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The identification and characterization of two populations of aggregating proteoglycans of high buoyant density isolated from post-natal human articular cartilages of different ages

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After chromatography on Sepharose CL-2B under associative conditions, high-buoyant-density human articular-cartilage proteoglycans were analysed biochemically and by radioimmunoassay with monoclonal antibodies to a core-protein-related epitope and to keratan sulphate. An examination of proteoglycans from individuals of different ages revealed the presence at 1 year of mainly a single polydisperse population containing chondroitin sulphate (uronic acid) and keratan sulphate. From 4 years onwards a smaller keratan sulphate-rich and chondroitin sulphate-deficient population appears in increasing amounts until 15 years. At the same time the larger population shows a progressive decrease in size from 1 year onward. By 23 years and after the proportion of keratan sulphate in the larger chondroitin sulphate-rich proteoglycan increases. Both adult proteoglycan populations are shown immunologically to aggregate with hyaluronic acid, with the smaller showing a greater degree of interaction. The larger population is richer in serine and glycine, and the smaller population contains more glutamic acid/glutamine, alanine, phenylalanine, lysine and arginine; its protein content is also higher. Whether the larger post-natal population represents a different gene product from the single polydisperse population found in the human fetus, which has a different amino acid composition, remains to be established. The smaller population, which represents approximately one-third the mass of the larger population in the adult, may represent a degradation product of the larger population, in which the hyaluronic acid-binding region and keratan sulphate-rich region are conserved.

INTRODUCTION

The high-buoyant-density proteoglycans of adult human articular cartilage are composed of a core protein to which are attached chondroitin 4- and 6-sulphates and keratan sulphate [1–3]. Many of these cartilage molecules possess the ability to bind specifically to hyaluronic acid to form macromolecular aggregates [4,5].

Human articular and bovine articular and tracheal cartilages all exhibit age-related changes in the chemistry of these aggregating proteoglycans. With increasing age such changes are characterized by a decrease in chondroitin sulphate-containing proteoglycan monomer size, an increase in keratan sulphate relative to chondroitin sulphate, an increase in 6-sulphation relative to 4-sulphation of chondroitin sulphate, a decrease in chain length of chondroitin sulphate and an increase in protein content [2,6–10]. In addition, immunological studies of human articular-cartilage proteoglycans have revealed that there are age-related differences that can be detected by measuring cell-mediated immune responses [11] or with monoclonal antibodies [12,13]. By the use of affinity chromatography, two distinct high-buoyant-density proteoglycan populations have been separated from the articular cartilage of a skeletally immature person, one with fetal biochemical characteristics and one with adult features [13]. Monoclonal antibodies that are specific for the fetal or the adult molecules have been used to demonstrate that with increasing fetal development and skeletal maturation and aging the fetal molecules are gradually replaced by the adult-specific molecules [13]. In this continuing study we demonstrate that these adult-specific molecules are in fact composed of two populations, and that after birth, and with increasing age, the smaller keratan sulphate-rich and chondroitin sulphate-deficient proteoglycan appears in increasing amount, in addition to the larger proteoglycan, which contains significant amounts of both keratan sulphate and chondroitin sulphate. The significance of these changes is discussed.

MATERIALS AND METHODS

Source of articular cartilage

Human articular cartilage was obtained at autopsy within 12 h of death from individuals with no macroscopic evidence of joint trauma, connective-tissue abnormality or arthritic disease [2]. All cartilage appearing macroscopically normal was removed from the proximal tibial plateau and distal femoral condyles. Any small areas of local degeneration were discarded. Juvenile material consisted of all the cartilages from the epiphyses of the distal femur and proximal tibia, excluding the growth plates.

Extraction and purification

Proteoglycan monomer was prepared as described previously, by isolating a D1 preparation by direct dissociative density-gradient centrifugation in CsCl and 4 M-guanidinium chloride [2]. Where indicated, gradient centrifugation was repeated once more to give a D1D1

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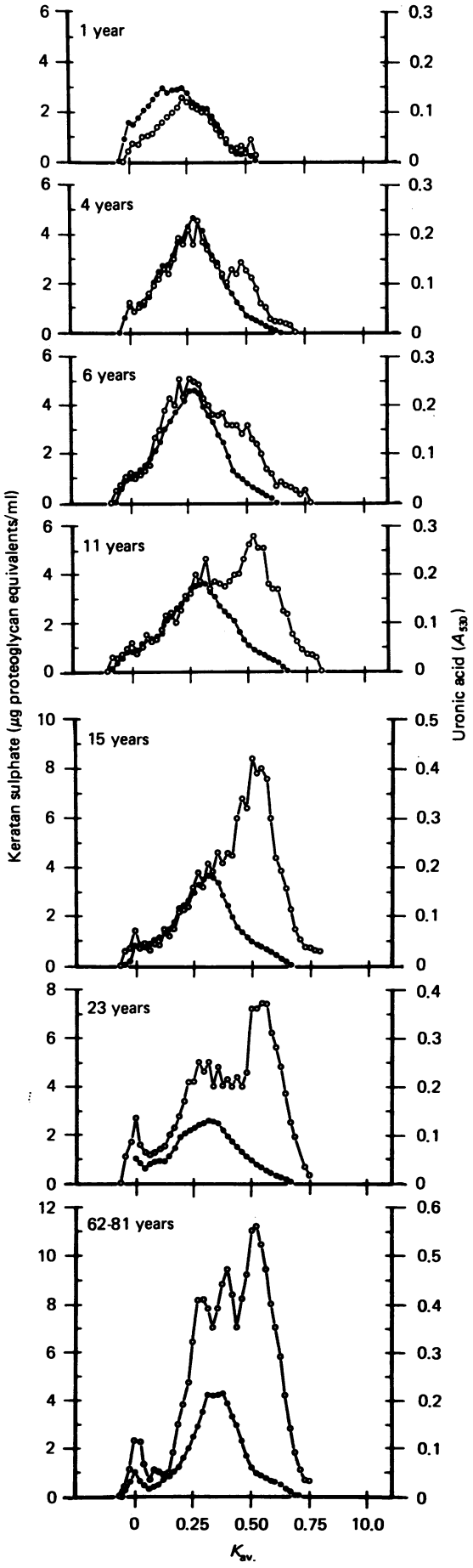

preparation. The preparations were dialysed at 4 °C, twice against 100 vol. of water, once against 100 vol. of 100 mM-sodium or -potassium acetate buffer, pH 6.0, and then exhaustively against water before being freeze-dried. Material was stored desiccated at 4 °C. The isolated D1 preparation represented 77% of the total extractable chondroitin sulphate (mean of eight preparations, range 63–91%).

Gel chromatography

For age analyses (Fig. 1) proteoglycan monomer at 2 mg dry wt./ml in 800 μl of 0.2 M-sodium acetate buffer, pH 5.5 (column buffer), was chromatographed on a Sepharose CL-2B (Pharmacia) column (111 cm × 1 cm diam.) with downward elution at 6 ml/h, and 1 ml fractions were collected. V_0 and V_t were determined from the elution profiles of uronic acid for monomer aggregated with hyaluronic acid and link protein (A1 preparation; see ref. [14] for definition) and glucuronolactone respectively. For biochemical and immunochemical analyses of separated proteoglycan populations (Fig. 2 and Tables 1 and 2), corresponding fractions were pooled from four separate chromatographic analyses, each employing 4 mg of proteoglycan per column.

Biochemical assays and reagents

These were all as described previously [13]. Hyaluronic acid was purified [15] and used to study proteoglycan aggregation as before [2].

Monoclonal antibodies

The antibody to keratan sulphate has been described elsewhere [16,17]. It closely resembles antibody KPC-190 [12] in that it recognizes keratan sulphate on cartilage proteoglycan monomer only when it is bound to core protein. It cross-reacts, however, with the keratan sulphate proteoglycan isolated from cornea but shows no reactivity with monomer isolated from the rat chondrosarcoma. Its reactivity with cartilage proteoglycan monomer is considerably diminished (by 80%) when monomer is treated with keratanase and even more (to 3% of original) when treated with papain. Antibody EFG-4 [12] recognizes an epitope that is specific for human adult cartilage proteoglycan monomer, and is associated with the core protein but not with the hyaluronic acid-binding region.

Radioimmunoassays

These were as described for AN9P1 [17] and EFG-4 [12], except that intact adult human proteoglycan monomer (50 years) was radiolabelled and used as a standard.

Fig. 1. Sepharose CL-2B chromatography of human cartilage proteoglycan monomer from persons of different ages

Distributions of uronic acid (●) and keratan sulphate determined by radioimmunoassay with antibody AN9P1 (○) are shown, the latter expressed as intact proteoglycan equivalents/ml. The 62–81-year-age preparation represents a pool of equal amounts of proteoglycan isolated as a D1D1 preparation, from five persons of ages 62–81 years.

RESULTS

Age changes

Proteoglycan monomers isolated from cartilages of different ages were chromatographed on Sepharose CL-2B. Column fractions were assayed for uronic acid by the carbazole method and for keratan sulphate by radioimmunoassay. The column profiles are shown in Fig. 1. At 1 year of age the leading edge of the keratan sulphate profile is slightly displaced to the right of the uronic acid profile, suggesting enrichment of keratan sulphate in the smaller molecules of the polydisperse proteoglycan population that contains uronic acid. The larger chondroitin sulphate-rich population exhibits a progressive decrease in size from 1 year onward. At and after 4 years the profiles for uronic acid and for keratan sulphate are similar for larger molecules, but a smaller keratan sulphate-rich and chondroitin sulphate-deficient population appears in increasing amounts until about 15 years. By 23 years the proportion of immunoreactive keratan sulphate to uronic acid in the larger chondroitin sulphate-rich proteoglycan increases. These observations reveal the appearance, with age, of a smaller proteoglycan population and changes with age in the larger population. We also observed the appearance of a small peak of keratan sulphate and uronic acid at the void volume at and after 15 years. This probably represents the presence of a small amount of aggregated proteoglycan as a result of limited contamination of the proteoglycan with hyaluronic acid.

Analyses of the two proteoglycan populations in adult cartilages

Proteoglycan monomers isolated from approximately equal amounts of human articular cartilage from five different persons in the 54–73-year age group were pooled and chromatographed as before on Sepharose CL-2B. As shown in Fig. 2(*a*) similar profiles were obtained for this age group compared with that shown in Fig. 1 for the 62–81-year group, in that the large population (pool A) was uronic acid- and galactosamine-rich and contained immunodetectable keratan sulphate, whereas the smaller population (pool B) was glucosamine-rich and deficient in uronic acid. These column fractions were also analysed with the monoclonal antibody EFG-4 (Fig. 2*b*). The same two populations were recognized by this antibody, indicating that its epitope is present in both populations, together with the epitope for AN9P1: it is also more concentrated in the smaller population (Fig. 2*c*). A comparison of the profiles for galactosamine, glucosamine and uronic acid (Fig. 2*a*) with those for keratan sulphate, determined by the use of antibody (Fig. 2*c*), revealed that fractions containing the smaller proteoglycan population (pool B) contained, relative to the larger molecules, less total glucosamine, although the antibody data revealed that there was more immunodetectable keratan sulphate in the fractions containing the smaller molecules (Fig. 2*c*). This difference may be because the monoclonal antibody to keratan sulphate recognizes a structural variant of keratan sulphate that is more common in the smaller population. In view of recent studies of antibodies to keratan sulphate [18], this could suggest that the smaller proteoglycan population contains a greater abundance of oversulphated keratan sulphate, with which our antibody reacts.

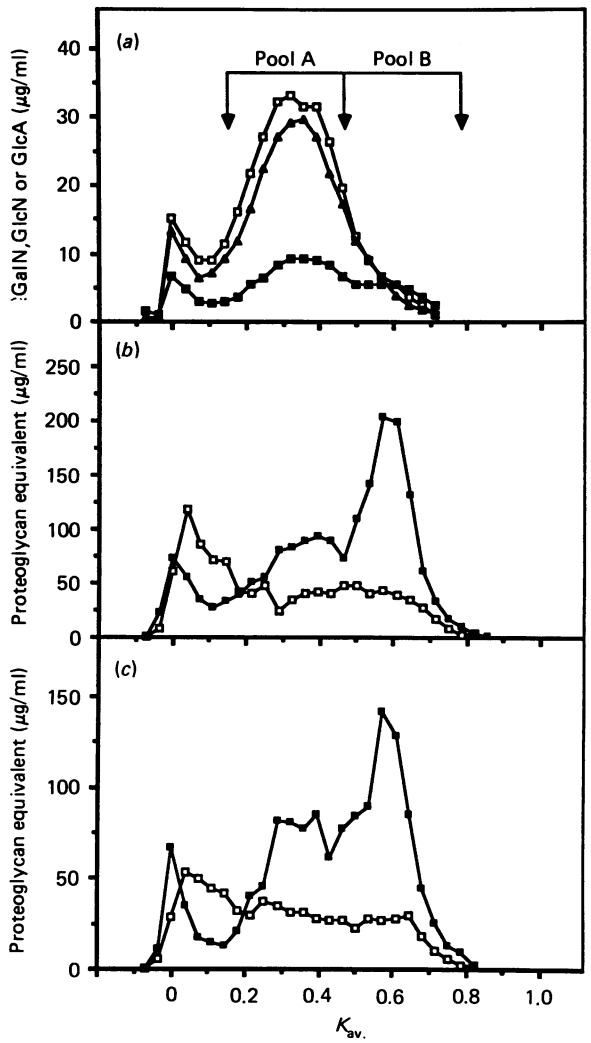

Fig. 2. Sepharose CL-2B chromatography of a mixture of equal amounts of human cartilage proteoglycan monomer isolated from five persons of ages 54–73 years to show comparative profiles for uronic acid, glucosamine, galactosamine and radioimmunoassays with antibodies EFG-4 and AN9P1

The distributions of glucuronic acid (GlcA, ▲), glucosamine (GlcN, ■) and galactosamine (GalN, □) are shown in (*a*). The fractions that were pooled for chemical analysis (Table 2) are indicated. Panel (*b*) shows the distribution of proteoglycan detected with monoclonal antibody EFG4 after chromatography with (□) and without (■) hyaluronic acid. Panel (*c*) shows the distribution of proteoglycan detected with monoclonal antibody AN9P1 after chromatography with (□) and without (■) hyaluronic acid.

When these proteoglycans were chromatographed after the addition of hyaluronic acid, different immunological profiles were observed (Figs. 2*b* and 2*c*). Both epitopes exhibited a smaller presence throughout the column and an overall decrease in the total amount of immunodetectable proteoglycan (Table 1). This is now known to be due to partial masking of these epitopes during aggregate formation (A. R. Poole, unpublished work), since it does not occur when these aggregated molecules are assayed under dissociative conditions after

Table 1. Aggregation of proteoglycans from adult human cartilage (54–73 years)

Aggregation was measured by the differences in the amount of proteoglycan detected in the fractions indicated after chromatography with and without hyaluronic acid. Pools A and B are the fractions indicated in Fig. 2.

	Aggregation (%)		Epitope detected after aggregation (% of total)
	Pool A	Pool B	
EFG-4	44.8	66.5	55.6
AN9P1	47.8	70.0	51.3

pretreatment with SDS and elevated temperature as described previously [19]. For this reason, aggregation was measured by determining the differences in the amount of proteoglycan detected in the included fractions (pools A and B) containing non-aggregated molecules. These results are indicated in Table 1. For fractions constituting pool A (large proteoglycan) and pool B (small proteoglycan) (as shown in Fig. 2) similar aggregation was observed with each antibody, although the smaller population appears to contain a greater proportion of aggregatable proteoglycan (Table 1). This may represent increased affinity of hyaluronic acid-binding region in smaller proteoglycans for hyaluronic acid rather than differences in the number of molecules with a hyaluronic acid-binding region.

Biochemical analyses of the large and the small proteoglycans

The column fractions containing the large and the small proteoglycans were pooled as indicated on Fig. 2(*a*), dialysed against deionized water and then freeze-dried. Analyses are shown in Table 2. The amino acid profiles indicate that the larger population, which represents approximately 3 times the mass of the smaller population, is richer in serine and glycine. The smaller population, however, contains more glutamic acid/glutamine, alanine, phenylalanine, lysine and arginine; its protein content is also higher. The increased content of uronic acid in the larger population is paralleled by an increased content of galactosamine, demonstrating its high content of chondroitin sulphate. The increased contents of glucosamine and sialic acid in the smaller population indicate an increased keratan sulphate content, as suggested immunologically. Compared with the composition of proteoglycan isolated as a high-buoyant-density preparation from fetal human cartilage at 24–27 weeks gestation, the two adult proteoglycans each had different amino acid and carbohydrate compositions (Table 2).

DISCUSSION

The present studies demonstrate that during development and aging human articular cartilage exhibits

Table 2. Amino acid analyses and chemical composition of high-buoyant-density proteoglycans from adult human cartilage (54–73 years)

Pool A and pool B were present in a weight ratio of 3.2:1. The data for fetal proteoglycan are reproduced from Table 3 of ref. [13] for 24–27-week-old fetal cartilage proteoglycan.

	Non-separated proteoglycans	2B large proteoglycan (pool A; Fig. 2)	2B small proteoglycan (pool B; Fig. 2)	Fetal proteoglycan
Amino acid compositions (residues/1000 residues)				
Asp	81	77	72	70
Thr	72	74	74	74
Ser	97	121	108	112
Glu	144	141	154	135
Pro	99	92	97	93
Gly	121	133	122	129
Ala	79	78	85	72
Val	73	71	65	70
Met	–	–	–	–
Ile	33	32	29	36
Leu	82	79	73	77
Tyr	23	20	21	22
Phe	31	28	35	45
His	12	11	12	15
Lys	19	14	17	17
Arg	35	29	36	31
Chemical composition (%)				
Protein	11.8	11.3	14.1	5.9
GlcN	6.4	5.2	10.0	1.4
GalN	18.3	21.0	13.1	25.0
UA	15.6	16.8	11.6	30.0
Sia	2.5	2.1	2.8	1.5
Chemical composition (molar ratio)				
GalN/GlcN	2.9	4.0	1.3	17.3

fundamental changes in the sizes and compositions of its proteoglycans of high buoyant density. Changes in composition have previously been suggested by earlier biochemical and immunological studies [11,13]. The presence in human adult cartilages of two distinct populations of different sizes and composition is now clearly recognizable. Previously, Bayliss *et al.* published a Figure which demonstrated the presence of uronic acid-rich and a smaller neutral-sugar-rich proteoglycan in human articular cartilage [20]. The present separation and characterization helps clarify earlier studies that reported their collective chemical and immunological compositions. Biochemical studies have also described the appearance with increasing age of a second smaller, lower-buoyant-density and more electrophoretically mobile proteoglycan in human [21,22] and bovine [10,23] cartilages. The smaller population has been identified as keratan sulphate-rich [21,23]. The present studies extend the earlier human observations by demonstrating the progressive increase in the proportion of the smaller of the two populations with development and aging and provide the first detailed description of the chemistry of these populations in human cartilage. A comparison of the amino acid compositions of the two adult human proteoglycan populations with those reported by Heinegård *et al.* [23] for two proteoglycan populations isolated from adult bovine nasal-septum cartilage reveals that there are some striking similarities even though these molecules are from different cartilages and species. Although the individual amino acid compositions are not identical for the large bovine proteoglycan compared with the large human proteoglycan and for the small bovine proteoglycan compared with the small human proteoglycan, the amino acid differences between the large and the small populations in each species are similar. Thus aspartic acid, serine and glycine contents are all higher in the larger populations, whereas the glutamic acid, proline, alanine, phenylalanine and arginine contents are greater in the smaller populations in each species. The protein contents are also greater in the smaller molecules in both species.

The age-related changes defined in this study also identify the initial appearance of a chondroitin sulphate- and keratan sulphate-containing larger proteoglycan during early development (defined immunologically and chemically) that progressively replaces a fetal-specific keratan sulphate-deficient proteoglycan that disappears by about 35 years, as reported previously [13]. The question naturally arises as to whether the larger adult population represents a modification of the large fetal proteoglycan of similar size, which we described recently [13]. This is thought to be unlikely, particularly since we have now shown that, in addition to the immunological differences, the amino acid compositions are not identical, particularly those of serine and phenylalanine. Hence the changes in the proteoglycan after birth, during maturation and in the adult would appear to represent the synthesis of a large separate 'adult-specific' proteoglycan, which may or may not represent a distinct gene product from that expressed in the fetus. The resolution of this problem awaits determination of the sequence data for the core proteins by cDNA analysis. There are, of course, considerable alterations in glycosylation as a result of post-translational modifications of the core protein. Our present studies also reveal that changes in the keratan sulphate component of the larger proteoglycan populations occur early in life and at after 23 years of age. Thus even the larger molecules continue to undergo changes with age. The larger chondroitin sulphate-rich proteoglycan probably represents the 'intact' adult-specific proteoglycan, and should perhaps be the standard with which comparisons are made between intact (native) proteoglycans in both biochemical and immunological assays, just as we have compared it with the single fetal proteoglycan.

It could be argued that the progressive appearance of the smaller aggregating keratan sulphate-rich adult proteoglycan indicates the appearance of a third gene product or a degradation product of the larger adult proteoglycan. There is reason to believe that the latter may be the more likely explanation, since the keratan sulphate-rich region is closest to the hyaluronic acid-binding region in bovine proteoglycan [14], and human proteoglycan probably has a similar structure. The production of an aggregating species enriched in keratan sulphate would occur if cleavage of the proteoglycan were in that part of the molecule more remote from the hyaluronic acid-binding region. The higher protein content of the smaller proteoglycan, its comparable aggregatability and decreased content of serine (attachment site for chondroitin sulphate) would favour this possibility.

During aging we have also noted other changes indicative of degradation of aggregating proteoglycans, namely the progressive accumulation of the hyaluronic acid-binding region essentially free of the keratan sulphate-rich region and the rest of the proteoglycan monomer [15], and the progressive partial cleavage of the link proteins [24], molecules that stabilize the binding of the proteoglycans to hyaluronic acid in aggregates. These observations together indicate that, as human cartilage matures and ages, changes occur that are suggestive of a progressive degradation of proteoglycans and their aggregates. These are detectable because of the retention of proteoglycans and hyaluronic acid-binding region that can still bind to hyaluronic acid. Thus the small keratan sulphate-rich proteoglycan may represent an intermediate in the degradation of the larger proteoglycan to the hyaluronic acid-binding region.

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