Proteoglycans of the human intervertebral disc

Electrophoretic heterogeneity of the aggregating proteoglycans of the nucleus pulposus

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Nuclei pulposi were dissected from lumbar discs of radiologically normal human spines of cadavers aged 17, 20 and 21 years. Proteoglycans were extracted with 4 M guanidine hydrochloride (dissociative conditions) with proteinase inhibitors and isolated as A1 fractions by associative density-gradient centrifugation. Aggregating and non-aggregating proteoglycans were separated by Sepharose 2B chromatography. Both aggregating and non-aggregating proteoglycans contained a keratan sulphate-rich region as isolated by chondroitinase/trypsin/chymotrypsin digestion and Sepharose CL-6B chromatography. Agarose/acrylamide-gel electrophoresis of individual fractions of a Bio-Gel A-50m dissociative-column separation of the aggregating proteoglycans revealed two, well-separated bands: S and F, the slower and faster migrating bands respectively. The non-aggregating proteoglycan fractions were eluted under associative conditions (0.5 M-sodium acetate, pH 6.8) and migrated as a single band in the electrophoretic system. The gel-electrophoretic heterogeneity of the aggregating proteoglycans was still evident after hydroxylamine fragmentation and removal of the hyaluronate-binding portion of the molecule. Dissociative density-gradient centrifugation of the aggregating proteoglycans partially separated the Band-S proteoglycans from the Band-F population. Subsequent dissociative chromatography of the high-buoyant-density Band F proteoglycans permitted discrimination of this band into two gel-electrophoresis-distinguishable populations (Bands F-1 and F-2). Enzyme-linked immunosorbent assays with a monoclonal antibody that recognized keratan sulphate demonstrated that the D1 fraction containing the Band F-1 proteoglycans was enriched in keratan sulphate compared with the total aggregating or non-aggregating pool of proteoglycans. The proteoglycans of young adult nucleus pulposus could then be ascribed to one of four structurally and/or electrophoretically distinct populations: (1) the non-aggregating population, which comprised about 70 % of the total extractable proteoglycans; (2) the aggregating pool, comprising: (a) Band F-1 proteoglycans, which had a relatively large hydrodynamic size, uronate/protein weight ratio, were enriched in keratan sulphate and had a high buoyant density; (b) Band S proteoglycans, which migrated slower in agarose/acrylamide gels, had a smaller hydrodynamic size, lower buoyant density and a lower uronate/protein ratio than the Band F-1 population; (c) Band F-2 proteoglycans, which were lower in buoyant density, smaller in hydrodynamic size and slightly faster in electrophoretic mobility than the Band F-1 proteoglycans.

INTRODUCTION

The proteoglycans of the nucleus pulposus of the intervertebral disc, like those of hyaline cartilage, contain glycosaminoglycan chains of chondroitin sulphate and keratan sulphate covalently attached to a polypeptide (Davidson & Woodhall, 1959; Lowther & Baxter, 1966; Heinegård & Gardell, 1967; Emes & Pearce, 1975). The keratan sulphate chains are clustered in a specific region (the keratan sulphate-rich region) or regions of the protein core of proteoglycans of young hyaline cartilage (Heinegård & Axelsson, 1977) and immature nucleus pulposus (Stevens *et al.*, 1979a; Heinegård *et al.*, 1979). Cartilage and disc proteoglycans, however, display notable structural differences (reviewed by McDevitt, 1981, 1988). Although over 80 % of the proteoglycans of cartilage can form aggregates with hyaluronic acid (Muir & Hardingham, 1975), only about 30 % of the nucleus

pulposus proteoglycans exhibit this property (Adams & Muir, 1976; Stevens *et al.*, 1979b; McDevitt *et al.*, 1981; Tengblad *et al.*, 1984).

Cartilage proteoglycans are markedly polydisperse with respect to M_r and chemical composition (Muir & Hardingham, 1975). Agarose/acrylamide-gel-electrophoretic separations, however, have demonstrated that this apparent polydispersity is due, in part, to the contribution of at least two structurally distinct populations (McDevitt & Muir, 1971; Roughley & Mason, 1976; Stanescu *et al.*, 1977; Bayliss & Ali, 1978; Roughley & White, 1980, 1983; Heinegård *et al.*, 1985). Immunological (Wieslander & Heinegård, 1981; Brennan *et al.*, 1983; Heinegård *et al.*, 1985) and biosynthetic studies (Triphaus *et al.*, 1980) support the concept of proteoglycan heterogeneity.

Aging involves the selective loss (Inerot *et al.*, 1978) and osteoarthritis the selective accumulation (Cox *et al.*,

Abbreviation used: ELISA, enzyme-linked immunosorbent assay.

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1985) in cartilage of proteoglycans that have a high content of the chondroitin sulphate-rich region. The relative proportion of the slower band in agarose/acrylamide-gel electrophoresis decreases with age (Inerot & Heinegård, 1983) and increases in osteoarthritis (Adams *et al.*, 1987). The proteoglycans in the slower band have relatively more chondroitin sulphate and less keratan sulphate than those in the faster band (Heinegård *et al.*, 1985). Isolation and structural characterization of the individual proteoglycan subpopulations is therefore important in elucidating the molecular events in aging and osteoarthritis.

Early work by Pedrini (1969) indicated that human nucleus pulposus proteoglycans were heterogeneous by cellulose acetate electrophoresis. Stanescu *et al.* (1980) and Cole *et al.* (1985) demonstrated two poorly resolved bands in the agarose/acrylamide gels in the total population of proteoglycans from baboon and dog nucleus pulposus respectively. It is not clear from these studies whether the two bands represented aggregating and non-aggregating proteoglycans respectively, or, alternatively, sub-sets of either one population.

Our study was undertaken: (a) to explore further the structural differences between aggregating and non-aggregating proteoglycans in human nuclei pulposi; (b) to establish whether either of these two populations was electrophoretically heterogeneous; (c) to identify the structural domains in proteoglycan molecules that confer heterogeneity on populations of these molecules.

EXPERIMENTAL

Materials

Guanidine hydrochloride (Absolute Grade) from Research Plus Inc. (Bayonne, NJ, U.S.A.) was used in the extraction procedures and dissociative density gradients. Guanidine hydrochloride (Practical Grade) from Sigma Chemical Co. (St. Louis, MO, U.S.A.) was purified with activated charcoal and used for dissociative column chromatography. The proteinase inhibitors (EDTA, benzamidine hydrochloride, *N*-ethylmaleimide, phenylmethanesulphonyl fluoride), hydroxylamine (Grade 1), Mes and DPCC (diphenylcarbonyl chloride)-treated trypsin were purchased from Sigma. α -Chymotrypsin and chondroitinase ABC were from Miles (Naperville, IL, U.S.A.); CsCl (density-gradient grade) and hyaluronic acid (human umbilical cord) were purchased from Gallard-Schlesinger (Carle Place, NY, U.S.A.). Sodium acetate and disodium EDTA were from Baker Chemical Co. (Brick Town, NJ, U.S.A.). Sepharose molecular-sieve gels were from Pharmacia Biotechnology Products (Piscataway, NJ, U.S.A.), and Bio-Gel A-50m, acrylamide, bisacrylamide, ammonium persulphate and alkaline phosphatase-linked goat anti-mouse antibody were purchased from Bio-Rad (Rockville Centre, NY, U.S.A.). Sea-Kem HE (High Electroendosmosis) agarose was from FMC Corporation (Rockland, ME, U.S.A.); tetramethylethylenediamine and carbocyanin DBTC (Stains-All) were from Eastman Kodak Co. (Rochester, NY, U.S.A.). Folin reagent was purchased from Fisher Scientific (Springfield, NJ, U.S.A.). Falcon PRO-BIND micro-well plates were obtained from Becton Dickinson Labware, Lincoln Park, NJ, U.S.A. A monoclonal antibody (5-D-4; Caterson *et al.*, 1983) that recognized keratan sulphate (Mehmet *et al.*, 1986) was kindly given by Dr. Bruce

Caterson (West Virginia University Medical Center, Morgantown, WV, U.S.A.).

Isolation of proteoglycan A1 fractions

Human spines were obtained at autopsy, and the non-pathological specimens were selected on the basis of their roentgenographic appearance. Three radiologically normal spines from humans aged 17, 20 and 21 years were employed in this study. The lumbar discs (L1/L2–L4/L5) were removed and dissected free of unwanted tissue. The lumbar nuclei pulposi from a given spine were pooled together and minced with a scalpel on ice-cooled glass. The tissue was then suspended in cold 4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 5.8, containing the following proteinase inhibitors: 0.1 M-6-amino hexanoic acid, 0.01 M-EDTA, 0.005 M-benzamidine hydrochloride, pH 5.8, and 1 mM-*N*-ethylmaleimide; 1 mM-phenylmethanesulphonyl fluoride was added immediately before use from a 100 mM stock in propan-2-ol (Oegema *et al.*, 1975; Pearson & Mason, 1977; Cox *et al.*, 1985); 10 ml of this solution was added per g of nucleus pulposus, and the tissue was extracted by gentle rotation at 4 °C for 24 h. The extract was centrifuged at 2500 rev./min for 20 min in a Sorvall GLC-2B centrifuge, and the pellets were washed with the extraction buffer. The pooled supernatants were filtered through glass wool and dialysed at 4 °C against 7 vol. of 0.05 M-sodium acetate, pH 6.8, containing the proteinase inhibitors. The density was adjusted to 1.4 g/ml by the addition of solid CsCl and the material centrifuged in a Beckman Ti60 rotor and Sorvall ultracentrifuge at 100 000 g_{av} for 48 h at 12 °C. The tubes were then frozen in liquid N₂, the bottom $\frac{2}{3}$ by volume (A1 fraction; Heinegård, 1972) was sawed off, and the thawed fractions were filtered through clean glass-wool plugs. The fractions were dialysed at 4 °C against two changes of 0.2 M-sodium acetate, pH 6.8, containing the proteinase inhibitors, then against two changes of distilled water, and finally freeze-dried.

Separation of aggregating and non-aggregating proteoglycans

Samples of A1 fractions (75 mg dry wt.) were incubated with 2% weight of hyaluronic acid uronic acid per weight of proteoglycan uronic acid at a concentration of 7.5 mg of proteoglycan/ml in 0.5 M-sodium acetate, pH 6.8, at 4 °C for 24 h. Each sample was then eluted with 0.5 M-sodium acetate, pH 6.8, from a Sepharose 2B column (180 cm $\times$ 2.4 cm) at 13 ml/h at 4 °C. Fractions (10 ml) were collected and analysed for uronic acid and protein. The separated proteoglycan aggregate and non-aggregating proteoglycans were dialysed against water, freeze-dried and analysed by agarose/acrylamide-gel electrophoresis.

Preparation of proteoglycans of different hydrodynamic sizes

Aggregating proteoglycans were chromatographed under dissociative conditions that abolished hyaluronate-proteoglycan aggregation. Freeze-dried samples (1–5 mg) of proteoglycan aggregates were dissolved in 4 M-guanidine hydrochloride/0.5 M-sodium acetate, pH 5.8, and applied to a column (100 cm $\times$ 0.9 cm) of Bio-Gel A-50m. The column was eluted with 4 M-guanidine hydrochloride/0.5 M-sodium acetate, pH 5.8 (dissociative conditions), at 1.05 ml/h at room temperature.

Fractions (1.0 ml) were collected and dialysed against water at 4 °C by the micro-procedure of Lau & Fujitaki (1981). Non-aggregating proteoglycans (1–5 mg dry wt.) were dissolved in 0.5 M-sodium acetate, pH 6.8, and chromatographed as described for the proteoglycan aggregates, except that the column was eluted with 0.5 M-sodium acetate, pH 5.8 (associative conditions). Dialysed fractions from both separations were monitored for uronic acid and protein and analysed by agarose/acrylamide-gel electrophoresis.

Hydroxylamine cleavage

A1 fractions of aggregating and non-aggregating proteoglycans (10 mg/ml) were treated with 1 M-hydroxylamine in 0.05 M-Mes, pH 6.5, for 120 h at 25 °C (Heinegård & Axelsson, 1977). The samples were dialysed against 0.05 M-Mes, pH 6.5, followed by 0.5 M-sodium acetate, pH 6.8. The samples were then applied to a Bio-Gel A-50m column (95 cm × 1.0 cm) and eluted at 1.5 ml/h in 0.5 M-sodium acetate, pH 6.8 at 4 °C. Fractions (1.0 ml) were monitored for uronic acid and protein and selected fractions were analysed by agarose/acrylamide-gel electrophoresis.

Chondroitinase/trypsin/chymotrypsin digestions

Keratan sulphate-rich fragments of aggregating and non-aggregating proteoglycans were prepared by the method of Heinegård & Axelsson (1977). Samples (20 mg of proteoglycan/ml of 0.1 M-Tris/0.1 M-sodium acetate, pH 7.3), were digested with chondroitinase ABC (0.01 nominal unit/mg of proteoglycan) at 37 °C for 5 h. DPCC-treated trypsin (2 µg/mg of proteoglycan) in the same buffer was added and incubated for an additional 10 h at 37 °C. This was immediately followed by addition of α -chymotrypsin (2 µg/mg of proteoglycan), and the incubation continued for a further 10 h at 37 °C. Samples were then stored at –80 °C and then chromatographed in a Sepharose CL-6B column (95 cm × 1.0 cm) in 0.5 M-sodium acetate, pH 6.8, at 5 ml/h. The 1 ml fractions were monitored for protein, uronic acid and hexose, and selected fractions were analysed by agarose/acrylamide-gel electrophoresis.

Dissociative equilibrium-density-gradient centrifugation

Freeze-dried samples of A1 fractions (5–10 mg) of aggregating and non-aggregating proteoglycans were mixed with 4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 5.8, and the proteinase inhibitors and solid CsCl added to yield a final density of 1.35 g/ml. Ultracentrifugation was performed in 12 ml-capacity tubes in an IEC swing-out rotor (488) and model B-60 ultracentrifuge at 100 000 g_{av} for 72 h at 20 °C. Fractions (12, of 1.0 ml) were aspirated from the top of each gradient with a Buchler Auto Densi-Flow instrument and the density of each was determined. Each fraction was dialysed at 4 °C by the micro-technique of Lau & Fujitaki (1981) against two changes of 0.2 M-sodium acetate, pH 6.8, and then against two changes of distilled water. All fractions were monitored for protein and uronic acid and then analysed by agarose/acrylamide-gel electrophoresis.

Agarose/acrylamide-gel electrophoresis

Samples were analysed by large-pore agarose (0.6 %)/acrylamide (1.2 %)-gel electrophoresis as described by McDevitt & Muir (1971). Sea-Kem HE agarose (Jahnke,

1983) was substituted for the BDH agarose previously employed. The gels were stained overnight with 0.005 % carbocyanin DBTC (Stains-All) in cold 50 % (v/v) formamide and destained in the dark in distilled water at 4 °C (Dahlberg *et al.*, 1969). A systematic pilot investigation established that proteins stain red, hyaluronic acid stains turquoise (λ_{max} 645 nm), the chondroitin sulphates stain purple (λ_{max} 555 nm for chondroitin 6-sulphate), and keratan sulphate stains blue (λ_{max} 550 and 580 nm) with carbocyanin DBTC (C. A. McDevitt, M. R. Jahnke & R. Torelli, unpublished work). The unconjugated dye had λ_{max} 510 nm. Two samples were run in the same tube gel by inserting a Teflon spacer 1–2 mm into the top of the gel.

Analytical procedures

Uronic acid and neutral sugars were measured by Heinegård's (1973) automated procedures. Protein was measured by the automated version of the Folin assay (Oosta *et al.*, 1978). Keratan sulphate was assayed by the inhibition ELISA technique of Rennard *et al.* (1980), by using the 5-D-4 monoclonal antibody (Caterson *et al.*, 1983). Micro-well plates were coated with human (60 years old) articular-cartilage proteoglycan (A1 fraction) that was digested with chondroitinase ABC. The coating proteoglycan was also used as a standard in the inhibition mixtures. Disc proteoglycans were digested with chondroitinase ABC for 4 h before assay. Alkaline phosphatase-conjugated goat anti-mouse antibody (dilution 1:750) was used to detect the quantity of primary antibody bound to the plates. Results were expressed in terms of mg equivalents of undigested human articular-cartilage proteoglycan/mg dry wt. of undigested disc proteoglycan. The keratan sulphate concentrations in the chromatographic fractions of the Bio-Gel A-50m separation of the hydroxylamine digests were assayed by radioimmunoassay (Caterson *et al.*, 1985) by using the 5-D-4 monoclonal antibody (Caterson *et al.*, 1983). Densities were determined in a 50 µl capillary pipette.

RESULTS

Experiments were conducted concurrently on proteoglycans from each of the three spines selected. Our results confirmed that the structures of the proteoglycans from the 17-, 20- and 21-year-old spines were very similar (Jahnke, 1983) and, for simplicity, in any one experiment we report the results obtained from one of the three tissue specimens.

The yield of proteoglycan from the nucleus pulposus was approx. 170 mg dry wt. of A1 fraction per disc. Uronic acid comprised about 15 % of the dry weight of the A1 preparations. Agarose/acrylamide-gel electrophoresis of the A1 preparations revealed a series of closely spaced but poorly resolved bands (Fig. 1b, gel A1) similar to that observed by Stanescu *et al.* (1980) with A1 fractions from baboon nucleus pulposus. We therefore attempted to separate the proteoglycans into populations that would migrate as clearly resolved bands in the electrophoretic system.

The A1 fraction was eluted from the preparative Sepharose 2B column (Fig. 1a) as aggregated proteoglycans in the void volume that were rich in protein, and as a second broad, retarded, non-aggregated peak that was rich in uronic acid. The retarded material comprised about two-thirds of the dry weight of the A1

Fig. 1. Agarose/acrylamide-gel electrophoresis of human nucleus pulposus proteoglycans fractionated by column chromatography

(a) Sepharose 2B gel chromatography of an A1 fraction after incubation with exogenous hyaluronic acid. The column was eluted with 0.5 M-sodium acetate, pH 6.8, and fractions were pooled as indicated by the bars. (b) Agarose/acrylamide-gel electrophoresis of unfractionated A1 fraction (left-hand gel), Pools 1 and 2 from (a) (centre gels) and hyaluronic acid (HA, right-hand gel). (c) Bio-Gel A-50m chromatography of aggregating proteoglycans [Pool 1 from (a)]. The column was eluted with 4 M-guanidine hydrochloride/0.5 M-sodium acetate, pH 5.8. (d) Selected column fractions from (c), as indicated by arrows (K_d values 0.00, 0.11, 0.22, 0.32, 0.42, 0.53, 0.64 and 0.76 respectively), were analysed by agarose/acrylamide-gel electrophoresis. (e) Bio-Gel A-50m chromatography of non-aggregating proteoglycans [Pool 2 in (a)]. The column was eluted with 0.5 M-sodium acetate, pH 6.8. (f) Four column fractions from (e), as indicated by arrows (K_d values 0.20, 0.42, 0.62 and 0.84 respectively), were analysed by agarose/acrylamide-gel electrophoresis. —, Uronic acid; ----, protein. See the text for details.

fraction. When the void-volume (Pool 1, Fig. 1a) and retarded (Pool 2, Fig. 1a) peaks were dialysed, freeze-dried and analysed by electrophoresis, each migrated on gels as a series of poorly resolved bands (Fig. 1b, gels 1 and 2). The diffuse staining towards the bottom of the gels is an artifact sometimes introduced in the casting of the gels, and was confined to the surface of the gel.

The relative hydrodynamic sizes of the excluded and retarded peaks were determined by chromatography on a Bio-Gel A-50m column under dissociative (Fig. 1c) and associative (Fig. 1e) conditions respectively. The void-volume material from the associative column was eluted under dissociative conditions as a single, very broad, retarded peak with K_d about 0.4. The retarded fractions of the non-aggregating proteoglycans were slightly less polydisperse, but of smaller size, with K_d about 0.6. An identical column profile with K_d 0.6 was obtained for the non-aggregating proteoglycans when

they were chromatographed under dissociative conditions (results not shown). The chromatographic behaviour of the void-volume and retarded peaks from the associative chromatographic separation was therefore consistent with them containing hyaluronate-proteoglycan aggregates and non-aggregating proteoglycans respectively. Selected fractions from the chromatographic separations in Figs. 1(c) and 1(e) were analysed by agarose/acrylamide-gel electrophoresis (Figs. 1d and 1f). The aggregating proteoglycans, except those at V_0 and near V_1 of the column, migrated as two bands (Fig. 1d); these will be referred to as Band S (slow-mobility band) and Band F (faster-mobility band) respectively. The electrophoretic mobilities of both bands increased with increasing K_d . The slower of the two bands (Band S) was fairly sharp throughout the column (gels 2–7, Fig. 1d). The faster band (Band F), in contrast, broadened in fractions after a K_d value of about

Fig. 2. Bio-Gel A-50m chromatography and agarose/acrylamide-gel electrophoresis of hydroxylamine-treated aggregating and non-aggregating proteoglycans

Hydroxylamine digests of aggregating (a) and non-aggregating (b) proteoglycans from the nucleus pulposus of a 20-year-old spine were chromatographed on a Bio-Gel A-50m column and eluted with 0.5 M-sodium acetate, pH 6.8. Arrows indicate column fractions analysed by agarose/acrylamide-gel electrophoresis. See Figs. 1(d) and 1(f) for electrophoresis of untreated proteoglycans. —, Uronic acid; ----, protein.

0.5 (gels 6 and 7, Fig. 1d) and appeared to be composed of two closely spaced bands. The material at the void volume (gel 1, Fig. 1d) migrated as a single band that was tentatively identified as hyaluronic acid on the basis of its low mobility and the characteristic turquoise colour with which it stained with carbocyanin DBTC. Only the faster broad proteoglycan band (Band F) was evident near the V_t of the column. All column fractions

of the non-aggregating proteoglycan separation, however, migrated as a single, sharp band, except for fractions near the V_t of the column (Fig. 1f). In summary, the aggregating proteoglycans were composed of at least two electrophoretically distinct populations, each of which was polydisperse in hydrodynamic size. The non-aggregating proteoglycans, in contrast, were composed of an apparently electrophoretically homogeneous population that was also polydisperse in size.

We used the hydroxylamine-cleavage procedure to explore the possibility that the structural feature that confers electrophoretic heterogeneity on the aggregating proteoglycans might reside in a small sub-region or domain in the molecule. Hydroxylamine has been shown to cleave the protein core of bovine tracheal (Heinegård & Axelsson, 1977) and bovine (Sweet *et al.*, 1979) and dog (Cox *et al.*, 1985) articular-cartilage proteoglycan aggregates between the keratan sulphate-rich and chondroitin sulphate-rich regions of the proteoglycan molecule. The complex composed of hyaluronic acid, the hyaluronic acid-binding site and the keratan sulphate-rich region is eluted in the void volume, whereas the chondroitin sulphate-rich region is retarded as a uronic acid-rich peak in Sepharose 2B chromatograms of the hydroxylamine digests (Heinegård & Axelsson, 1977; Sweet *et al.*, 1979; Cox *et al.*, 1985). The hydroxylamine digest of aggregating disc proteoglycans was eluted as two peaks from a Bio-Gel A-50m column (Fig. 2a): (a) a void-volume peak that was rich in protein but not in carbazole-positive material, unlike the isolated keratan sulphate-rich region (see below); (b) a broad, uronic acid-rich, retarded peak. Aggregating proteoglycans incubated under identical conditions in Mes buffer without hydroxylamine were eluted as a single void-volume peak (results not shown; Jahnke, 1983). Gel-electrophoretic analysis of the void-volume peak of the hydroxylamine digest (Fig. 2a) revealed a turquoise band that migrated only a few millimetres into the gel, similar to hyaluronate standards, and a very faint pinkish band, similar to that obtained with proteins. The second band probably corresponds to the protein-rich hyaluronic acid-binding fragments of the void-volume peak. We could not detect any blue- or purple-staining band, characteristic of the keratan sulphate-rich region (M. R. Jahnke & C. A. McDevitt, unpublished work), in the electrophoretic analysis of the void-volume material of the column. Radioimmunoassays for keratan sulphate confirmed this interpretation: the broad, retarded, uronic acid peak contained 97.6 % of the keratan sulphate that was eluted from the column. The hydroxylamine therefore cleaved the protein-rich hyaluronate-binding region from the remaining glycosaminoglycan attachment region of the molecule. Fractions from the retarded chromatographic peak migrated as two sharp bands in electrophoresis gels (Fig. 2a), the proportions of which changed according to the K_d value of the fraction analysed. The structural differences that conferred electrophoretic heterogeneity on the aggregating proteoglycans were therefore located in the glycosaminoglycan-attachment portion of the molecule. The chromatographic elution profile of the hydroxylamine digest of the non-aggregating proteoglycans (Fig. 2b), in contrast, was unchanged compared with that of the starting material (Fig. 1e), suggesting that these proteoglycans were not cleaved in the incubation. Moreover, individual column fractions still migrated as a single band in the electrophoretic gels (Fig.

Fig. 3. Sepharose CL-6B chromatography of aggregating and non-aggregating nucleus pulposus proteoglycans after digestion with chondroitinase ABC, trypsin and chymotrypsin

Samples of aggregating (a) and non-aggregating (b) nucleus pulposus proteoglycans were digested sequentially with chondroitinase ABC, trypsin and chymotrypsin and eluted with 0.5 M-sodium acetate, pH 6.8, from a column of Sepharose CL-6B. —, Uronic acid; ----, protein; ····, neutral sugar. See the text for details.

2b). In summary, the main glycosaminoglycan attachment region of the aggregating proteoglycans was still electrophoretically heterogeneous after removal of the hyaluronic acid-binding portion of the molecule with hydroxylamine.

Chondroitinase/trypsin/chymotrypsin digests of aggregating (Fig. 3a) and non-aggregating (Fig. 3b) proteoglycans were eluted from Sepharose CL-6B as a broad, hexose-rich, retarded peak analogous to the keratan sulphate-rich region found by Heinegård & Axelsson (1977) and Stevens *et al.* (1979a), and a second large peak comprising small degradation products, including disaccharides, at the V_t of the column. We did not, however, note a bimodal distribution in the keratan sulphate-rich fragments, as Heinegård *et al.* (1979) reported with fragments generated from young bovine nucleus pulposus proteoglycans. The keratan-sulphate-rich region of the aggregating proteoglycans was slightly

Fig. 4. CsCl-density-gradient centrifugation of aggregating and non-aggregating nucleus pulposus proteoglycans

Aggregating and non-aggregating proteoglycan samples were subjected to CsCl-density-gradient centrifugation under dissociative conditions (4 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 5.8, with proteinase inhibitors). (a) Distribution of aggregating proteoglycans from a 17-year-old spine after CsCl-density-gradient centrifugation. The gradient fractions are numbered from highest to lowest buoyant densities, from right to left. Alternate fractions D1, D3, D5, D7, D9 and D11 were analysed by composite agarose/acrylamide-gel electrophoresis. Each gel is shown above the appropriate fraction. (b) The distribution of non-aggregating proteoglycans from the same donor after CsCl-density-gradient centrifugation. The gradient fractions are numbered as in (a). Alternate gradient fractions were analysed by composite agarose/acrylamide-gel electrophoresis and are shown above the appropriate fractions. —, Uronic acid; ----, protein; ····, density. Additional experimental details are given in the Experimental section.

larger in hydrodynamic size and had a markedly higher protein/hexose weight ratio than did that of the non-aggregating proteoglycans (Figs. 3*a* and 3*b*). When the keratan sulphate-rich region from the aggregating proteoglycan was pooled, concentrated and rechromatographed on a column of Sephacryl S-200 in 4 M-guanidine hydrochloride, the protein and uronic acid co-eluted (results not shown). The enriched protein content of the keratan sulphate-rich region of the aggregating proteoglycan could not therefore be attributed to non-covalently linked protein. Individual fractions from the keratan sulphate-rich-region peaks (results now shown) of the aggregating and non-aggregating proteoglycans migrated similarly in agarose/acrylamide electrophoretic gels as a very broad band that had a higher mobility than that of the intact proteoglycans. The triple enzyme digestion therefore generated a product that was particularly polydisperse, but not apparently heterogeneous, as assessed by gel electrophoresis.

Dissociative density-gradient centrifugation was used in an attempt to separate the electrophoretically heterogeneous populations of the aggregating proteoglycans (Fig. 4*a*). A systematic investigation (Jahnke, 1983) established that a starting density of 1.35 g/ml gave optimum separations for analysis by electrophoresis. The D1 fraction, the only fraction that was richer in uronic acid than protein, contained 36% of the dry weight and 16% of the uronic acid of the starting material. The bands detected in agarose/acrylamide gels by carbocyanin DBTC (Fig. 4*a*) were broader than those observed in individual column fractions (Fig. 1*d*), presumably because the proteoglycans in each gradient fraction were polydisperse in size. Three major electrophoretically distinguishable bands were evident in the gradient fractions. The bottom fraction (D1) migrated as a single broad band that appeared similar in relative mobility to Band F of Fig. 1*d*. Fractions of lower buoyant density (D2–D7) displayed three bands: a broad slow-mobility band and two closely spaced bands of higher mobility, the slower of which appeared similar in mobility to the single band of the D1 fraction. The two closely spaced faster bands were unambiguously distinguishable in the

original gel before photography. Fractions of lowest buoyant density (D9–D11) displayed a variety of bands apparently unique to these fractions. However, as only 3% of the total uronic acid in the gradient was recovered in these low-buoyant density fractions, these proteoglycans must represent a small proportion of the total aggregating population.

The distribution of uronic acid and protein in the dissociative density-gradient separation of non-aggregating proteoglycans is shown in Fig. 4*b*. The distribution was similar to that of aggregating proteoglycans (Fig. 4*a*), except that the uronic acid/protein weight ratio of the non-aggregating proteoglycans was higher than that of the aggregating proteoglycans of identical buoyant density.

Electrophoresis of the fractions of highest and intermediate buoyant density (D1–D7), in contrast with the aggregating proteoglycans, revealed a single major band with a very faint minor band of higher mobility (Fig. 4*b*). The upper fraction of the gradient (D11) was similar to the corresponding fraction in the aggregating proteoglycan separation, in that it contained minor bands of unique electrophoretic mobility.

Table 1 summarizes the keratan sulphate contents, as assessed by ELISA assays, of the non-aggregating proteoglycans and the aggregating proteoglycans before and after dissociative density-gradient purification. The total aggregating pool of proteoglycans had slightly higher keratan sulphate contents than the total non-aggregating pool. Dissociative density-gradient subfractionation resulted in a marked increase in the keratan sulphate contents of the high-buoyant-density fractions (D1 and D2) at the expense of the lower density fractions (D3–D9).

The relationship of the three major bands evident in the dissociative density gradient to Band S and Band F of Fig. 1*d* was explored by comparing the electrophoretic behaviour of identical column fractions of the total aggregating proteoglycans and those of the D1 fractions. The total aggregating population of proteoglycans (i.e. Peak 1 from Fig. 1*a*) was slightly smaller in hydrodynamic size (Fig. 5) on a dissociative Bio-Gel

Table 1. Keratan sulphate contents of proteoglycan fractions, assayed by ELISA

An A1 proteoglycan fraction of 60-year-old normal human articular cartilage was digested with chondroitinase ABC and used both to coat the micro-well plates and as a standard in the inhibition assay. A monoclonal antibody (5-D-4) that recognizes keratan sulphate was employed. Results were expressed in terms of mg equivalents of undigested human articular-cartilage proteoglycan (dry weight). The ratio of the dry weights of the non-aggregating to the aggregating pool was 2:1. About 60% of the keratan sulphate of the total pool of extracted proteoglycans is therefore in the non-aggregated pool.

Fraction	Description of fraction	Keratan sulphate content (mg equivalents of human articular-cartilage proteoglycan/mg dry wt. of disc proteoglycan)
Fraction 1, Fig. 1 <i>a</i>)	Total aggregating pool	0.470
Fraction 2, Fig. 1 <i>c</i>)	Total non-aggregating pool	0.353
D1 fraction, Fig. 4 <i>a</i>)	Mainly Band F-1	0.771
D2 fraction, Fig. 4 <i>a</i>)	F-1, F-2; some S	0.734
D3–D9 fractions, Fig. 4 <i>a</i>)	Small quantities of F1; F2 and S	0.139

Fig. 5. Agarose/acrylamide-gel electrophoresis of Bio-Gel A-50m column fractions of aggregating proteoglycans before and after dissociative density-gradient centrifugation

Bio-Gel A-50m chromatograms of the aggregating proteoglycan population (right-hand chromatographic profile with arrows from gels 1a and 2a) and the high-buoyant-density subfraction from the dissociative density gradient (left-hand chromatographic profile with arrows from gels 1b and 2b). Loads of 3 mg dry wt. of aggregating proteoglycan and 2 mg dry wt. of high-buoyant-density fraction were applied to the gel. Selected column fractions with K_d values of 0.3 and 0.4 from either the total aggregating sample or the D1 subfraction were analysed by composite agarose/acrylamide-gel electrophoresis, shown above the column profiles. Gels: 1a, total aggregating proteoglycan sample (K_d 0.3); 1b, D1 fraction (K_d 0.3); 1a/1b, split gel of both samples; 2a, total aggregating proteoglycan sample (K_d 0.4); 2b, D1 subfraction, (K_d 0.4); 2a/2b, split gel of both samples. —, Uronic acid. See the text for details.

A-50m column than the subset of that population of high buoyant density (D1) from the dissociative density-gradient centrifugation. Two fractions of different hydrodynamic size from the two column separations, fractions 49 and 59, were compared by agarose/acrylamide-gel electrophoresis (Fig. 5). Although fraction 49 of the total

aggregating population migrated as two bands (Bands S and F) as before (gel 1a, Fig. 5), the same column fraction of the material that was purified as high-buoyant-density proteoglycans in the dissociative-gradient centrifugation migrated as a relatively sharp single band (gel 1b, Fig. 5). Gel 1a/1b (Fig. 5) had both specimens loaded on the same gel with a spacer in between. The high-buoyant-density proteoglycans (hereafter referred to as Band F-1) had a migration similar to the trailing edge of the faster of the two bands of the unfractionated material. It is very clear from these gels that Band S of the total aggregating population was entirely absent from the high-buoyant-density fraction. Gels 2a, 2b and 2a/2b (Fig. 5) depict the electrophoretic bands obtained from a fraction (tube 59) of slightly smaller hydrodynamic size. The density-gradient-purified material (gel 2b) migrated as two bands (identified as F-1 and F-2), the faster of which (F-2) corresponded in mobility to the leading edge of the faster band of the total aggregating pool (gel 2a). Thus the high-buoyant-density fraction of the aggregating proteoglycans was composed predominantly of Band F-1 proteoglycans, with a small proportion of the smaller-size Band F-2 proteoglycans. Chromatography on Sepharose CL-6B of trypsin/chymotrypsin/chondroitinase ABC digests of the D1, D2 and mixed upper fractions of the dissociative gradient confirmed that the keratan sulphate-rich region was present throughout the gradient (results not shown; Jahnke, 1983).

In summary, normal human nucleus pulposus contains four structurally distinct populations of proteoglycans, three of which have functional hyaluronic acid-binding sites. Band F-1 proteoglycans constitute the major aggregating population, are relatively rich in uronic acid, and have a large hydrodynamic size and high buoyant density. The Band S population contains molecules of different sizes that presumably have similar charge-to-mass ratios. The proteoglycans of Band S have a slower electrophoretic mobility, are more polydisperse in hydrodynamic size, lower in uronic acid and higher in protein content than the Band F-1 population. Band F-2 proteoglycans, the least abundant of the aggregating subpopulations, are very small in size and migrate in electrophoresis slightly faster than the Band F-1 proteoglycans.

DISCUSSION

Our study with radiologically normal young-adult nucleus pulposus proteoglycans has demonstrated that: (1) aggregating proteoglycans contain three electrophoretically distinct populations; (2) electrophoretic heterogeneity is a property of the glycosaminoglycan-attachment region of aggregating proteoglycans, as removal of the hyaluronic acid-binding region by hydroxylamine produces carbohydrate-rich fragments that still display distinguishable bands; (3) chondroitinase ABC/trypsin/chymotrypsin digestion eliminated electrophoretic heterogeneity; (4) non-aggregating proteoglycans do not exhibit electrophoretic heterogeneity as the aggregating proteoglycans do, and lack a hydroxylamine-sensitive protein domain at the extremity of the core protein; (5) all proteoglycan populations, except possibly the Band F-2 proteoglycans, contain a keratan sulphate-rich region.

Previous investigations have demonstrated that the

total extractable proteoglycans of the human nucleus pulposus are markedly more polydisperse in size than are those of young animal hyaline cartilage (Rosenberg *et al.*, 1982). Our results clarify this anomaly of disc proteoglycans: the apparent polydispersity is due, in part, to the existence of electrophoretically, and therefore structurally, distinct populations that overlap in hydrodynamic size and buoyant density. The experimental approach that we adopted clearly eliminates the possibility that the different bands in the electrophoretic gels were merely extremes of a homogeneous polydisperse continuum.

The three major distinct aggregating proteoglycans share some properties in common. They all possess a functional hyaluronic acid-binding site. Our study eliminates the possibility of a major sub-population in Bands F-1 and S that is predominantly a chondroitin sulphate proteoglycan with minimal quantities of keratan sulphate. The Band F-1 proteoglycans are distinctive in being the only aggregating population containing more uronic acid than protein.

Most of the non-aggregating proteoglycans of the young adult discs in our study migrated as a single band in electrophoretic gels. The structural property that confers electrophoretic heterogeneity on the aggregating proteoglycans, including those that have had their hyaluronic acid-binding site removed by hydroxylamine, is not present in the non-aggregating proteoglycans. DiFabio *et al.* (1987), in contrast, observed three electrophoretic bands in the non-aggregating proteoglycans of older spines. It should be emphasized that the spines investigated by DiFabio *et al.* (1987) were from individuals aged 64, 67 and 72 years, in contrast with the 17–21-year-old group in our study. Aging of the disc therefore appears to be associated with altered catabolic and/or synthetic mechanisms that give rise to a different set of non-aggregating proteoglycans. An increased degradation of proteoglycans has been noted in the aging of the dog disc (Cole *et al.*, 1986) and apparently precedes degeneration of the human nucleus pulposus (Pearce *et al.*, 1987).

It is noteworthy that the hydroxylamine cleaved the protein core of the aggregating disc proteoglycans between the hyaluronic acid-binding site and the glycosaminoglycan-attachment region of the molecule, as assessed by column chromatography and gel electrophoreses. The non-aggregating proteoglycans, in contrast, were unaffected in their column-chromatographic or gel-electrophoretic properties by hydroxylamine. This is the first direct evidence that the non-aggregating proteoglycans totally lack a binding site, whether functional or non-functional. Moreover, as hydroxylamine cleaves between the protein core of hyaline-cartilage proteoglycans between the keratan sulphate-rich and chondroitin sulphate-rich regions of the molecule (Heinegård & Axelsson, 1977; Sweet *et al.*, 1979; Cox *et al.*, 1985), possibly at asparaginyl-glycyl peptide bonds (Bornstein & Balian, 1970), our experiment suggests that the respective core proteins of the proteoglycans of the human disc and animal cartilage are structurally different in this region of the molecule.

The proteoglycans of the nucleus pulposus of the intervertebral disc undergo considerable extracellular processing that is different from that experienced by hyaline-cartilage proteoglycans (McDevitt *et al.*, 1981). Most of the newly synthesized proteoglycans in the dog

(McDevitt *et al.*, 1981) and human (Oegema *et al.*, 1979) nucleus pulposus are large molecules with functional hyaluronic acid-binding sites. With time in the extracellular matrix, however, these proteoglycans become progressively smaller and lose their capacity to aggregate with hyaluronic acid (McDevitt *et al.*, 1981). Thus proteinase cleavage of the binding site and core protein gives rise to the majority of the very large proportion of non-aggregating proteoglycans in the mature disc. This interpretation is consistent with the work of Bayliss *et al.* (1986), who found a topographical variation in the rate of proteoglycan synthesis throughout the disc, the lowest rates being in the centre of the nucleus pulposus. The Band S and Band F-2 aggregating proteoglycans may represent products of degradation *in vivo* of the larger Band F-1 population. Alternatively, their respective core proteins may have different amino acid sequences, and all or some of them may be converted in time into the non-aggregating pool by proteinase cleavage. Only amino acid or the coding mRNA nucleotide sequences of the protein cores of the different populations can unequivocally resolve these possibilities.

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