Characterization of newly synthesized proteoglycans from rabbit menisci in organ culture

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Rabbit menisci were incubated with $\text{Na}_2^{35}\text{SO}_4$ in short-term organ culture to label newly synthesized proteoglycans. The radioactive products present in both tissue and culture medium were characterized separately with respect to distribution after ultracentrifugation in CsCl isopycnic density gradients, hydrodynamic size, interaction with hyaluronic acid, and glycosaminoglycan composition (types, size and content). Analysis of proteoglycan size by gel-filtration chromatography of the most-dense CsCl fractions (A1) on Sephacryl S-500 (associative conditions) resolved three species. A peak with K_{av} approx. 0.7 was present in each chromatogram, and constituted the principal component in tissue extracts. Two other peaks with K_{av} values of approx. 0.2 and 0.45 were also found. When the A1 fraction from tissue was subjected to CsCl -density-gradient ultracentrifugation under dissociative conditions, 71% of the recovered radioactivity was present in the most dense (A1D1) fraction. Incubation with hyaluronic acid of either A1 or A1D1 fraction from associative extract did not alter the apparent size of the labelled product, indicating a lack of aggregate formation. Meniscal proteoglycans showed an unusual and marked tendency to adsorb irreversibly to agarose and agarose-containing gel-filtration-chromatography media. High-pressure liquid-chromatographic analyses indicated that the sulphated glycosaminoglycans consisted of chondroitin 6-sulphate (72%), chondroitin 4-sulphate (19%) and dermatan sulphate (5%). Endo- β -galactosidase (keratanase) digestion of the material failed to detect the presence of keratan sulphate. Of the labelled glycosaminoglycans, 95% was eluted from Sephacryl S-400 as a single symmetrical peak with a K_{av} of 0.5. The results of studies with tissue extracts and culture medium were similar.

Abbreviations used: GAG(s), glycosaminoglycan(s); C6S, chondroitin 6-sulphate; C4S, chondroitin 4-sulphate; KS, keratan sulphate; DS, dermatan sulphate; HA, hyaluronic acid; h.p.l.c., high-pressure liquid chromatography; GdmCl, guanidinium chloride; $\Delta\text{Di}0\text{S}$, 2-acetamido-2-deoxy-3-O-(β -gluco-4-enepyranosyluronic acid)-D-galactose; $\Delta\text{Di}4\text{S}$, 2-acetamido-2-deoxy-3-O-(β -D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose; $\Delta\text{Di}6\text{S}$, 2-acetamido-2-deoxy-3-O-(β -D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose; GBSS, Gey's balanced saline solution; BNS, bovine nasal septum; PG, proteoglycan(s); UA, uronic acid.

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The menisci of the knee are fibrocartilagenous semilunar-shaped elements located between the femoral condyles and the tibial plateau. They assume a weight-bearing role in the joint, and previous studies have shown that between 30 and 60% of the total load across the joint is transmitted through these structures (Shrive, 1974; Krause *et al.*, 1976).

Proteoglycans are a heterogeneous subclass of glycoconjugates consisting of a protein core to which GAG chains are covalently attached. The

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number of GAG chains per core protein may vary from 1 to 150 (Christner *et al.*, 1979; Hascall & Hascall, 1981). GAGs most common to the protein core of connective-tissue proteoglycans are of the sulphated variety. They are C6S, C4S, KS and DS. Individual proteoglycans may form large aggregates by attaching via their globular end (the hyaluronate-binding region) to the unsulphated GAG, HA (Hardingham & Muir, 1972; Hascall & Heinegård, 1974; Hassell *et al.*, 1979; Yanagishita *et al.*, 1979; Oldberg *et al.*, 1979; Roughley *et al.*, 1981).

The properties of the classical 'cartilage' proteoglycans have been described by using hyaline cartilage as the source. In this tissue the proteoglycans have been extensively characterized. However, only a few published studies have examined the proteoglycan or glycosaminoglycan composition of meniscal fibrocartilage in any depth, the majority concerning the human meniscus (Solheim, 1965; Habuchi *et al.*, 1973; McNicol & Roughley, 1980; Roughley *et al.*, 1981). The most recent paper described two types of proteoglycans from adult human meniscus. One was of high buoyant density, capable of interacting with HA and lacked DS. This type was termed 'cartilage-like'. The second type was of lower buoyant density, did not interact with HA, and contained DS.

Adams & Muir (1981, 1982) have characterized the proteoglycans of the canine meniscus. They found the major glycosaminoglycan to be C6S. Substantial amounts of C4S and some non-sulphated chondroitin, as well as DS, were found. They detected no KS. The intact proteoglycans were smaller, had smaller chondroitin sulphate chains and were more protein-rich than were those of hyaline cartilage. However, they were able to aggregate equally well with HA.

The rabbit has been used as a model to study various diseases affecting the knee, such as osteoarthritis (Moskowitz *et al.*, 1973; Langenskiöld *et al.*, 1979) and immune synovitis (Goldberg *et al.*, 1974). Because the menisci have an important mechanical function, changes in their biochemical and structural composition should be evaluated when studying a model of joint disease. However, there has not been any previous study published which has analysed the proteoglycans of rabbit menisci. Nor have others attempted to characterize newly synthesized proteoglycans. Such data should be useful in monitoring early biochemical changes in the meniscal fibrocartilage during the development and progression of joint disease in the various rabbit models.

The purpose of the present study was to determine the biochemical characteristics of the proteoglycans of the normal rabbit meniscus. Because of the minute amount of material obtainable from

rabbit menisci, and as it has been demonstrated in other systems that chondrocytes grown in short-term organ culture retain their phenotypic expression (Lane & Brighton, 1974; Malemud *et al.*, 1978; Benya & Nimni, 1979; Sokoloff, 1980), a short-term 'in vitro' organ-culture approach was used.

Experimental procedures

Materials

All media, as well as GBSS, were purchased from Grand Island Biological Co., Grand Island, NY, U.S.A. $\text{Na}_2^{35}\text{SO}_4$ (>99% radionuclidic purity) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. CsCl, LiCl, EDTA, sodium acetate, formic acid and methanol (h.p.l.c. grade) were purchased from Fisher Scientific Co., Fair Lawn, NJ, U.S.A.; GdmCl from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.; 6-aminohexanoic acid and benzamidinium hydrochloride from Eastman Kodak, Rochester, NY; and Spectrapor dialysis tubing (6000–8000 M_r cut-off) from Pierce Chemical Co., Rockford, IL, U.S.A. Keratanase, *Streptomyces* hyaluronidase and chondroitinase AC and ABC were obtained from Miles Laboratories, Kankakee, IL, U.S.A. Papain was purchased from Worthington Biochemical Co., Freehold, NJ, U.S.A. Sephacryl S-400 and S-500 and Sepharose CL-2B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Umbilical-cord HA and disaccharide standards ($\Delta\text{Di}0\text{S}$, $\Delta\text{Di}4\text{S}$, $\Delta\text{Di}6\text{S}$) were obtained from Sigma, St. Louis, MO, U.S.A. Type I reagent-grade water for h.p.l.c. was prepared by using a Barnstead NANOpure cartridge system. Bovine nasal-septum proteoglycan aggregate (A1) was kindly given by Dr. Arnold Caplan, Department of Biology, Case Western Reserve University.

Animals

All rabbits were New Zealand White virgin females weighing between 1.5 and 2 kg. A total of 23 rabbits were used for these studies.

Tissue isolation and organ culture

Rabbits were killed by CO_2 euthanasia. The knee joints were exposed under aseptic conditions. The lateral and medial menisci were removed and placed into a beaker of sterile GBSS. The meniscal horns as well as adherent synovium and joint capsule were dissected away under a laminar-flow hood using a scalpel blade and curved microdissection scissors. The remaining meniscal fibrocartilage was pooled and cut into four to eight pieces. Organ cultures were grown in 15 ml of sulphate-free DMEM (Srivastava *et al.*, 1974)

supplemented with 0.1% penicillin, 0.1% streptomycin, 0.1% mycostatin, 1% fungizone and $\text{Na}_2^{35}\text{SO}_4$ (10 $\mu\text{Ci}/\text{ml}$). Approx. 16 pieces of tissue were placed in 100mm-diam. $\times$ 20mm-high plastic culture dishes (Falcon Plastics, Los Angeles, CA, U.S.A.), which were then incubated at 37°C for 48h under CO_2/air (1:9). In one experiment, cultures were removed at 1, 6, 10 and 48h.

Preparative procedures

After culture the tissue pieces were separated from the culture medium and washed twice with GBSS. Tissues were initially extracted under associative conditions (A extract) [0.5M-GdmCl, pH 5.8, plus proteinase inhibitors (0.1M-EDTA, 5mM-benzamidine hydrochloride and 0.2M-6-aminohexanoic acid)] for 24h at 4°C. The tissue was subsequently extracted for an additional 24h under dissociative conditions (D extract) (4M GdmCl, pH 5.8, plus proteinase inhibitors). The tissue residue was digested with papain and residual radioactivity measured.

The medium as well as the A and D tissue extracts were dialysed exhaustively against distilled water with proteinase inhibitors at 4°C with Spectrapor dialysis tubing. The retained material was then flash-frozen and freeze-dried.

Isopycnic-CsCl-density-gradient centrifugation

Freeze-dried material was reconstituted in either an associative CsCl buffer [0.5M-sodium acetate, ρ_0 (starting density of the solution at 27°C) = 1.69g/ml, pH 5.8, or a dissociative CsCl buffer (4M-GdmCl in 0.1M-sodium acetate, ρ_0 = 1.50g/ml), pH 5.8, plus proteinase inhibitors. These were centrifuged at 110000g (r_{av} 8.35cm) at 12°C for 48h in a Beckman L5-50 ultracentrifuge with an SW 50.1 rotor. The resulting gradients were divided into four approximately equal fractions by siphoning from the top of the tube using a Pasteur pipette attached to a peristaltic pump. The above-mentioned fractions were labelled A1–A4 for associative gradients and D1–D4 for dissociative gradients. The fractions were then dialysed against 0.5M-sodium acetate, pH 5.8, at 4°C for gel-filtration chromatography. In certain experiments the A1 fraction had its density adjusted to 1.50g/ml and molarity adjusted to 4M by the addition of CsCl and 8M-GdmCl respectively. These fractions were then centrifuged as described above. The fractions so derived were labelled A1D1–A1D4 and dialysed against 0.5M-sodium acetate. Residual hyaluronic acid that may have been present in A1D1 fractions was removed by digestion with *Streptomyces* hyaluronidase as described by Faltz *et al.* (1979).

Column chromatography

Sephacryl CL-2B, Sephacryl S-500 and S-400 columns (6.6mm $\times$ 100cm) were eluted with either 0.5M-sodium acetate, pH 5.8 (associative buffer) or 4M-GdmCl, pH 5.8 (dissociative buffer) at a flow rate of 9ml/h. Effluent fractions (0.9ml) were collected from the column. The void volume (V_0) was determined with Dextran Blue 2000 and BNS proteoglycan aggregate (A1), and the total volume (V_t) with $\text{Na}_2^{35}\text{SO}_4$. Agarose columns (6.6mm $\times$ 10cm) were eluted with associative buffer and fractions (1ml) were collected. DEAE-cellulose columns (6.6mm $\times$ 10cm) were first eluted with 3 column vol. of 0.05M-Tris buffer, pH 7.2, and then an equal amount of 1M-LiCl/0.05M-Tris, pH 7.2.

Characterization of proteoglycans

Size. The hydrodynamic size distribution of the meniscal proteoglycans was determined by chromatography on Sephacryl S-500. A1 fractions were co-chromatographed with proteoglycan aggregate as carrier. Column effluents were mixed with Aquasol (1:9, v/v) and assayed for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer. Unlabelled material was detected by continuous monitoring of A_{230} with a Gilford model 250 spectrophotometer.

Interaction with hyaluronic acid. The properties of meniscal PG in respect of their aggregation with HA *in vitro* under associative conditions (0.5M-sodium acetate, pH 5.8) were assayed by the method of Hardingham & Muir (1972) as follows. Meniscal PG (A1 and A1D1) as well as BNS PG (A1D1) were mixed with HA in a ratio (HA/PG) of 1:100 on the basis of UA. Because the UA content of individual gradient fractions from meniscal material was below detectable limits, the amount of UA per fraction was calculated for these as follows. Before CsCl-density-gradient centrifugation the total amount of UA in the extract was determined by the method of Bitter & Muir (1962). The total amount of radioactivity was also determined. After centrifugation, the percentage of radioactivity in each fraction was determined and assumed to be representative of the UA distribution. On the basis of these results the amount of UA in the A1 and A1D1 fractions was calculated and the appropriate amount of HA was added. The mixture was incubated with gentle rolling for 48h at 4°C and then chromatographed on a Sephacryl S-500 column under associative conditions. Under these conditions, approx. 95% of the BNS A1D1 PG formed aggregates and was eluted in the void volume.

Characterization of GAGs. GAGs were released from meniscal A1 PG by papain digestion and β -

elimination by the method of Srivastava *et al.* (1974). Digested peptides were separated from GAG by chromatography on DEAE-cellulose. The size distribution of the GAG chains was determined by chromatography on Sephacryl S-400 under associative conditions. The GAG composition of A1D1 was determined in part by h.p.l.c. analysis of chondroitinase derived disaccharides by the method of Lee & Tiekemann (1979), a Varian model 5000 h.p.l.c. apparatus with a Varian MicroPak-NH₂ column (0.4 cm × 30 cm; 10 µm particle size) being used. Peaks for the reference disaccharides (ΔDi4S, ΔDi6S, ΔDi10S) were detected by utilizing a fixed-wavelength (254 nm) detector connected to a Spectra-Physics 4100 integrating printer/plotter. Radiolabelled disaccharides from meniscal GAGs were detected by liquid-scintillation counting of the column effluent. The amount of each disaccharide was determined by calculating the total radioactivity in each elution peak. Percentages were calculated on the basis of the total radioactivity eluted from the column.

KS was assayed by keratanase digestion and ethanol precipitation of meniscal GAGs as previously described (Keiser & Malmud, 1983).

Results

Extraction of ³⁵S-labelled material

Between 31 and 43% of the incorporated radio-label was found in the medium. Approx. 50% of the non-dialysable radiolabel extracted from the tissue was recovered in 0.5 M-GdmCl. The remainder was extracted in 4 M-GdmCl. In all experiments, less than 3% of the total label remained in the tissue residue and was considered unextractable material.

Proteoglycan distribution in CsCl density gradients

Associative gradients. PG extracted from rabbit meniscal fibrocartilage with associative (0.5 M-GdmCl) or dissociative (4 M-GdmCl) buffers distributed similarly when subjected to isopycnic-CsCl-density-gradient centrifugation under associative conditions (Table 1). Approximately one-third of the radiosulphate was recovered in each of the A1 and A4 fractions. The remaining one-third was distributed equally between the A2 and A3 fractions. The PG from the medium showed a somewhat different distribution, with slightly more than one-third of the ³⁵S being in the

Table 1. *Density-gradient distribution: associative gradients*

Tissue was labelled in organ culture for 48 h in medium containing Na₂³⁵SO₄. At the end of the labelling period, medium was removed, and the tissue residue was extracted for 24 h at 4°C with 0.5 M-GdmCl plus proteinase inhibitors. The extract was removed and the tissue was re-extracted for another 24 h with 4 M-GdmCl. After dialysis, samples were freeze-dried and reconstituted in 0.5 M-GdmCl, containing CsCl to bring the density of the solution to 1.69 g/ml. Samples were then centrifuged at 110 000 *g* at 12°C as described by Hascall & Sajdera (1969). Values are means from four experiments. All values are within 1 s.e.m.

Fraction	Average density (g/ml)	³⁵ S radioactivity (%)		
		Tissue extracts		Culture medium
		0.5 M-GdmCl	4 M-GdmCl	
A1	1.80	33	28	35
A2	1.68	16	15	20
A3	1.59	14	16	25
A4	1.48	37	41	20

Table 2. *Density-gradient distribution: dissociative conditions*

Culture, labelling and extraction was the same as that in Table 1. The A1 fraction was derived from 0.5 M-GdmCl-extracted material as described in the Experimental procedures section. After freeze-drying, the material was reconstituted in 4 M-GdmCl containing CsCl to bring the density of the solution to 1.50 g/ml, then centrifuged as described by Hascall & Sajdera (1969). All values are within 1 s.e.m.

Fraction	Average density (g/ml)	³⁵ S radioactivity (%)	
		4 M-GdmCl extract (tissue) (n = 4)	A1 (tissue) (n = 2)
D1	1.61	45	71
D2	1.49	22	19
D3	1.42	15	9
D4	1.33	18	1

A1 fraction, as with the tissue extracts, but the remaining two-thirds being more or less equally distributed between the other three fractions of lower density (A2–A4).

Dissociative gradients. Dissociatively extracted PG from rabbit meniscus distributed with a greater amount of radiolabel in the denser fractions than did associatively extracted material (Table 2). Almost one-half of the ^{35}S was found in

the D1 fraction, with the remaining radiosulphate being distributed approximately evenly between the D2, D3 and D4 fractions. A1 fractions derived from associatively extracted meniscal fibrocartilage and subsequently subjected to CsCl centrifugation under dissociative conditions gave a distribution in which most of the radioactivity (71%) was found in the A1D1 fraction, with the majority of the remainder (19%) in the A1D2.

Fig. 1. *Sephacryl S-500* chromatography of rabbit meniscal PG

CsCl-density-gradient fractions were prepared as described in the Experimental procedures section. All fractions were dialysed exhaustively against 0.5M-sodium acetate, pH 5.8 (associative buffer) at 4°C. Portions (0.5 ml) were applied to a column (6.6 mm × 100 cm) and eluted with associative buffer. Radiosulphate activity was measured by liquid-scintillation spectrometry. In this typical experiment, the A1 fractions from medium and dissociatively (D) extracted tissue demonstrated a polydisperse pattern. The A1 fraction from associatively (A) extracted tissue showed only a peak with a K_{av} of 0.7. The appearance of peaks with smaller K_{av} values is seen in A2–A4 fractions of A and D extracts.

Proteoglycan characterization by gel-permeation chromatography

Chromatography on Sephacryl S-500 of culture medium as well as associative (A) and dissociative (D) extracts of tissue from menisci labelled for 48 h with $\text{Na}_2^{35}\text{SO}_4$ is shown in Fig. 1. The A1 fractions contained up to three groups of apparent monomers, which could be differentiated on the basis of their hydrodynamic volume. PG from the A extract were eluted as a single peak with a K_{av} of 0.7. Those from the D-extract were eluted as a major peak ($K_{av} = 0.7$) and a minor peak ($K_{av} = 0.45$). Three elution peaks were detected from medium: a major peak ($K_{av} = 0.45$) and two lesser peaks ($K_{av} = 0.3$ and 0.7). Chromatography of the same fractions on a dissociative column did not alter the elution profile (i.e. no change in K_{av}), indicating that the peaks seen under associative conditions did not represent small HA-mediated aggregates. Chromatography of CsCl fractions from medium and tissue extracts of organ-cultured menisci labelled for 1, 6, 10 and 48 h showed that all three peaks were present at each time period and that these peaks increased in amount during the culture period (results not shown).

Chromatography of the less-dense CsCl fractions (A2–A4) generally showed a less polydisperse profile (Fig. 1). In some instances the hydrodynamic volume of the PG appeared to increase (smaller K_{av}) from the most-dense (A1) to the least-dense (A4) fractions.

Aggregation properties with HA

Elution profiles of A1 and A1D1 PG from A extracts on Sephacryl S-500 before and after incubation with umbilical-cord HA is shown in Fig. 2. Addition of HA at a ratio (HA/PG) of 1:100 failed to decrease the K_{av} (i.e. increase the hydrodynamic volume) of the radioactive peaks of either A1 or A1D1 material, indicating a lack of HA-mediated PG aggregation. Although not shown, A1 and A1D1 PG from culture medium also failed to aggregate when incubated with exogenous HA.

GAG characterization

Sephacryl S-400 chromatography. The size of GAGs from associative and dissociative tissue extracts and medium was analysed by Sephacryl S-400 chromatography. Of the radioactivity from each of the A1 fractions, 95% was recovered as a symmetrical peak, which was eluted with a K_{av} of approx. 0.5 (Fig. 3). A small peak (5%) with a K_{av} of 0.76 was also seen in the A and D extracts. The elution profile became more polydisperse in the less-dense (A2–A4) fractions. Of special interest is the appearance of larger GAG chains in the less-dense fractions in certain instances. This was

Fig. 2. Sephacryl S-500 chromatography of rabbit meniscal PG from the A1 (a) and A1D1 (b) fractions with and without HA

A1 and A1D1 fractions were prepared and chromatographed on Sephacryl S-500 before (Δ) and after ($\square$) incubation with HA (see the Experimental procedures section). The addition of HA did not shift the position of elution peaks, and the K_{av} in all cases remained 0.7, indicating a lack of aggregation. This Figure shows data from tissue-derived proteoglycans. The profiles for medium-derived proteoglycans were similar.

especially prominent with the culture-medium-derived GAGs.

H.p.l.c. Dissacharides were derived by chondroitinase AC and ABC digestion of papain-released GAGs from tissue and medium A1D1 fractions. H.p.l.c. analysis indicated the presence of four radioactive elution peaks (Fig. 4). Three of the peaks coincided with those of the standards. A fourth peak was eluted later, but with a somewhat variable retention time (between 8 and 12 min). This peak represented approx. 10 and 3% of the radioactivity from tissue- and medium-derived material respectively. Lacking identity of this fourth peak, we are not able to determine whether material eluted at these later, variable retention

Fig. 3. *Sephacryl S-400 chromatography of GAG chains*

Rabbit meniscal GAGs were derived by papain digestion and β -elimination as described in the Experimental procedures section. Portions (0.5 ml) were applied to columns and eluted with associative buffer. The A1 fractions from both tissue and medium were eluted with most of the radiolabel ($K_{av} = 0.5$) and consist mainly of C6S and C4S chains. The minor peaks at K_{av} 0.76 may represent DS. Fractions A2–A4 showed more polydispersity. Larger-sized GAG chains were seen in some of the less-dense fractions.

times represented different components or only one component. Retention times of the other peaks were quite consistent and reproducible.

Radioactive peaks which were co-eluted with standards after chondroitinase AC digestion indicated the presence of C6S (72%) and C4S (19%) (Figs. 4a and 4c). The Δ Di6S peak represents the minimum amount of C6S present, as we have found that the radiolabelled material that is eluted coincident with the Δ Di10S standard is actually derived from the Δ Di6S material (Norby *et al.*, 1983). Thus the total amount of C6S present was obtained by adding the radioactivity under the Δ Di10S and Δ Di6S peaks. After digestion of an equal amount of A1D1-derived material with chondroitinase ABC, the amount of radioactivity eluted in the Δ Di4S position increased, indicating the presence of DS (5%), an epimer of C4S (Hoffman *et al.*, 1958; Fransson & Havsmark, 1970).

Keratanase digestion of both medium- and tissue-derived A1D1 GAG, followed by ethanol

precipitation, failed to indicate the presence of KS.

Proteoglycan-agarose interaction. Chromatography of meniscal A1 or D1 PG on agarose-containing gels (e.g. Sepharose CL-2B) or agarose itself resulted in a significant amount of label being adsorbed on to the gel matrix (Table 3). Only 53–70% of the radiolabel could be recovered. This was not true of hyaline-cartilage-derived proteoglycans, where 96–98% of the material was recovered. Meniscal PG did not adsorb on non-agarose-containing gels such as Sephacryl, as demonstrated by a 98% recovery.

Discussion

It has been previously demonstrated that the proteoglycans of human and canine menisci exhibit certain properties different from hyaline cartilages of the same species (McNicol & Roughley, 1980; Roughley *et al.*, 1981; Adams & Muir, 1982). In the present paper we report that the PG of the rabbit meniscus synthesized *in vitro* likewise

Fig. 4. *H.p.l.c. of chondroitinase-derived disaccharides from rabbit meniscal GAGs*

GAGs were released from medium and tissue A1D1 PG with papain and the analysis was carried out as described in the Experimental procedures section. Continuous lines (—) represent reference standards of each disaccharide as indicated. These were measured as A_{254} . The discontinuous lines (----) represent ^{35}S -labelled rabbit meniscal disaccharides measured as radioactivity (c.p.m.). (a) Tissue-derived, chondroitinase AC-digested; (b) tissue-derived, chondroitinase ABC-digested; (c) medium-derived, chondroitinase AC-digested; (d) medium-derived, chondroitinase ABC-digested.

Table 3. *Proteoglycan-agarose interaction*

Samples were eluted from columns with 0.5M-sodium acetate, pH 5.8 (associative buffer) and/or 4M-GdmCl (dissociative buffer). Rabbit meniscal PG consistently exhibited adsorption when chromatographed on columns packed with either agarose-containing gels or agarose itself. This was true when columns were eluted with either associative or dissociative buffer. Adsorption was not seen with the other PG types tested, nor with meniscal PG on non-agarose-containing gels.

Cartilage PG type	Chromatography gel	Amount recovered as ^{35}S radioactivity or UA (%)
Rabbit meniscus	Sephacryl S-500*	98
	Sephacryl CL-2B†	53–66
	Agarose	70
Hyaline cartilage‡	Sephacryl CL-2B†	96–98

* Contains no agarose.

† Contains agarose.

‡ BNS, rabbit growth plate and articular cartilage of rabbit, pig and dog.

demonstrate structural and physical differences from their hyaline cartilage counterparts. Previous authors, however, have for the most part placed major emphasis on the 'hyaline-cartilage-like' nature of meniscal PG. Habuchi *et al.* (1973), on the other hand, have presented results indicating that meniscal GAGs represent a separate family that may be specifically adapted to the function of fibrocartilages. Our data also suggests that greater weight should be given to the importance of the differences seen between the two types of proteoglycan.

The PG of rabbit menisci distributed in both associative and dissociative CsCl density gradients with a pattern that is quite distinct from that displayed by proteoglycans of hyaline cartilage (Garg & Swann, 1981; Santer *et al.*, 1981; Rostand *et al.*, 1982). These different patterns were not due to our methodology, because articular-cartilage as well as growth-plate-cartilage PG isolated from the same animals distributed as expected, with 76–88% of the radiol sulphate in the A1 or D1 fraction (results not shown).

Results obtained from Sephacryl S-500 chromatography under both associative and dissociative conditions indicate (1) that rabbit meniscal PG are smaller than their counterparts from hyaline-type cartilages, and (2) that, even under associative conditions, they exist as monomers, many of which are capable of sedimenting into fractions of relatively high density. The smaller size of these PG is in agreement with the findings of others (Roughley *et al.*, 1981; Adams & Muir, 1982) and is consistent with the need for these molecules to fit within the closely spaced and regularly arranged collagen fibres of the fibrocartilage matrix. Such a relationship between PG size and matrix geometry has been proposed for other tissues, such as corneal stroma (Hascall & Hascall, 1981).

Neosynthetic rabbit meniscal PG appear to be rather dense, as they are capable of penetrating an A1 fraction (average density approx. 1.80 g/ml) in the monomeric form. The density of a PG is dependent on the amount of carbohydrate substituted on the core protein and/or its degree of sulphation (Ito *et al.*, 1982). In the present study we did not look at the amount of carbohydrate substitution or degree of sulphation of these proteoglycans. However, Roughley *et al.* (1981) have previously presented results that are compatible with a PG in human menisci that is more glycosylated than those found in hyaline cartilage.

Another unusual feature of rabbit meniscal PG is that, unlike those isolated from typical hyaline cartilages, the hydrodynamic volume of the molecules from the tissue tended to increase in going from fractions of higher to lower density. This was

not an artefact of our methodology, as PG extracted from the articular cartilage of the same animals distributed chromatographically in the expected fashion after CsCl-density-gradient ultracentrifugation. These results indicate that the PG in the more-dense fractions are more compact and probably contain a higher carbohydrate-to-protein ratio. This again is consistent with previous findings (Roughley *et al.*, 1981). Those in the less-dense fractions probably contain less carbohydrate, but are larger, perhaps by virtue of possessing longer GAG chains. This notion is supported by the fact that gel-exclusion chromatography of the meniscal GAGs (Fig. 3) indicated the presence of larger chains in the fractions of lower density.

Even within the A1 fractions themselves, however, there was a certain degree of polydispersity with respect to size of the PG. The PG most consistently noted was the one with a K_{av} of 0.7. In A extracts it was the only one present. In D extracts and medium it appeared together with peaks having K_{av} values of 0.45 and 0.3. These last two PG may represent larger monomers or perhaps dimers and trimers of the basic monomer with the K_{av} of 0.7. Sheehan *et al.* (1978) have demonstrated by light-scattering and ultracentrifugation that PG subunits from pig laryngeal cartilage can self-associate in the absence of HA. A similar phenomenon may have occurred in the present study.

Unlike results obtained by others with dog and human menisci (Roughley *et al.*, 1981; Adams & Muir, 1982), we could not demonstrate interaction of rabbit meniscal PG with HA. HA was added in a ratio reported to be optimal for hyaline-cartilage PG-aggregate formation (Hardingham & Muir, 1972; Franzén *et al.*, 1981). The lack of aggregation was not caused by the presence of small amounts of endogenous HA being bound to HA-binding region, since the proteoglycans from *Streptomyces*-hyaluronidase-digested A1D1 fractions, which should be devoid of any HA fragments, also failed to aggregate under associative conditions. There are at least two interpretations of these data. First, rabbit meniscal PG may be synthesized in organ culture without an HA-binding region. Alternatively, the HA-binding region may be non-functional and thus not capable of interacting with HA under the assay conditions employed. Aggregation is considered critical for fixing the proteoglycans in the tissue matrix (Hascall & Hascall, 1981). Therefore an alternative means of stabilizing meniscal PG in fibrocartilage must be envisaged if these molecules in fact lack or contain a non-functional HA-binding region, as is the case with some other PG types (Axelsson & Heinegård, 1978; Hassell *et al.*, 1979; Yanagishita *et al.*, 1979; Yanagishita & Hascall, 1979). It is possible

that, in meniscal fibrocartilage, there is a substitute role played by fibronectin, chondronectin or some other glycoprotein in stabilization of the proteoglycans. This has yet to be explored.

Why rabbit meniscal PG bind to agarose and agarose-containing gels (Table 3), whereas the other PG types do not, eludes explanation at this time.

Digestion of ^{35}S -labelled rabbit meniscal GAGs with specific endohydrolases demonstrated that these proteoglycans contain C6S (72%), with lesser amounts of C4S (19%) and DS (5%). The presence of these GAGs in meniscal fibrocartilage is consistent with previous reports (Habuchi *et al.*, 1973; Roughley *et al.*, 1981; Adams & Muir, 1981). We were unable to detect KS. Canine menisci also seem to lack KS (Adams & Muir, 1981), whereas human menisci contain this GAG (Roughley *et al.*, 1981). This may be related either to differences in the weight-bearing role of the menisci in bipeds versus quadrupeds or simple species differences.

In summary, the data indicate that rabbit meniscal PG have properties that distinguish them from those of hyaline cartilages. These differences may be of greater importance biologically than has been previously appreciated. As such, the proteoglycans of hyaline and fibrocartilage may require different physical and structural characteristics to fulfil their functional role in the matrix.

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