articular cartilage with 4M-guanidinium chloride showed that there was an age-related increase in their content of protein and keratan sulphate. 2. The hydrodynamic size of the dissociated proteoglycans also decreased with advancing age, but there was little change in the proportion that could aggregate. 3. Results suggested that some extracts of aged-human cartilage had an increased content of hyaluronic acid compared with specimens from younger patients. 4. Dissociated proteoglycans, from cartilage of all age groups, bind to hyaluronic acid and form aggregates in direct proportion to the hyaluronic acid concentration. 5. Electrophoretic heterogeneity of the dissociated proteoglycans was demonstrated on polyacrylamide/agarose gels. The number of proteoglycan species observed was also dependent on the age of the patient.

Proteoglycans are a major component of hyaline cartilages and can be extracted with dissociating solvents such as 4M-guanidinium chloride (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969). In cartilage, most of the proteoglycans exist as aggregates formed by the non-covalent association of proteoglycan subunits with hyaluronic acid (Gregory, 1973; Hardingham & Muir, 1972, 1974b; Hascall & Heinegård, 1974a,b). The macromolecular complex formed by this association is further stabilized by two link-proteins, which also bind to the aggregate. Proteoglycan subunits consist of a protein backbone of variable length to which are attached variable proportions of chondroitin sulphate and keratan sulphate. This results in a polydisperse population of macromolecules in which the molecular weights of individual subunits decrease in proportion to their chondroitin sulphate contents and chondroitin sulphate/protein ratios (Hascall & Sajdera, 1970; Tsiganos *et al.*, 1971; Heinegård & Hascall, 1974; Hardingham *et al.*, 1976; Rosenberg *et al.*, 1976; Heinegård, 1977).

Although age-related variations in the size and chemistry of proteoglycans from pig and dog articular cartilage have been reported (Šimůnek & Muir, 1972; Inerot *et al.*, 1978), results from similar studies on human articular cartilage have not. Perricone *et al.* (1977) were unable to detect any aggregates in extracts of normal aged-human articular cartilage, whereas Bayliss & Ali (1978) demonstrated aggregates of human cartilage proteoglycans and their dissociation, by using preformed CsCl density gradients. The results presented here describe the

chemical and structural properties of human articular-cartilage proteoglycans and are compatible with a large degree of polydispersity in subunit size and heterogeneity in composition at all ages.

Experimental

Materials

All reagents were of analytical grade, except glucuronolactone and carbazole, and were supplied by BDH Chemicals, Poole, Dorset, U.K. Acetylacetone was redistilled (b.p. 133–134°C). Hyaluronic acid (human umbilical cord) was obtained from BDH and Visking tubing from Scientific Instruments Centre, London W.C.1, U.K.

Analytical methods

Uronic acid was determined by the Bitter & Muir (1962) modification of the carbazole method of Dische (1947), with glucuronolactone as standard. Samples for hexosamine analysis were hydrolysed in 5M-HCl at 100°C for 4 h. Galactosamine/glucosamine ratios were determined by the method of Antonopoulos (1966), with glucosamine hydrochloride and galactosamine hydrochloride as standards. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Preparative methods

Extraction of proteoglycans. Human articular cartilage was obtained fresh from the operating theatre and either used immediately or stored at –20°C until required. Normal cartilage consisted of

fracture-of-the-neck-of-the-femur specimens or amputation specimens. The articular surface of each specimen was rinsed with cold sterile 0.9% NaCl before removal of full-thickness cartilage with a scalpel. Samples of cartilage were then prepared by powdering in liquid nitrogen by using a Spex Freezer Mill (Spex Industries Inc., Metuchen, N.J., U.S.A.). A grinding time of 15s for 2g of cartilage resulted in optimum extraction (Bayliss & Ali, 1978).

Proteoglycans were extracted with 4M-guanidinium chloride buffered at pH 7.0 with 0.1M-sodium phosphate. Cartilage was suspended in 10 times its weight of 4M-guanidinium chloride buffered with 0.1M-sodium phosphate, pH 7.0, and rolled gently on a Dorchester mixer (Raven Scientific Ltd., Haverhill, Suffolk, U.K.) at 4°C for 24h. The extracts were clarified by centrifuging for 15min at 1000g on a bench centrifuge, and the supernatant was removed and the residue washed with a small volume of cold buffered 4M-guanidinium chloride. The extract and washings were combined and dialysed against 9vol. of 0.05M-sodium phosphate buffer, pH 7.0, to return the proteoglycans to associative conditions. The proteoglycans in 0.4M-guanidinium chloride were then purified as described below.

Digestion of cartilage residue. The cartilage residues after extraction were washed with sterile 0.9% NaCl and then digested with papain. The digestion mixture consisted of 200 μ l of 2 $\times$ crystallized papain (BDH) in 100ml of 0.2M-sodium acetate, pH 5.0, containing 0.02M-cysteine and 0.004M-EDTA. The enzyme was activated by incubating the solution at 56°C for 30min. A portion of the activated enzyme solution (5ml/g wet wt. of cartilage) was added to the residue and incubated at 56°C for 24h. Some of the cartilage residues from elderly patients were not fully digested after this time and a further 2.5ml of freshly prepared enzyme was added and digestion continued for a further 24h. Any insoluble residue was removed by centrifugation for 10min at 1000g and the supernatant analysed for uronic acid.

Associative density-gradient centrifugation of proteoglycans. Solid CsCl was added to the dialysed extract until the density was 1.5g/ml. Equilibrium density-gradient centrifugation was then performed in an MSE Superspeed 50 centrifuge with an 8 $\times$ 25ml angle rotor at 95000g_{av}. for 48h at 10°C. After centrifugation the gradients were fractionated by upward displacement into a bottom fraction (A₁, 5ml; ρ_{av} = 1.58g/ml) and a top fraction (A₂, 13ml; ρ_{av} = 1.48g/ml). The fractions were dialysed for 24h against 0.05M-sodium phosphate buffer, pH 7.0, and stored at -20°C until required.

Dissociative density-gradient centrifugation of proteoglycans. The bottom 5ml from the associative density gradient was mixed with an equal volume of 8M-guanidinium chloride buffered with 0.1M-

sodium phosphate, pH 7.0. Solid CsCl was then added to give a final density of 1.5g/ml. Centrifugation was carried out as described for the associative gradients and fractions (2ml) were collected in the same way. Each fraction was stored frozen after dialysis against 0.05M-sodium phosphate buffer, pH 7.0.

Gel chromatography. Proteoglycan samples (1ml) containing 300–500 μ g of uronic acid in 0.1M-sodium phosphate buffer, pH 7.0, containing 0.1M-NaCl, were applied to a column (150cm $\times$ 0.8cm) of Sepharose 2B (Pharmacia, Uppsala, Sweden) and eluted with the same buffer at 3ml/h at 4°C by using a peristaltic pump, or applied to a column (150cm $\times$ 0.8cm) of Bio-Gel A-150m (Bio-Rad Laboratories Ltd., Bromley, Kent, U.K.) and eluted at 1.5ml/h. Gel chromatography at pH 3.0 was carried out on a column (150cm $\times$ 0.8cm) of Sepharose 2B equilibrated with 0.1M-sodium citrate/phosphate buffer containing 0.1M-NaCl. In each case fractions (1ml) were collected and their uronic acid and/or protein content was determined. Proteoglycan aggregates and glucuronolactone were used as markers of the void volume and total volume of the columns respectively.

When gel chromatography was carried out in the presence of 4M-guanidinium chloride, Sepharose 2B was suspended in 4M-guanidinium chloride buffered with 0.1M-sodium phosphate buffer, pH 7.0. The column (150cm $\times$ 0.8cm) was eluted with buffered 4M-guanidinium chloride by using a hydrostatic head of 40cm. Fractions (1ml) were collected and dialysed against water to remove the guanidinium chloride and then assayed for their uronic acid content.

Recombination of fractions from dissociative density gradients. A sample of each fraction was dialysed separately for 24h against 4M-guanidinium chloride buffered with 0.1M-sodium phosphate, pH 7.0. Fractions were then mixed in the required proportions and dialysed for a further 24h against 0.1M-sodium phosphate, pH 7.0, containing 0.1M-NaCl. The extent of interaction was measured, by gel chromatography on Sepharose 2B, as the increase in material eluted in the region of the void volume of the column. This was estimated by cutting and weighing a tracing of the uronic acid elution profile (Hardingham & Muir, 1974b).

Interaction of proteoglycans and hyaluronic acid. Samples of proteoglycan (300–500 μ g of uronic acid) in 0.1M-sodium phosphate buffer, pH 7.0, containing 0.1M-NaCl were mixed with various concentrations of hyaluronic acid (based on uronic acid content) and allowed to interact at 22°C for 4h. The interaction was assessed by gel chromatography on Sepharose 2B as described above.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The 10% polyacrylamide gels and the

method of sample preparation used were as described by Weber & Osborn (1969). Electrophoresis was carried out on samples containing 10–25 μ g of protein by using a Shandon disc-electrophoresis chamber with a current of 5mA/tube and 0.05% Bromophenol Blue as a marker. The protein bands were then stained with Coomassie Blue.

Polyacrylamide/agarose-gel electrophoresis. Electrophoresis on large-pore polyacrylamide/agarose gels was performed by the method of McDevitt & Muir (1971). Proteoglycan samples (2–3 μ g of uronic acid) in 12 μ l of a 40% (w/v) sucrose solution containing 0.05% (w/v) Bromophenol Blue were layered on the surface of each gel. Electrophoresis was carried out for 4 h (2.5 mA/tube, voltage gradient 10V/cm). A sample of chondroitin sulphate (bovine tracheal) was included in each tube as an internal reference. Gels were stained with 0.2% Toluidine Blue in 0.1M-acetic acid and destained in 3% (v/v) acetic acid for 1 h and then in water.

Digestion with hyaluronidase. A sample of fraction D₁(82) in 0.05M-sodium acetate, pH 5.0, was digested for 4 h at 37°C with hyaluronidase (ex *Streptomyces*) (Calbiochem Ltd., Bishops Stortford, Herts., U.K.). A portion of the enzyme (2 turbidity-reducing units/20 μ l) was added to the mixture after each hour of incubation. The digest was then dialysed against

0.1M-sodium phosphate buffer, pH 7.0, for 24 h and chromatographed on a column (150cm $\times$ 0.8cm) of Sepharose 2B as described above.

Results

Extraction and purification of proteoglycans from normal cartilage

Samples of human articular cartilage from patients of various ages were powdered and extracted with 4M-guanidinium chloride, pH 7.0, as described by Bayliss & Ali (1978). Some 70–80% of the tissue uronic acid was extracted from all samples. After purifying by equilibrium density-gradient centrifugation, 80–90% of the uronic acid was recovered in the bottom 5 ml (A₁ fraction) of each gradient (Table 1). The proteoglycan subunits in fraction A₁ were separated from hyaluronic acid and the link-protein(s) by a further density-gradient centrifugation in 4M-guanidinium chloride. Analyses of dissociated fractions D₁–D₉ are given in Tables 1 and 2. For ease of discussion the age of the patient to whom the fractions refer is given in parentheses after the fraction number, e.g. D₁₋₃(20) refers to the combined fractions D₁₋₃ derived by dissociative gradient centrifugation from a 20-year-old patient.

Table 1. Analysis of fractions obtained by equilibrium density-gradient centrifugation of proteoglycans under associative and dissociative conditions

Fractions were as described in the text.

Fraction	Content (% of total)		Protein (Lowry)/uronic acid (wt. ratio)	Galactosamine/glucosamine (molar ratio)	Uronic acid excluded from Sepharose 2B (% of total)
	Uronic acid	Protein			
A ₁ (13)	87.0	32.6	1.20	3.10	68
A ₂ (13)	13.0	67.4	16.90	1.57	—
A ₁ (20)	83.8	41.6	1.83	1.76	58
A ₂ (20)	16.2	58.4	13.11	1.29	—
A ₁ (34)	82.7	36.0	3.39	0.72	60
A ₂ (34)	17.3	64.0	29.12	0.50	—
A ₁ (52)	85.8	55.7	4.67	0.58	67
A ₂ (52)	14.2	44.3	22.70	0.71	—
A ₁ (80)	86.5	58.0	3.03	0.89	67
A ₂ (80)	13.5	42.0	20.50	0.71	—
A ₁ (82)	87.9	53.0	4.61	0.49	65
A ₂ (82)	12.1	47.0	26.01	0.47	—
D ₁ (13)	73.0	26.1	0.52	4.71	6
D ₂ (13)	9.8	9.4	—	2.11	—
D ₁ (20)	70.3	29.2	0.74	2.18	6
D ₂ (20)	11.1	11.0	—	1.33	—
D ₁ (34)	62.6	22.0	1.53	1.48	14
D ₂ (34)	15.4	11.0	—	0.58	—
D ₁ (52)	63.6	21.3	1.00	0.98	27
D ₂ (52)	13.2	10.5	—	0.61	—
D ₁ (80)	60.0	17.1	1.02	1.25	18
D ₂ (80)	15.2	10.1	—	0.60	—
D ₁ (82)	60.8	18.2	1.26	1.07	44
D ₂ (82)	12.9	10.6	—	0.59	—

Table 2. Analysis of proteoglycans obtained by equilibrium density-gradient centrifugation under dissociative conditions
Fractions were as described in the text.

Fraction	Density (g/ml)	Age (years)	...	Uronic acid (% of total)						Protein (Lowry)/uronic acid (wt. ratio)					
				13	20	34	52	80	82	13	20	34	52	80	82
D ₁	1.61			73.0	70.3	62.6	63.6	60.0	60.8	0.52	0.74	1.53	1.00	1.02	1.26
D ₂	1.57			9.8	11.1	15.4	13.2	15.2	12.9	1.39	1.77	3.06	2.40	2.37	3.46
D ₃	1.54			5.0	5.7	7.1	7.3	8.2	7.7	1.85	2.83	5.11	3.99	3.88	5.57
D ₄	1.51			3.0	4.3	4.9	4.2	5.5	5.0	2.78	3.25	6.71	5.50	4.96	6.90
D ₅	1.49			2.1	2.8	3.2	2.9	3.8	3.9	4.07	4.90	8.89	7.74	8.84	9.03
D ₆	1.47			1.8	1.8	2.3	2.6	3.0	3.0	5.33	6.89	14.90	8.87	9.97	12.50
D ₇	1.45			1.5	1.5	1.8	2.2	1.8	2.6	8.62	8.00	16.85	11.40	17.93	14.13
D ₈	1.43			1.2	1.0	1.6	1.6	1.5	1.9	10.11	13.33	23.36	24.28	25.20	20.30
D ₉	1.42			2.1	1.1	1.1	1.8	1.0	1.7	28.96	20.69	35.06	31.06	33.36	39.72

Analysis of aggregated proteoglycans

Although fraction A₁ accounted for approximately the same proportion of uronic acid on each gradient, there were large variations in the protein content and galactosamine/glucosamine molar ratios of proteoglycan aggregate preparations from cartilage of young and aged patients (Table 1). Fraction A₁ from the mature patients contained 2.5–3.5 times as much protein as fractions A₁(13) and A₁(20).

Amino acid analysis of fractions A₁(13), A₁(34) and A₁(80) showed that this was not the result of an increase in aromatic amino acids, which would give an abnormally high value in the Lowry *et al.* (1951) protein assay. There did not appear to be any major differences in the amino acid content of these specimens, which also excluded the possibility of an additional protein component in close association with the proteoglycans from aged-human cartilage. As found for other cartilages, serine, glutamine and glycine were the predominant amino acids in each case. There was a similar increase in the glucosamine content of the A₁ fractions with advancing age, indicating a higher keratan sulphate and/or hyaluronic acid content. Gel chromatography of the A₁ fractions on a column (150cm×0.8cm) of Sepharose 2B showed that, although there were variations in composition, approximately the same proportion of each was excluded from the column, indicating very-high-molecular-weight complexes at all ages (Fig. 1).

Analysis of disaggregated proteoglycans

The distribution of uronic acid and protein on the dissociative density gradients of each specimen studied is shown in Tables 1 and 2. Fractions D₁(13) and D₁(20) accounted for 73 and 70% respectively of the uronic acid on the gradient. However, in the older age group this fraction represented only 60% of the uronic acid. Similarly, although 29% of the protein was in fraction D₁(20), only 18% was present in fraction D₁(82). The proteoglycans in fraction D₁

from mature patients also had a higher protein and keratan sulphate content than did those in D₁(13) and D₁(20). This change in chemistry of the dissociated proteoglycans with aging is seen in all fractions of the dissociative gradients. Thus at all ages there is an increase in the protein content of the proteoglycans in proceeding from the dense to the less-dense regions of the gradient (Table 2). However, all the proteoglycan fractions derived from the gradient of an aged patient have a higher protein/uronic acid weight ratio than the corresponding fractions of the 13- or 20-year-old patient.

Comparison of the proteoglycans from each of the dissociative gradients was initially carried out on the combined fractions D₁₋₃ (80–85% of the total uronic acid), even though this might possibly have included some hyaluronic acid from the middle of the gradient (Hardingham & Muir, 1974b). When fractions D₁₋₃(13) and D₁₋₃(20) were eluted on a column of Sepharose 2B, 74 and 85% of the proteoglycans were retarded by the gel respectively, indicating that the majority were disaggregated (Fig. 1 and Table 1). In contrast, the elution pattern of identical fractions from the other gradients suggested that very little disaggregation had occurred. It was concluded that mixing with hyaluronate in fraction D₃ might have resulted in some reaggregation, and comparison was made of fraction D₁ from each gradient in an attempt to avoid this. The proteoglycans in fractions D₁(13) and D₁(20) were completely retarded on Sepharose 2B, but a considerable proportion of those in fractions D₁(34), D₁(52) and D₁(80), and especially D₁(82), were still excluded from the column (Fig. 1 and Table 1). Nevertheless, where disaggregation could be observed it was apparent that the retarded proteoglycans were poly-disperse and those derived from the cartilage of mature individuals were of smaller average size than those from the 13- and 20-year-old patients. This finding was confirmed by chromatography of D₁(13)

Fig. 1. Gel chromatography on Sepharose 2B of aggregated and disaggregated proteoglycans. Samples containing 300–500 μ g of uronic acid were applied to a column of Sepharose 2B (150cm $\times$ 0.8cm) and eluted as described in the text. The uronic acid content of each 1 ml fraction was determined. V_0 and V_t mark the void volume and total volume of the column respectively.

Fig. 2. Gel chromatography of proteoglycans on (a) Bio-Gel A-150m and (b) Sepharose 2B. (a) Samples of fractions $D_1(13)$ (—) and $D_1(52)$ (---) were applied to a column of Bio-Gel A-150m (150cm $\times$ 0.8cm) and eluted as described in the text. (b) Samples of fractions $A_1(20)$ (—) and $A_1(80)$ (---) were dialysed against 0.1M-sodium citrate/phosphate buffer, pH 3.0, and applied to a column of Sepharose 2B (150cm $\times$ 0.8cm) eluted with the same buffer. Fractions (1 ml) were analysed for uronic acid.

and $D_1(52)$ on a column (150cm $\times$ 0.8cm) of Bio-Gel A-150m.

Although gel chromatography on Sepharose 2B differentiated between aggregated and disaggregated proteoglycans, there was still considerable overlap of the two species, especially for proteoglycans from younger individuals. The effectiveness of using the same column size with Bio-Gel A-150m was therefore examined. Bio-Gel has a much higher exclusion limit than Sepharose 2B and Fig. 2(a) shows that, although it was necessary to halve the flow rate, a clearer separation of excluded and retarded material was obtained. Whereas 27% of the proteoglycans in fraction $D_1(52)$ were excluded from Sepharose 2B (Fig. 1), only 15% were excluded from Bio-Gel

(---) were applied to a column of Bio-Gel A-150m (150cm $\times$ 0.8cm) and eluted as described in the text. (b) Samples of fractions $A_1(20)$ (—) and $A_1(80)$ (---) were dialysed against 0.1M-sodium citrate/phosphate buffer, pH 3.0, and applied to a column of Sepharose 2B (150cm $\times$ 0.8cm) eluted with the same buffer. Fractions (1 ml) were analysed for uronic acid.

A-150m. In addition to emphasizing the polydispersity of the dissociated proteoglycan preparations, the Bio-Gel elution profiles also confirmed the smaller size of proteoglycans from mature patients.

An attempt was made to study the size range of all the proteoglycans within an A_1 preparation by using the disaggregating effect of low pH (Sajdera & Hascall, 1969; Hardingham & Muir, 1974a; Bayliss & Ali, 1978). Samples of the $A_1(20)$ and $A_1(80)$ preparations were dialysed against 0.1M-sodium citrate/phosphate buffer, pH 3.0, containing 0.1M-NaCl and eluted on a column of Sepharose 2B (150cm $\times$ 0.8cm). Fig. 2(b) shows that in both cases the proteoglycans were polydisperse, but once again there was an increase in the proportion of lower-molecular-weight species in fraction $A_1(80)$.

Evidence for variations in hyaluronic acid content

It was observed that a greater percentage of the proteoglycans in fraction D_1 from some mature individuals was excluded from Sepharose 2B than the corresponding fractions from the 13- and 20-year-old patients (Fig. 1). This implied that either (i) the excluded material represented a fraction of proteoglycans that did not disaggregate or (ii) hyaluronic acid and link-protein had not been separated from the proteoglycans.

To examine the first suggestion, fractions $D_{1-3}(20)$ and $D_{1-3}(82)$ were chromatographed on a column (150cm $\times$ 0.8cm) of Sepharose 2B equilibrated with 4M-guanidinium chloride, pH 7.0 (Fig. 3). Fraction

Fig. 3. Gel chromatography on Sepharose 2B in 4M-guanidinium chloride of disaggregated proteoglycans. Samples of fraction $D_{1-3}(20)$ (—) and fraction $D_{1-3}(82)$ (----) were applied to a column of Sepharose 2B (150cm $\times$ 0.8cm) eluted with 4M-guanidinium chloride/0.1M-sodium phosphate, pH 7.0. The uronic acid content of 1ml fractions was measured after dialysis against 0.1M-sodium phosphate buffer, pH 7.0.

$D_{1-3}(20)$ gave an elution profile almost identical with that given by the same fractions eluted from Sepharose 2B equilibrated with buffer, except that the excluded peak was absent. Similarly fraction $D_{1-3}(82)$ was completely retarded on the Sepharose 2B column equilibrated with 4M-guanidinium chloride, demonstrating that all the proteoglycans in fraction $D_{1-3}(82)$ could be disaggregated. This experiment also confirmed that proteoglycans from the cartilage of the 82-year-old patient were of smaller hydrodynamic size than those from the 20-year-old patient.

The second possibility was examined by studying the effect of recombining fractions from dissociative gradients (Fig. 4). Thus when fraction $D_{1-3}(20)$ was mixed with fraction $D_{4-9}(20)$, in the proportions in which they occurred on the gradient, 50% of the proteoglycans were excluded from Sepharose 2B (Fig. 4b). In the same way, when fraction $D_{1-3}(20)$ was mixed with fraction $D_{4-9}(82)$ in the same proportion as fraction $D_{4-9}(20)$ (based on uronic acid), 64% of the proteoglycans were excluded from the column (Fig. 4c). The higher protein content of the proteoglycans in fraction $D_{4-9}(82)$ than in fraction $D_{4-9}(20)$ was also reflected in the aggregates formed

Fig. 4. Gel chromatography on Sepharose 2B of reaggregated proteoglycan fractions

Proteoglycans were fractionated as shown in Table 1 and recombined as described in the text. They were dialysed against 0.1M-sodium phosphate buffer, pH 7.0, containing 0.1M-NaCl and applied to a column of Sepharose 2B (150cm $\times$ 0.8cm). Fractions (1ml) were analysed for uronic acid (—) and protein (----). (a) Fraction $D_{1-3}(20)$; (b) fraction $D_{1-3}(20) + D_{4-9}(20)$; (c) fraction $D_{1-3}(20) + D_{4-9}(82)$.

during recombination. This demonstrated that the factors promoting aggregation were present in the same fractions on both dissociative gradients. Electrophoresis of fractions A_1 , D_1 and D_9 from the 20-, 52- and 82-year-old patients on sodium dodecyl sulphate/polyacrylamide gels also confirmed that fractions $D_1(52)$ and $D_1(82)$ were not contaminated with link-protein(s); no protein bands were present in fraction D_1 . Only fractions A_1 and D_9 contained the two protein bands that are characteristic of the link-protein(s). A number of slow-migrating species were also observed, but were not characterized further. Similar bands were observed in bovine nasal-cartilage extracts by Roughley *et al.* (1978).

As a result of these experiments, and of consideration of the work of Hardingham & Muir (1974*a,b*), it was concluded that the most likely explanation for the proteoglycans eluting in the void volume in fraction D_1 from adults was contamination with hyaluronic acid. This suggested that there might be an increase in the hyaluronic acid content in the cartilage extracts from some mature patients to such an extent that it overlapped with fraction D_1 . To investigate this hypothesis further fraction $D_1(20)$, which is completely retarded by Sepharose 2B (Fig. 1), was recombined with fraction $D_{4-7}(20)$. The amount of fraction $D_{4-7}(20)$ used was adjusted to account for 50% (v/v) of its proportion of the dissociative gradient so that complete reaggregation would not occur. When chromatographed on Sepharose 2B, 30% was excluded from the column (Fig. 5*a*). However, when fraction $D_1(20)$ was mixed with fraction $D_{4-7}(52)$ or $D_{4-7}(82)$, in the same proportions as fraction $D_{4-7}(20)$ (based on uronic acid content), 48 and 56% respectively were excluded from the same column (Figs. 5*b* and 5*c*). This confirmed that fractions $D_{4-7}(52)$ and $D_{4-7}(82)$ were richer in the interacting component than fraction $D_{4-7}(20)$.

It was not possible to isolate the interacting component from fractions $D_1(52)$ or $D_1(82)$ because of the small sample available. However, when fraction $D_1(82)$ was digested with fungal hyaluronidase, which is specific for hyaluronic acid, and subsequently chromatographed on Sepharose 2B all the proteoglycans were retarded on the column and no excluded material was observed.

Interaction of dissociated proteoglycans with hyaluronic acid

The interaction of hyaluronic acid with fraction D_1 and its effect on the hydrodynamic size of the dissociated proteoglycans was assessed by gel chromatography on Sepharose 2B. There was a linear relationship between the amount of hyaluronic acid added and the percentage of total uronic acid excluded from the column, up to a concentration of 0.7–0.8% (w/w) hyaluronic acid (based on uronic

Fig. 5. Gel chromatography on Sepharose 2B of proteoglycan fractions from dissociative density gradients. Proteoglycan fractions were recombined and eluted on a column of Sepharose 2B (150cm×0.8cm) as described in the text. Fractions (1 ml) were analysed for uronic acid. (a) Fraction $D_1(20)+D_{4-7}(20)$; (b) fraction $D_1(20)+D_{4-7}(52)$; (c) fraction $D_1(20)+D_{4-7}(82)$.

acid content). When the hyaluronic acid concentration was further increased the amount of proteoglycan excluded did not change. This pattern was similar for the two ages studied; however, the total uronic acid excluded from Sepharose 2B was less for fraction $D_1(80)$ (Fig. 6). These experiments are further proof that the proteoglycans of normal human articular cartilage do form aggregates in the same way as described for cartilages from other sources.

Electrophoretic heterogeneity of dissociated proteoglycans

Electrophoresis on composite polyacrylamide/agarose gels demonstrated that the proteoglycans in fraction D_1 were heterogeneous (Fig. 7*b*). In fraction $D_1(13)$ there were two Toluidine Blue-staining bands. The slower-moving of these was very broad and stained strongly. The second band was much fainter and narrower and was only clearly visible at higher sample concentrations than that shown. In contrast fractions $D_1(52)$, $D_1(80)$ and $D_1(82)$ each contained three bands. The most rapidly moving of these corresponded with the faster band in fraction

Fig. 6. Interaction of disaggregated proteoglycans with hyaluronic acid

Increasing proportions of hyaluronic acid (human umbilical cord) were mixed with a constant amount of fraction D₁(13) (●) or of fraction D₁(80) (○). Samples were applied to a column of Sepharose 2B (150cm×0.8cm) and the proportion of uronic acid excluded from the gel was determined as described in the text.

D₁(13), but was more intensely stained. The other two bands were also strongly metachromatic, but both had a higher mobility than the slower-migrating species in fraction D₁(13). Aggregate preparations A₁(13) and A₁(80) were excluded from the gel, although in the latter case there was a faintly staining band with the same mobility as the fast species in the dissociated fractions.

Discussion

Previous reports have indicated that there is an increase in the protein and glucosamine content of pig articular-cartilage proteoglycans with increasing age (Brandt & Muir, 1969, 1971a,b; Šimůnek & Muir, 1972). Similar observations were made for human rib cartilage and dog articular cartilage (Rosenberg *et al.*, 1965; Inerot *et al.*, 1978). Although detailed observations have been made of the total tissue chemistry of human articular cartilage (Anderson *et al.*, 1964; Miles & Eichelberger, 1964; Stockwell & Scott, 1967; Maroudas *et al.*, 1969, 1973), little is known about the composition of the purified proteoglycans. The present results show that with advancing age there is an increase in the protein and keratan sulphate content of the extractable proteoglycans of human articular cartilage. These findings confirm recent studies of similar extracts by using preformed CsCl density gradients (Bayliss & Ali, 1978).

Fig. 7. Electrophoresis (a) of proteoglycan fractions on sodium dodecyl sulphate/polyacrylamide gels and (b) on polyacrylamide/agarose gels of proteoglycans obtained by equilibrium density-gradient centrifugation under dissociative conditions

(a) Proteoglycan fractions from dissociative equilibrium density gradients were electrophoresed on neutral 10% polyacrylamide gels with 0.1% (w/v) sodium dodecyl sulphate (Weber & Osborn, 1969). Electrophoresis was carried out at 5 mA/gel for 4 h. (A) Fraction A₁(20); (B) fraction A₁(52); (C) fraction A₁(82); (D) fraction D₉(20); (E) fraction D₉(82). The gels have been aligned to show components of corresponding mobility. The two link-proteins are arrowed. (b) Purified proteoglycan aggregates were dissociated in 4M-guanidinium chloride and fractionated on dissociative equilibrium density gradients. Samples of fraction D₁ were applied to composite polyacrylamide/agarose gels prepared by the method of McDevitt & Muir (1971) and subjected to electrophoresis at 2.5 mA/gel for 4 h. (A) Fraction D₁(13); (B) fraction D₁(80); (C) fraction D₁(52); (D) fraction D₁(82). A sample of chondroitin sulphate (bovine tracheal) was included in each gel to act as an internal reference (arrowed).

Proteoglycan structure is based on a protein core with an invariant globular hyaluronic acid-binding region rich in keratan sulphate and a region of variable length, which contains a variable degree of substitution with chondroitin sulphate. Thus within any preparation the proteoglycans range in size from those of high buoyant density, with a low protein/uronic acid ratio and relatively high molecular weight, to those of low buoyant density, with a high protein/uronic acid ratio and lower molecular weight (Heinegård & Hascall, 1974; Hardingham *et al.*, 1976; Rosenberg *et al.*, 1976; Heinegård, 1977). Each of the dissociative gradients described here shows this change in chemistry of high- and low-density proteoglycans. Furthermore, the decrease in size and change in composition of human proteoglycans with increasing age is also consistent with the proposed model of proteoglycan structure. Although this change is small, it is significant when one considers, for example, that the protein/uronic acid ratio of fraction D₁(80), albeit higher than that of D₁(13), is lower than that of D₂(13). On the basis of the model described by Hardingham *et al.* (1976) and their observations on the relationship between buoyant density and proteoglycan composition and subunit size, such a small change in buoyant density might not be expected to result in a very large decrease in proteoglycan size. The polydispersity of the proteoglycans, when chromatographed on Sepharose 2B, could also obscure quite considerable changes in molecular weight. It should also be appreciated that 30 and 40% of the proteoglycans from immature and mature specimens respectively are not present in the D₁ preparations. However, the very high protein content of these proteoglycans suggested that they would be smaller than the proteoglycans in the corresponding D₁ fractions. This was confirmed by gel chromatography of fractions A₁(20) and A₁(80) at pH 3.0. The higher keratan sulphate content of proteoglycans from mature cartilage is also consistent with a decrease in molecular weight (Hardingham *et al.*, 1976). In this way our observations agree with the findings of Inerot *et al.* (1978) on dog articular cartilage.

After the first decade of life there is little change in the chondroitin sulphate content of human articular cartilage. Therefore the increase in protein/uronic acid weight ratio of aged-cartilage proteoglycans implies an absolute increase in protein content in addition to a decreased substitution of core protein with chondroitin sulphate. As a consequence there must also be an increase in the total number of proteoglycan subunits in mature cartilage. The high keratan sulphate content of these extracts also suggests this, the invariant hyaluronic acid-binding region of proteoglycans being rich in keratan sulphate (Heinegård & Axelsson, 1977). However, it is still not clear what proportion of keratan sulphate

is distributed in the variable regions of the core protein, or how this may change with site and age.

The variation in proteoglycan size and chemistry must ultimately influence the aggregates that they form. Sepharose 2B chromatography cannot distinguish between aggregates of different sizes, but our results do show that approximately the same proportion of proteoglycans is aggregated at the ages studied. Thus, because there is a fixed number of proteoglycan-binding sites per length of hyaluronic acid (Hardingham & Muir, 1973; Hascall & Heinegård, 1974b), this would suggest that there may be more hyaluronic acid in aged-cartilage extracts to accommodate the increased number of proteoglycan subunits. It is therefore of interest that the recombination experiments suggest that the preparations from 52- and 82-year-old patients may have a higher content of hyaluronic acid. Whether the molecular weight of hyaluronic acid also changes is not known, but the importance of hyaluronic acid chain length in regulating aggregate size has been demonstrated (Hascall & Heinegård, 1974a; Swann *et al.*, 1976).

Perricone *et al.* (1977) have reported that the proteoglycans extracted from morphologically normal human articular cartilage do not aggregate. Our results show very clearly that this is not the case and that cartilage specimens of a variety of ages contain a large proportion of aggregated proteoglycans. The discrepancy between their results and ours is possibly a consequence of the different extraction procedures. We have shown that proteoglycans are degraded when diced cartilage is extracted at 20°C and pH 5.8, and only after controlled powdering of cartilage was this effect eliminated (Bayliss & Ali, 1978). Perricone *et al.* (1977) also assumed that degradation was limited simply because their proteoglycans were larger than chondroitin sulphate; even the autolysis products of human articular cartilage, at pH 5.0, are larger than chondroitin sulphate (Bayliss & Ali, 1978) and do not interact with hyaluronic acid (M. T. Bayliss, unpublished work). Enzymic degradation of the keratan sulphate-rich hyaluronic acid-linkage region of the proteoglycans would account for the simultaneous absence of link-proteins and the high galactosamine content of their 'aggregate' preparation compared with ours. It might also explain why they were able to isolate such a large proportion of proteoglycans at densities over 1.72 g/ml.

The high protein content of human proteoglycans from aged individuals might account, in part, for the dry weight of articular cartilage not accounted for by collagen and glycosaminoglycans (Muir *et al.*, 1970). The results of these authors also indicated that the discrepancy in dry weight was less in cartilage from younger individuals and varied through the cartilage depth. It is significant therefore that proteoglycans from the basal zones of human articular cartilage are

richer in protein than those extracted from other zones (M. T. Bayliss, unpublished work).

Although gel chromatography suggests that the dissociated proteoglycans are polydisperse in size, their gel-electrophoretic profile shows that they are heterogeneous and confirms that distinct species of proteoglycan exist in human articular cartilage. Heterogeneity has been observed for the proteoglycans of human epiphyseal and baboon articular cartilage (Stanescu & Maroteaux, 1975a,b; Stanescu *et al.*, 1977), bovine nasal cartilage (Pearson & Mason, 1977; Roughley & Barrett, 1977; Roughley, 1977) and human costal cartilage (Pearson & Mason, 1978). However, in foetal cartilage only one electrophoretic band was present, whereas in the baboon and bovine proteoglycan preparations two bands were observed. The proportion of each species also varied with buoyant density and composition. A third, faster-migrating species of proteoglycan was found in the latter cartilage extracts, but it was isolated only in low-density fractions and was considered to represent non-aggregated proteoglycans (Stanescu *et al.*, 1977). Roughley (1977) has also shown for bovine nasal proteoglycans that there is a variation in hexosamine molar ratio with size and electrophoretic mobility. Thus the age-related change in electrophoretic profile observed for human cartilage proteoglycans may reflect the increased keratan sulphate content of aged-human cartilage.

The mechanical properties of articular cartilage are known to be influenced by proteoglycan content. Kempson *et al.* (1970) have shown that there is a good correlation between the stiffness and glycosaminoglycan content of human articular cartilage. Of particular significance was their finding that at a given concentration keratan sulphate influenced the stiffness to a greater extent than did chondroitin sulphate. The swelling pressure of normal cartilage is also partly dependent on the high osmotic pressure of the proteoglycan polymer solution (Ogston, 1970; Ogston & Wells, 1972; Maroudas, 1973, 1975). Maroudas (1976) has also shown that swelling pressure varies through the cartilage depth. More recently it has been demonstrated that the compliance of articular cartilage increases with age (Armstrong *et al.*, 1977). These findings emphasize the possible effects that age-related changes in proteoglycan structure could have on the mechanical properties of cartilage.

As a result of our experience in handling osteoarthrotic cartilage specimens we considered it important to obtain a clearer understanding of normal cartilage proteoglycans before attempting to explain the pathological changes. In many studies of normal human articular cartilage, there is considerable variation between samples, even those of the same age. It is not surprising therefore to find the same variability expressed at the macromolecular level, as in cartilage of the 52- and 82-year-old described here,

further emphasizing the need for cautious and extensive sampling, especially of pathological material.

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