Proteoglycans of the Intervertebral Disc

HOMOLOGY OF STRUCTURE WITH LARYNGEAL PROTEOGLYCANS

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The structure of the proteoglycans from normal pig nucleus pulposus and relatively normal human annulus fibrosus and nucleus pulposus was investigated in detail and the results were compared with the current structural model of proteoglycans of hyaline cartilage. Like proteoglycans of cartilage, those of intervertebral disc contain keratan sulphate and chondroitin sulphate attached to a protein core; they are able to aggregate to hyaluronic acid; the protein core likewise has three regions, one lacking glycosaminoglycans, another rich in keratan sulphate and a third region rich in chondroitin sulphate. However, disc proteoglycans contain more keratan sulphate and protein and less chondroitin sulphate and are also considerably smaller than cartilage proteoglycans. In proteoglycans of human discs, these differences appeared to be due principally to a shorter region of the core protein bearing the chondroitin sulphate chains, whereas in proteoglycans of pig discs their smaller size and relatively low uronic acid content were due to shorter chondroitin sulphate chains. There were subtle differences between proteoglycans from the nucleus and annulus of human discs. In the latter a higher proportion of proteoglycans was capable of binding to hyaluronate.

Much of what is known about the structure of proteoglycans, in general, has come from investigations of those of bovine nasal (Hascall & Sajdera, 1969; Thyberg *et al.*, 1975; Heinegård & Axelsson, 1977) and pig laryngeal (Hardingham *et al.*, 1976) cartilage. From the accumulated data of these studies, a structural model of the hyaline-cartilage proteoglycans has been proposed (for review see Muir, 1978). With the introduction of efficient dissociative extraction procedures (Sajdera & Hascall, 1969) and their purification by equilibrium density-gradient centrifugation methods (Hascall & Sajdera, 1969), attention has been turned to proteoglycans in other connective tissues such as intervertebral discs, because sufficient yields may thus be obtained without the use of high-speed homogenization.

The intervertebral disc has been classified as being 'cartilage-like' (Walmsley, 1964) because of a number of similarities with cartilage. Type II collagen is the sole collagen of nucleus pulposus (Eyre & Muir, 1975a) and hyaline cartilage (Eyre & Muir, 1975b). Moreover, there are similarities in the structure of the proteoglycans of each tissue (Hascall & Sajdera, 1970; Emes & Pearce, 1975). Both contain keratan sulphate and chondroitin sulphate attached to a protein core (Pearce & Grimmer, 1976) and they likewise interact with hyaluronic acid to form aggregates (Adams & Muir, 1976; Emes & Pearce,

1975). However, there are subtle differences between the proteoglycans of cartilage and intervertebral discs, such as lower susceptibility to papain and to Pronase (Hopwood & Robinson, 1974a) and in the relative proportions of keratan sulphate and chondroitin sulphate that they contain, suggesting that they may not be identical. Detailed knowledge of the structure of the disc proteoglycans and the extent of homology with hyaline-cartilage proteoglycans is needed before changes that may occur with aging (Gower & Pedrini, 1969) or disc degeneration (Mitchell *et al.*, 1961) can be interpreted. In the present work, proteoglycans from pig and human discs were studied in comparison with the structural model proposed for cartilage proteoglycans.

Materials

All reagents were of analytical grade except glucosamine hydrochloride, galactosamine hydrochloride, carbazole, acetylacetone and guanidinium chloride. The guanidinium chloride was purified with activated charcoal and Celite (Hopkin and Williams, Chadwell Heath, Essex, U.K.). Acetylacetone was redistilled at 138°C. Hyaluronic acid (from umbilical cord), 2×crystallized papain (EC 3.4.22.2) and 4×crystallized chymotrypsin (EC 3.4.21.1) were obtained from BDH Chemicals, Poole, Dorset, U.K. Chondroitin

6-sulphate (from shark cartilage), chondroitin 4-sulphate (from whale cartilage), chondroitinase ABC (EC 4.2.2.4), 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-*O*-sulphate-D-galactose (Δ Di-6S) and 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulphate-D-galactose (Δ Di-4S) were from Miles Laboratories, Slough, U.K. Trypsin (EC 3.4.21.4), type XI, was purchased from Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K.; amino acid standards were from Calbiochem Co., Hereford, U.K.; leech hyaluronidase was from Biotrics Inc., Arlington, MA, U.S.A.; Luma Gel was from Lumac Systems, Basel, Switzerland. Sepharose 2B and 6B and Sephadex G-10, G-25 and G-200, obtained from Pharmacia, Uppsala, Sweden, were used for column chromatography.

Methods

Analytical methods

Uronic acid content was measured by an automated procedure (Heinegård, 1973) of the modified (Bitter & Muir, 1962) carbazole reaction (Dische, 1947), with glucuronolactone as standard. Total hexosamine content was determined by the Boas (1953) modification of the Elson & Morgan (1933) technique. Glucosamine/galactosamine molar ratios were determined, after hydrolysis in 8M-HCl for 3 h at 95°C (Swann & Balazs, 1966), by automated ion-exchange chromatography (Hardingham & Muir, 1974). Neutral sugars were measured by g.l.c., as silyl derivatives of their methyl glycosides, by the method of Clamp *et al.* (1967). Mannitol was used as an internal standard. Sulphate was determined by the benzidine procedure (Antonopoulos, 1962) after hydrolysis in 25% (v/v) formic acid for 24 h at 100°C. Protein was measured by an automated modification (Heinegård, 1973) of the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V; Armour Pharmaceuticals, Eastbourne, Sussex, U.K.) as standard, and by summation of the individual amino acids. Total amino acid analyses were performed on a Locarte amino acid analyser by using a single-column elution system, after hydrolysis in 6M-HCl for 24 h at 105°C in sealed glass tubes under nitrogen (Hardingham & Muir, 1974). No corrections for destruction of amino acids during hydrolysis were made. Hydroxyproline was measured by the method of Woessner (1961) after hydrolysis in 6M-HCl at 105°C for 24 h in sealed glass tubes under nitrogen.

Preparative methods

Extraction of proteoglycans. Intact pig spines were obtained fresh from the slaughterhouse (T. Walls and Co., Southall, Middx., U.K.), and human spines

were obtained approx. 8 h *post mortem*. Human spines, which were between 15 and 30 years of age, were kept frozen until used. Only the lumbar intervertebral discs were chosen for investigation and were classified grades 0–1 by the nomenclature of Lewin (1964) for extent of disc degeneration. Discs were dissected from the vertebral bodies, care being taken to avoid contamination from cartilage endplates or adjoining tendon. The nucleus pulposus was separated from the annulus fibrosus and the annulus adjacent to the nucleus removed. The outer annulus and the nucleus from the human specimens were analysed for their proteoglycan content, but only the nucleus pulposus of pig discs was investigated. The nucleus and annulus were minced into small pieces with a scalpel and suspended in 10 vol. (w/v) of 4.0M-guanidinium chloride/0.05M-sodium acetate, pH 5.8, containing 0.1M- ϵ -amino-n-hexanoic acid, 0.01M-EDTA, 0.005M-benzamidine hydrochloride hydrate and 0.001% (w/v) of soya-bean trypsin inhibitor. Extraction was carried out for 48 h at 4°C with samples gently shaking on a Rocking Roller (Luckham Ltd., Burgess Hill, Sussex, U.K.). The residual tissue was removed from the extract by filtering through glass wool. With one 28-year-old human disc this residual tissue was further washed with water, suspended in a solution containing 0.005M-EDTA, 0.05M-NaCN and 0.01M-cysteine hydrochloride, and digested with papain (5%, w/w, of wet residual tissue) for 4 h at 60°C. The uronic acid content of the papain hydrolysate was measured by the carbazole reaction (Bitter & Muir, 1962).

Purification of proteoglycans

Equilibrium density-gradient centrifugation. A sample of the 4M-guanidinium chloride extract was dialysed in the cold against 7 vol. of 0.05M-sodium acetate, pH 5.8, containing 0.1M- ϵ -amino-n-hexanoic acid, 0.01M-EDTA, 0.005M-benzamidine hydrochloride hydrate and 0.001% (w/v) soya-bean trypsin inhibitor for 2 days at 4°C. To the dialysed extract containing approx. 2.5 mg of proteoglycans/ml was added solid CsCl until the density of the solution was 1.45 g/ml. Associative equilibrium density-gradient centrifugation was performed as previously described (Hardingham *et al.*, 1976) with an MSE 65 centrifuge and 8 $\times$ 25 ml angle-head rotor at 95000g_{av} for 48 h at 15°C. After centrifugation, the tubes were frozen and cut into two fractions: a bottom fraction (A1) of 4.6 ml and a top fraction (A2) of 13.7 ml. To the A1 fraction was added an equal volume of 7.5M-guanidinium chloride/0.05M-sodium acetate, pH 5.8, and solid CsCl until a density of 1.45 g/ml was obtained. The solution was centrifuged under dissociative conditions as described by Hardingham *et al.* (1976). After centrifugation, the tubes were

again cut into two unequal fractions, the bottom 4.6 ml (A1-D1) and the top 13.7 ml (A1-D2). The A1-D1 fraction, which contained the proteoglycans, was exhaustively dialysed against 0.05 M-sodium acetate, pH 6.8, and stored at -20°C for further investigation.

Preparative Sepharose 2B chromatography. Samples of the A1-D1 proteoglycan fraction (15 ml) containing 1.0–1.5 mg of uronic acid/ml were mixed with 0.4 mg of hyaluronic acid and applied to a column (150 cm $\times$ 5 cm) of Sepharose 2B, which was eluted at 4°C with 0.5 M-sodium acetate, pH 6.8, at 16 ml/h by using a peristaltic pump (Watson Marlow, Falmouth, Cornwall, U.K.). Fractions (9.6 ml) were collected and their uronic acid and protein contents determined. The fractions containing uronic acid that were excluded from and retarded by the gel were separately pooled, concentrated by vacuum dialysis, exhaustively dialysed against water and freeze-dried.

Cetylpyridinium chloride fractionation of proteoglycans. A sample of the 4 M-guanidinium chloride extract was dialysed against water for 2 days at 4°C . Sodium acetate (2 M) was added to a final concentration of 0.15 M, followed by 10% (w/v) cetylpyridinium chloride, dropwise, until maximum precipitation of proteoglycans was obtained. Samples were centrifuged at $1200g_{av.}$, the supernatant was removed and the precipitate washed three times by gentle stirring with 0.05% (w/v) cetylpyridinium chloride in 0.005 M-sodium sulphate. The precipitate was redissolved in 50 ml of 80% (v/v) propanol and the proteoglycans were reprecipitated as the sodium salt with the addition of 2 ml of saturated sodium acetate and 5 vol. of ethanol. After standing for 17 h at 5°C , samples were centrifuged at $1200g_{av.}$ and 5°C . The supernatant was decanted and the precipitate dried under vacuum and applied to a preparative Sepharose 2B column (see above).

Sedimentation coefficients of intervertebral-disc proteoglycans

Purified A1-D1 proteoglycans from human annulus fibrosus, pig and human nucleus pulposus containing 100–400 μg of uronic acid/ml were dissolved in 0.5 M-guanidinium chloride (GnHCl) containing 0.05 M-sodium acetate and 0.001 M-EDTA, pH 5.8. Umbilical-cord hyaluronic acid was then added to give a final concentration of approx. 2% (w/w) of the total uronic acid. Sedimentation-velocity centrifugations were carried out at 20°C and 40 000 rev./min (115 000 $g_{av.}$) in an MSE Centriscan analytical centrifuge with a six-place analytical rotor, single-sector quartz cells and 20 mm centre-pieces. Schlieren patterns of the sedimenting proteoglycans were scanned at 2 min intervals. The sedimentation coefficient at zero concentration ($s_{20,0.5\text{M-GnHCl}}^0$) was

obtained by extrapolation of the values of s against uronic acid content by linear regression analysis (Rosenberg *et al.*, 1973).

Molecular-weight determination of disc chondroitin sulphate

The average molecular weights ($\bar{M}_w$) of chondroitin sulphate chains released from A1-D1 proteoglycans by β -carbonyl elimination (Anderson *et al.*, 1965) were determined by the gel-filtration method of Wasteson (1969). Proteoglycans (4 mg) were dissolved in 1 ml of 0.5 M-NaOH and gently shaken for 17 h at 4°C or for 5 days at room temperature. The samples were neutralized with 0.5 M-HCl, and then 0.5 ml was applied to a column (120 cm $\times$ 1 cm) of Sephadex G-200 equilibrated with 0.2 M-NaCl containing 0.05 M-Tris/HCl, pH 7.0. Uronic acid was analysed in each 0.5 ml fraction, the elution positions of the glycosaminoglycans were determined, and the results compared with standard samples of chondroitin 6-sulphate and chondroitin 4-sulphate of known molecular weight. The void volume (V_0) and the column's total volume (V_t) were determined by the respective addition of aggregated ^3H -labelled pig laryngeal proteoglycans and $^3\text{H}_2\text{O}$, and assay of radioactivity. Luma Gel (5 ml) and water (1.5 ml) were added to each fraction and radioactivity (d.p.m.) was measured on a Searle mark III liquid-scintillation spectrometer. Background counts corresponding to buffer alone were subtracted from the observed d.p.m. of each fraction.

Molecular-weight ($\bar{M}_n$) estimations of chondroitin sulphate were also performed by the end-label method (Robinson & Hopwood, 1973; Hopwood & Robinson, 1973), by assuming one reducing sugar per glycosaminoglycan chain. Proteoglycans (5 mg) were dissolved in 1 ml of 0.5 M-KOH/0.02 M-NaB 3 H $_4$ (2.5 mCi/mmol) and shaken at 4°C for 10 days. At the end of the reaction, excess borohydride was destroyed by acidification with acetic acid (50 μl). The glycosaminoglycan chains released by alkali were desalted on a column (60 cm $\times$ 1.5 cm) of Sephadex G-10, eluted with 0.1 M-NH $_4$ HCO $_3$ and freeze-dried. They were then resuspended in 1% (w/v) cetylpyridinium chloride/0.05 M-Na $_2$ SO $_4$ in order to solubilize keratan sulphate and glycoprotein oligosaccharides and then applied to a micro-cellulose column (6 cm $\times$ 0.5 cm). The column was washed twice with 5 ml of 1% (w/v) cetylpyridinium chloride/0.05 M-Na $_2$ SO $_4$ followed by 1.0 M-MgCl $_2$. Uronic acid and radioactivity were measured in each 5 ml fraction. Specific radioactivity of the borohydride reducing solution (d.p.m./ μmol of substrate) was assessed by reducing a standard, xylose (12 μM), to xylitol under the same conditions. Xylitol was isolated by chromatography, on the same column of Sephadex G-10, quantified by the g.l.c. method of

Clamp *et al.* (1967) and radioactivity/ μ mol of xylose determined. Pig laryngeal proteoglycan with chondroitin sulphate of average molecular weight 15000 (Tsiganos & Muir, 1969) was used as a control.

Identification of aggregation of disc proteoglycans with disc hyaluronic acid

The associative density-gradient fraction (A1) from a human annulus preparation was dialysed exhaustively against water at 4°C and freeze-dried. Samples containing 3 mg of uronic acid were taken. The first was dissolved in 1.5 ml of 0.5 M-sodium acetate, pH 6.8; the second was dissolved in 1.5 ml of 0.1 M-Tris/HCl, pH 7.0, and incubated at 37°C for 4 h with 18 μ g of leech hyaluronidase. The third sample, dissolved in 0.1 M-Tris/HCl, pH 7.0, was also incubated at 37°C for 4 h, but without leech hyaluronidase. The three samples were applied separately in succession to a column (160 cm $\times$ 1.3 cm) of Sepharose 2B eluted with 0.5 M-sodium acetate, pH 6.8, and the uronic acid content of each 4 ml fraction was determined.

Isolation of a protein-rich region from human disc proteoglycans

Human disc proteoglycans (120 mg), isolated by equilibrium density-gradient centrifugation under dissociative conditions, were dissolved in 20 ml of 70% (w/v) formic acid and treated with 1% (w/v) CNBr for 24 h at room temperature. Excess CNBr was removed by adding 500 ml of water and freeze-drying. The sample was redissolved in 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, solid CsCl added until a density of 1.45 g/ml was obtained and the sample was then centrifuged as described by Heinegård (1977) in an MSE 65 centrifuge with an 8 $\times$ 25 ml angle-head rotor at 95000 g_{av} for 48 h at 15°C. The tubes were frozen and cut into four roughly equal portions. The solutions from each were dialysed against water, with preheated dialysis tubing, and the uronic acid and protein contents determined in each fraction.

Identification of keratan sulphate- and chondroitin sulphate-rich regions in the intervertebral-disc proteoglycans

The procedure of Heinegård & Axelsson (1977) was employed. Proteoglycans (80 mg) from human annulus fibrosus and human and pig nucleus pulposus, purified by equilibrium density-gradient centrifugation under dissociative conditions, were dissolved in 0.1 M-sodium acetate/0.1 M-Tris/HCl, pH 7.3, and incubated for 5 h at 37°C with 1 unit of chondroitinase ABC. Trypsin (200 μ g) and chymotrypsin (200 μ g) were then added and the solution was incubated at 37°C overnight. The digests were applied to a column (120 cm $\times$ 2.4 cm) of Sepharose 6B, eluted with 0.5 M-sodium acetate, pH 6.8, and the uronic acid content of each 6.4 ml fraction was determined. Fractions containing uronic acid-positive peaks were pooled and either desalted on a Sephadex G-25 column (30 cm $\times$ 3 cm), eluted with 10% (v/v) ethanol, or exhaustively dialysed against water. The peaks were analysed for uronic acid, hexosamine, galactose and xylose contents, and glucosamine/galactosamine molar ratios determined. In addition, the lowest-molecular-weight peaks from each chromatogram were analysed for quantities of isomeric chondroitin sulphate disaccharides by the paper-chromatographic technique of Saito *et al.* (1968).

Results

Recovery of uronic acid after guanidinium chloride extraction and equilibrium density-gradient centrifugation

When uronic acid was measured in the 4.0 M-guanidinium chloride extract and in the papain digest of the residue from a 28-year-old human disc, more than 92% and 75% of the total was found in the guanidinium chloride extracts of the nucleus pulposus and annulus fibrosus respectively. After equilibrium density-gradient centrifugation, under dissociative conditions, at a starting density of 1.45 g/ml, the uronic acid (Table 1) was principally

Table 1. Uronic acid content of fractions obtained by equilibrium density-gradient centrifugation of proteoglycans extracted from a 28-year-old human disc

For experimental details see the text.

Region of disc	Fraction	Density (g/ml)	Uronic acid contents	
			(mg)	(% of total)
Nucleus	Top (associative: A2)	<1.53	2.7	4
	Top (dissociative: A1-D2)	<1.53	8.2	11
	Bottom (dissociative: A1-D1)	>1.53	64.0	85
Annulus	Top (associative: A2)	<1.53	1.4	3
	Top (dissociative: A1-D2)	<1.53	5.0	11
	Bottom (dissociative: A1-D1)	>1.53	40.0	86

Fig. 1. Gel chromatography on Sepharose 2B of monomeric proteoglycans (A1-D1) in the presence of 1% (w/w) hyaluronic acid

Proteoglycans were obtained from: (a) a 15-year-old human nucleus; (b) a 15-year-old human annulus; (c) a 30-year-old human nucleus; (d) a 30-year-old human annulus; (e) a pig nucleus; (f) a 15-year-old human larynx. The uronic acid (—) and protein (----) contents of the fractions were determined. V_0 and V_t mark the void volume and the total volume of the column respectively.

located in the bottom A1-D1 fraction. The starting density chosen was lower than 1.6g/ml, which is normally used for purification of laryngeal hyaline-cartilage proteoglycans, because the higher density gave poor recovery of the disc proteoglycans at the bottom of the gradient.

Sepharose 2B chromatography

Gel chromatography of dissociated proteoglycan fractions A1-D1 showed a small excluded peak, suggesting that not all endogenous hyaluronic acid had been removed. On addition of umbilical-cord hyaluronate to the preparations a maximum of excluded material resulted, and although all disc preparations possessed some ability to bind to hyaluronate, as judged by the change in elution profile, the proportion that did so varied with each specimen (Fig. 1) and was greater in the annulus (Figs. 1b and 1d) than in the nucleus (Figs. 1a and 1c) of a given disc.

Like the proteoglycans of laryngeal cartilage (Fig. 1f), the majority of proteoglycans of the 15-year-old annulus interacted with hyaluronate, and were excluded on gel chromatography (Fig. 1b). This was not so with proteoglycans of older human discs (Figs. 1c and 1d).

The proportions of excluded material in proteoglycans prepared by equilibrium density-gradient centrifugation under associative conditions were very similar to those seen when hyaluronate was

Fig. 2. Gel chromatography on Sepharose 2B of proteoglycans purified by cetylpyridinium chloride precipitation. Proteoglycans were obtained from: (a) a 30-year-old human nucleus; (b) a 30-year-old human annulus. The uronic acid (—) content, protein (----) content and A_{260} (...) of the fractions were determined. V_0 and V_t mark the void volume and the total volume of the column respectively.

added to proteoglycans prepared under dissociative conditions. It was therefore concluded that in the former the proteoglycans excluded from the gel were bound to endogenous hyaluronate.

Proteoglycans from the annulus and nucleus of the same 30-year-old disc that were isolated by cetylpyridinium chloride precipitation also contained considerable amounts of excluded material (Fig. 2), which had low absorption at 260 nm, and hence were not complexed with cetylpyridinium chloride. The similar elution profiles on Sepharose 2B chromato-

graphy of the disc proteoglycans prepared by two different methods suggested that the excluded material was not an artifact of preparation.

Chemical analysis of intervertebral-disc proteoglycans

Disc proteoglycans, purified by density-gradient centrifugation and further fractionated on Sepharose 2B, were analysed for carbohydrate, protein and sulphate content and the results compared with analyses previously reported for proteoglycans of pig laryngeal cartilage (Table 2). There were striking

Table 2. *Composition of proteoglycans of intervertebral disc*

Proteoglycans were extracted from a 30-year-old human disc and a 8-month-old pig disc and from pig laryngeal cartilage. Protein content was obtained from summation of amino acid analysis.

	Content (% of dry weight)					
	Human nucleus		Human annulus		Pig nucleus	Pig laryngeal cartilage
	Excluded by Sepharose 2B	Retarded by Sepharose 2B	Excluded by Sepharose 2B	Retarded by Sepharose 2B		
Protein	12	10	15	12	11	7
Uronic acid	13	17	12	16	22	28
Galactose	11	11	10	10	—	5†
Sulphate	15	13	14	13	11	13
Hexosamine*	17	17	20	21	20	25
Glucosamine/ galactosamine molar ratio	1.0:1.1	1.0:2.0	1.0:0.9	1.0:1.7	1.0:4.9	1.0:12.5
Protein/glucos- amine weight ratio	1.5:1.0	1.8:1.0	1.4:1.0	1.6:1.0	3.2:1.0	3.7:1.0

* No correction for destruction of hexosamine during hydrolysis was made.

† Tsiganos & Muir (1969).

Table 3. *Amino acid composition of human intervertebral-disc proteoglycan fractions separated by gel chromatography on Sepharose 2B and proteoglycans of pig nucleus*

Proteoglycans were extracted from a 30-year-old human disc and a 8-month-old pig disc. Results are compared with analyses of pig laryngeal-cartilage proteoglycans (Hardingham *et al.*, 1976). Each value given is the average of at least two separate analyses.

Amino acid	Content (residues/1000 residues)					
	Human nucleus		Human annulus		Pig nucleus	Pig laryngeal cartilage
	Excluded	Retarded	Excluded	Retarded		
Asp	66	64	67	76	65	77
Thr	85	91	83	85	64	58
Ser	84	98	81	94	133	123
Glu	133	135	132	132	162	145
Pro	118	124	106	112	92	92
Gly	95	113	98	105	128	136
Ala	84	76	86	74	74	77
Val	80	75	76	74	59	69
Ile	35	36	34	36	38	38
Leu	76	83	77	86	74	78
Tyr	33	16	36	21	19	16
Phe	36	27	40	32	26	27
His	14	11	16	14	13	8
Lys	21	26	22	29	15	16
Arg	41	29	50	34	37	41

differences in protein, uronic acid, galactose and hexosamine contents, indicating that proteoglycans of intervertebral discs contained considerably more protein and keratan sulphate. Moreover, the composition of proteoglycans of pig nucleus resembled the composition of proteoglycans of human nucleus more closely than that of pig laryngeal-cartilage proteoglycans, even though the proteoglycans of pig nucleus were somewhat richer in chondroitin sulphate than those of human nucleus. On gel chromatography there were subtle differences in composition between excluded and retarded fractions of disc proteoglycans. The excluded material contained less uronic acid and galactosamine, suggesting that there was either slightly less chondroitin sulphate or more keratan sulphate in this fraction. Disc proteoglycans that did not bind to hyaluronate contained less protein than those that could.

Amino acid composition of intervertebral-disc proteoglycans

The amino acid compositions of the hyaline cartilage and all disc proteoglycan preparations (Table 3) had a high content of threonine, serine, glutamic acid, proline and glycine. Human nucleus and annulus proteoglycans were similar in amino acid composition, although again there were differences between the excluded and retarded fractions separated by Sepharose 2B chromatography. The proteoglycans that did not bind to hyaluronate contained higher amounts of serine and glycine and lower amounts of arginine, phenylalanine, tyrosine and alanine. In contrast with the gross compositions (see above), the amino acid composition of pig nucleus proteoglycans resembled that of pig laryngeal-cartilage proteoglycans rather than that of proteoglycans of human discs.

Table 4. *Sedimentation coefficients of intervertebral-disc and laryngeal-cartilage proteoglycans*

Proteoglycans were extracted from a 30-year-old human disc and a 8-month-old pig disc and larynx. *s* values are of non-aggregated and aggregated proteoglycans. The proportion of aggregates in the preparation from human nucleus was too low to determine the sedimentation coefficient accurately.

Tissue	$s_{20, 0.5M-GnHCl}^0$ (S)	
	Monomer	Aggregate
Human nucleus	5.4	—
Human annulus	7.2	30
Pig nucleus	11.0	39
Pig laryngeal cartilage*	24.1	146

* T. E. Hardingham (unpublished work).

Fig. 3. *Gel chromatography on Sepharose 2B of proteoglycans (A1) from human annulus treated with leech hyaluronidase*

(a) Before incubation with hyaluronidase; (b) after incubation for 4h with leech hyaluronidase; (c) incubation for 4h without enzyme. The uronic acid (—) content of the fractions was determined. *V*₀ and *V*_t mark the void volume and the total volume of the column respectively.

Sedimentation coefficients of intervertebral-disc proteoglycans

On analytical ultracentrifugation with added hyaluronic acid, intervertebral-disc proteoglycans generally showed a bimodal pattern attributable to aggregates and monomers. However, after 60 min or longer, some degree of heterogeneity was observed in the monomeric proteoglycans. The sedimentation coefficients of the aggregates of the disc proteoglycans were considerably lower (Table 4) than those of hyaline-cartilage proteoglycans. This difference

Table 5. *Analysis of fractions obtained after dissociative density-gradient centrifugation of CNBr-treated disc proteoglycans*

After CNBr cleavage, proteoglycan fractions were separated by equilibrium density-gradient centrifugation under dissociative conditions. (Note: before cleavage, 22% of the proteoglycans from this specimen were excluded on Sepharose 2B in the presence of added hyaluronate.)

Fraction no.	Volume (ml)	Hexuronic acid		Protein		Protein/hexuronic acid ratio (w/w)
		(ml)	(% of total)	(mg)	(% of total)	
1 (Bottom)	3.3	18.3	53	7.2	27	1.0:2.5
2	4.5	10.1	29	8.7	33	1.0:1.2
3	4.3	4.6	13	6.1	23	1.0:0.8
4 (Top)	5.0	1.4	4	4.3	16	1.0:0.3

Table 6. *Amino acid composition of density-gradient fractions of CNBr-treated human disc proteoglycans.*

For experimental details see the text.

Amino acid	Fraction ...	Content (residues/1000 residues)			
		1	2	3	4
Asp		62	56	78	95
Thr		84	103	93	86
Ser		115	105	96	88
Glu		156	149	142	126
Pro		87	99	91	72
Gly		136	118	102	92
Ala		86	86	83	84
Val		76	70	68	69
Ile		33	30	35	39
Leu		82	74	74	80
Tyr		14	18	27	33
Phe		25	27	32	33
His		8	12	14	18
Lys		12	19	26	34
Arg		24	32	39	51

appeared to be due primarily to lower sedimentation coefficients of the monomeric disc proteoglycans. Thus, the s_{20}^0 in 0.5M-guanidinium chloride of dissociated proteoglycans of pig nucleus was only half of that of dissociated proteoglycans of laryngeal cartilage, indicating that the proteoglycans of the intervertebral disc were of considerably smaller size. The smaller molecular size of proteoglycans of human nucleus and annulus was also evident on gel-filtration chromatography (Figs. 1a, 1b and 1f).

Molecular-weight estimation of disc chondroitin sulphate chains

The chondroitin sulphate released from proteoglycans of pig nucleus, 30-year-old human annulus, and nucleus from proteoglycans by NaOH treatment at 4°C for 17 h had K_{av} 0.52, 0.47 and 0.32 respectively when chromatographed on Sephadex G-200. This corresponded to average molecular weights ($\bar{M}_w$) of

9000, 17000 and 20000 for the chondroitin sulphate chains in these respective tissues. After 5 days' treatment with NaOH at room temperature, no appreciable degradation or 'peeling' was observed, since there was no overall change in the average molecular weight.

Average molecular weights ($\bar{M}_n$) of chondroitin sulphate from the same tissues estimated by the end-group method were somewhat lower than those estimated by Sephadex G-200 chromatography. Nevertheless, the molecular weight of the chondroitin sulphate from the pig nucleus proteoglycans was found by two independent methods to be about half that of chondroitin sulphate chains of proteoglycans of pig laryngeal cartilage or human disc. With use of an alkali/borohydride solution, which gave a specific radioactivity of 1.88×10^7 d.p.m./ μ mol of xylitol, the molecular weights for chondroitin sulphate from pig nucleus, pig laryngeal cartilage, human nucleus and human annulus were estimated as 7900, 15200, 14600 and 14300 respectively.

Identification of aggregation of disc proteoglycans with disc hyaluronate

Proteoglycans purified by equilibrium density-gradient centrifugation under associative conditions (A1 fractions) contained no hydroxyproline and hence were free of contaminating collagen. On chromatography on Sepharose 2B, about one-third of the total uronic acid of a preparation from annulus fibrosus was excluded from the gel (Fig. 3a), whereas after incubation with leech hyaluronidase (Fig. 3b) none of the uronic acid-positive material was excluded. A sample of the same preparation when incubated under identical conditions, but without enzyme, gave the same elution profile (Fig. 3c) as the starting material (Fig. 3a). It is concluded that the excluded fraction from Sepharose 2B chromatography of the disc proteoglycans obtained after associative centrifugation consisted of proteoglycans that were aggregated and bound to endogenous hyaluronate rather than to collagen.

Fig. 4. Gel chromatography on Sepharose 6B of disc proteoglycans after incubation with chondroitinase, trypsin and chymotrypsin. Proteoglycans were obtained from: (a) pig nucleus; (b) 30-year-old human nucleus; (c) 30-year-old human annulus. The uronic acid (—) content was determined in each fraction.

Isolation of protein-rich fragment from disc proteoglycans

Human intervertebral-disc proteoglycans, like those of hyaline cartilage, were found to possess a region of the core protein that was essentially free of glycosaminoglycans. When treated with CNBr and then fractionated by density-gradient centrifugation under dissociative conditions, disc proteoglycans yielded a fraction at the top of the gradient (fraction 4), which contained little uronic acid (Table 5), but which accounted for 16% of the total protein. Amino acid analysis of this fraction revealed higher contents of aromatic and basic amino acids than in the fraction rich in uronic acid, which separated at the bottom of the gradient (Table 6).

Identification of chondroitin sulphate-rich and keratan sulphate-rich regions in pig nucleus proteoglycans

Pig nucleus proteoglycans (A1-D1) after digestion with chondroitinase ABC, chymotrypsin and trypsin separated on Sepharose 6B chromatography into three uronic acid-positive peaks (Fig. 4a). Although the majority of uronic acid was in peaks B and C, 60% and 40% of the total glucosamine and galactose respectively were present in peak A. From the carbohydrate analyses (Table 7) of these fractions, it was concluded that peak A was derived from a region rich in keratan sulphate, since (1) glucosamine and galactose, the sugars of the repeating disaccharide of keratan sulphate, were the principal sugars of this peak, (2) there was less uronic acid and galactosamine, the major sugars of chondroitin sulphate, and (3) almost no xylose was detected, the carbohydrate which links chondroitin sulphate to the protein core. Peak B was derived from the region of the disc proteoglycan rich in chondroitin sulphate, since (1) uronic acid and galactosamine were present, (2) almost all the xylose was located in this fraction and (3) much less glucosamine than galactosamine was present, even after chondroitinase treatment.

Table 7. Carbohydrate analysis of disc proteoglycan after proteolysis, separated on Sepharose 6B into three fractions (A, B and C). For details see the text.

Fraction	Content (% of total)								
	Pig nucleus			Human annulus			Human nucleus		
	A	B	C	A	B	C	A	B	C
Uronic acid	3	29	68	18	5	77	12	4	83
Xylose	9	91	None	59	41	None	84	16	None
Galactose	49	51	None	88	12	None	91	8	None
Hexosamine	13	15	72	43	4	53	44	6	50
Glucosamine/galactosamine molar ratio	1.0:0.3	1.0:2.3	All galactosamine	1.0:0.2	1.0:0.5	All galactosamine	1.0:0.2	1.0:0.6	All galactosamine

Peak C was identified as chondroitin 6-sulphate disaccharides, which had been released by chondroitinase because most of the uronic acid was in this fraction and galactosamine was the only hexosamine present. Essentially all the disaccharides chromatographed with standard Δ Di-6S on paper chromatography.

Human nucleus and annulus proteoglycans, on the other hand, when digested under the same conditions as used for proteoglycans of pig nucleus gave somewhat different elution profiles on Sepharose 2B (Figs. 4b and 4c). Peak A was much larger and peak B smaller in chromatograms of both nucleus and annulus proteoglycans. Although more than 50% of the total xylose was located in peak A, nevertheless, in both fractions of peak B the proportion of xylose relative to the total hexosamine was high. Peak C consisted of Δ Di-6S, as in peak C derived from pig nucleus proteoglycans, and no Δ Di-4S was detected in peak C from either human nucleus or annulus proteoglycans. These data were consistent with there being a considerably smaller chondroitin 6-sulphate-rich region in the human disc proteoglycans.

Discussion

Any attempt to deduce the structure of intervertebral-disc proteoglycans should take account of the considerable heterogeneity of these macromolecules. Thus, at best, it is possible only to formulate a general structure representative of the majority of proteoglycans in the tissue. Moreover, postulated structures based on data obtained from extractable proteoglycans may not be appropriate for the 10–20% of unextractable proteoglycans. However, with these reservations it is possible to assess the degree of homology between cartilage and disc proteoglycans.

In both tissues the proteoglycans contain variable amounts of keratan sulphate and chondroitin sulphate. In young human discs, at least the majority of proteoglycans are capable of aggregation and therefore should possess a hyaluronic acid-binding region similar to that in proteoglycans of hyaline cartilage (Hardingham *et al.*, 1976). This is in agreement with the isolation of a fragment of the core protein after CNBr treatment that contained relatively few glycosaminoglycan chains and possessed an amino acid composition similar to that of the hyaluronate-binding region of cartilage proteoglycans (Heinegård & Hascall, 1974). However, the proportion of aggregated proteoglycan isolated from mature human intervertebral discs, particularly from the nucleus pulposus, was much less. This was unlikely to be due to a deficiency of hyaluronate (Hardingham & Adams, 1976), but to partial degradation or deficiency of the hyaluronic acid-binding region of the protein core, as suggested from the difference in

amino acid composition and lower protein content of these proteoglycans.

The location of the keratan sulphate-rich region of the pig nucleus proteoglycans was not established in the present investigation, but the separation into fractions richer in keratan sulphate or in chondroitin sulphate after trypsin and chymotrypsin treatment is analogous to that reported for bovine nasal-cartilage proteoglycans (Heinegård & Axelsson, 1977). However, with human disc proteoglycans, since the fractions were not clearly separated by gel chromatography, the chondroitin sulphate-rich region was probably shorter than in hyaline-cartilage proteoglycans.

Fig. 5. Models of proteoglycan structure

(a) Proteoglycans typical of those in hyaline cartilage, consisting of a protein core with three distinct regions: a hyaluronate-binding region, a keratan sulphate-rich region, and a chondroitin sulphate-rich region. (b) Proteoglycans with the normal number, but with shorter, chondroitin sulphate chains. (c) Proteoglycans with protein core of normal length, but with fewer chondroitin sulphate chains. (d) Proteoglycans in which the chondroitin sulphate-rich region is shortened, but the chains are of normal length.

Despite their homology there were quantitative differences in physical parameters and chemical analyses between disc and laryngeal proteoglycans. Although sedimentation coefficients are not exclusively based on molecular weight, the much lower sedimentation coefficients of disc proteoglycans and the more retarded elution from Sepharose 2B indicate that they are considerably smaller than proteoglycans of laryngeal cartilage or bovine (Rosenberg *et al.*, 1976) and dog (McDevitt, 1978) articular cartilage. A further difference was that the disc proteoglycans contained more protein and keratan sulphate and less chondroitin sulphate than did cartilage proteoglycans.

From the similarities and differences between cartilage and disc proteoglycans, three models for the general structure of disc proteoglycans that aggregate are suggested (Fig. 5). In the first the length of the protein core and the number of keratan sulphate and chondroitin sulphate chains attached to it are the same as in cartilage proteoglycans (Fig. 5a), but the chondroitin sulphate chains are shorter (Fig. 5b). This model appears to be more appropriate for proteoglycans of pig rather than human intervertebral discs, because the chondroitin sulphate chains released from proteoglycans of young pig discs were found by two different methods to be considerably shorter than those of pig laryngeal-cartilage proteoglycans, whereas the core proteins were similar in amino acid composition. Moreover, the disc proteoglycans were digested by trypsin and chymotrypsin in a similar manner to cartilage proteoglycans (Heinegård & Hascall, 1974). The ratio of protein to keratan sulphate content (as judged by the weight ratio of protein:glucosamine) is also similar (Table 2), indicating that the smaller size of disc proteoglycans was not due to less protein or less keratan sulphate.

In contrast, chondroitin sulphate chains of human disc proteoglycans released on alkaline β -elimination were of similar length to those of hyaline cartilage (Tsiganos & Muir, 1969), and hence the smaller size but higher protein and keratan sulphate content of human disc proteoglycans must be due to there being fewer chondroitin sulphate chains in the molecule. In Fig. 5(c) the core protein is of similar length to that in cartilage proteoglycans (Fig. 5a), but with fewer chondroitin sulphate chains set further apart. This model, however, does not explain why human disc proteoglycans are less susceptible to papain, Pronase, trypsin and chymotrypsin than are cartilage proteoglycans (Hopwood & Robinson, 1974a), since less substitution by glycosaminoglycans should render the core protein more rather than less susceptible to proteolytic digestion. An alternative structure is shown in Fig. 5(d), where the core protein itself is shorter, although retaining the hyaluronate-binding and keratan sulphate-rich regions. This

model would account for the lower protein:glucosamine ratio of human compared with disc proteoglycans (Table 2), and would result in a different amino acid composition with higher contents of threonine to which keratan sulphate is attached (Hopwood & Robinson, 1974b) and lower contents of amino acids such as glycine and serine prevalent in the chondroitin sulphate linkage region (Baker *et al.*, 1972).

The differences in structure between proteoglycans of young pig and mature human discs may result from subtle differences in the function of intervertebral discs in quadrupeds and bipeds or may simply be the general result of aging. The common features of smaller size and lower chondroitin sulphate content that distinguish disc from cartilage proteoglycans may provide the disc with physical properties somewhat different from those of hyaline cartilage, such as lower compressive stiffness. The specialized function of the disc is to be moderately flexible while at the same time absorbing and redistributing mechanical forces in the spine.

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