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Extraction and Characterization of Proteoglycan from Human Meniscus

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This study consists of (1) the extraction of proteoglycan from the human meniscus under dissociative conditions, (2) an investigation of the changes that occur in the abundance and structure of this proteoglycan with age and (3) a comparison of these findings with those for human articular-cartilage proteoglycan. Adult meniscus was found to possess proteoglycan molecules of similar size and glycosaminoglycan content to those present in cartilage, although tissue concentrations were considerably lower. In addition, age-related changes, with respect to the occurrence of keratan sulphate and the sulphation of chondroitin sulphate chains, were common to both tissues. The presence of aggregated proteoglycan was demonstrated, although specific interaction with hyaluronic acid was not conclusively shown biochemically. Differences were, however, noted in the structure of the proteoglycan between the two tissues: dermatan sulphate was found in the meniscus proteoglycan preparation and the core proteins exhibited some dissimilarities. A proteoglycan structure of this type would be compatible with its participation in meniscus elasticity, especially as the material is localized in a specific area.

The human knee joint contains two menisci, one located in each of the medial and lateral compartments of the joint between the femoral condyles and tibial plateaus. They are semi-lunar in shape with the inner edge being free and the outer edge being attached peripherally to the joint capsule. The precise function of the meniscus has been long debated, particularly with respect to the roles it may play in weight-bearing, nutrition and lubrication of the articular cartilage, and joint stability. Biomechanical studies suggest, however, that the menisci may be important weight-bearing structures in the knee joint (Shrive, 1974; Seedhom *et al.*, 1974; Walker & Erkman, 1975; Krause *et al.*, 1976). They are interposed between the articular surfaces, thereby increasing the contact area available for load transmission (Kettlekamp & Jacobs, 1972) and are potentially available to act as shock absorbers.

The ability of articular cartilage to withstand compressive loading is conferred mainly by its proteoglycan content (Kempson *et al.*, 1976). The functional properties of cartilage proteoglycans are due mainly to their specialized structure, consisting of a long central core protein to which many anionic glycosaminoglycan chains are attached (Muir, 1969). The size of these molecules is further increased by specific interaction with hyaluronic acid (Hardingham & Muir, 1973; Hascall & Heinegård, 1974). The elastic properties of the cartilage are dependent on the large size and high anionic charge of the proteoglycan (Hascall, 1977). If the meniscus is to be effective in a weight-bearing role, it would be advantageous for it to contain cartilage-like proteoglycan.

The purpose of this study is to isolate meniscus proteoglycan by the techniques employed in the isolation of cartilage proteoglycan, and to ascertain the changes that might occur in the abundance and structure of such a molecule with age.

Experimental

Materials

Guanidinium chloride and CsCl were obtained from BDH Chemicals, Poole, Dorset, U.K. Phenylmethanesulphonyl fluoride, iodoacetic acid, papain, bacterial collagenase (type I) and hyaluronic acid (from human umbilical cord) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Pepsin was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Chondroitinases ABC and AC and their disaccharide degradation products of chondroitin sulphate were from Miles Laboratories, Elkhart, IN, U.S.A. Acrylamide and *NN'*-methylenebisacrylamide were from Eastman Kodak Co., Rochester, NY, U.S.A. Cellulose t.l.c. plates were obtained from Mandel Scientific Co., Ville St. Pierre, Montreal, Que., Canada. Pepstatin was a gift from the U.S.–Japan Medical Science Program.

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Source of tissue

Human menisci were removed from the knee joint at autopsy, within 18h of death. Joints were chosen that appeared macroscopically normal and in which the menisci were of normal appearance and attachment. Both medial and lateral menisci were used. Foetal and newborn tissues were collected from foetuses and newborn babies of less than 3000g and were pooled. Likewise tissue from adults aged 40–70 years was pooled in decades. In all other cases, the tissue under investigation was from single individuals. The tissue was carefully trimmed to ensure the complete removal of all synovium and capsular tissue, and then finely diced. The human articular cartilage was obtained at autopsy from the distal femur of individuals in the 50–60 year-age group. All samples were stored at −20°C before extraction of proteoglycans.

Extraction of proteoglycan

Diced menisci were extracted with 10vol. of 4M-guanidinium chloride/0.1M-sodium acetate, pH6.0, containing 1mM-EDTA, 1mM-iodoacetic acid, 1mM-phenylmethanesulphonyl fluoride and 5μg of pepstatin/ml as inhibitors of metallo-, thiol, serine and carboxy proteinases respectively. Extraction was for 70h at 4°C. The extracts were filtered through Whatman 54 hardened filter paper, and the residues washed with extraction medium. CsCl was added to the filtrate to give a density of 1.50g/ml, and guanidinium chloride was added such that its concentration remained at 4M. This solution was then subjected to density-gradient centrifugation (Hascall & Sajdera, 1969) at 10°C for 48h at 100000$g_{av.}$ ($r_{av.}$ 6.3cm) in a Sorval T865 rotor. The gradient was fractionated and assayed for density, uronic acid and absorbance at 280nm. The fractions were divided into three pools according to their density: pool D1 contained fractions having a density greater than 1.54g/ml, pool D2 contained fractions having a density between 1.54 and 1.43g/ml and pool D3 contained fractions having a density less than 1.43g/ml. D1 contained most of the uronic acid content of the extract, although a small increase was observed in D2 at density 1.48g/ml, and D3 contained most of the protein content of the extract. The pooled material was converted to its potassium salt, and then dialysed exhaustively against water at 4°C before being freeze-dried. The D1 preparation from the articular cartilage was obtained in a similar manner.

Analysis of tissue residues

The tissue residue was washed exhaustively with water and then subjected to degradation with bacterial collagenase at a concentration of 4mg/g of tissue. The degradation was performed in 0.2M-Tris/HCl, pH7.0, containing 1mM-$CaCl_2$, at 40°C for 16h. The solution was made 5mM with respect to EDTA and dithiothreitol, and papain was added at a concentration of 4mg/g of tissue. Incubation was continued for a further 16h, at which time all meniscus samples had been solubilized, except for the pooled tissue from 61–70 year olds. In addition, the foetal/newborn and 4 year-old samples exhibited a fine fibrillar suspension. The digest was clarified by centrifugation at 500$g_{av.}$ ($r_{av.}$ 13.1cm), and 4vol. of ethanol was added to the supernatant. The dried precipitate was redissolved in water for measurement of uronic acid content.

Proteolytic degradation

The proteoglycan (D1) was subjected to degradation by either papain or pepsin (Roughley, 1978). Degradation was performed at a D1 concentration of 2mg/ml in 0.2M-sodium acetate, pH5.0; 5mM-EDTA and 5mM-cysteine were added to the buffer used for papain digestion. The enzymes were added at a concentration of 10μg/ml of D1, and two equal additions were made, one at the commencement of incubation and one after 4h. Incubation was allowed to proceed for 18h at 40°C; then pepsin was inhibited by the addition of pepstatin (10μg/ml), and papain by the addition of iodoacetic acid (10mM). The resulting solutions were subjected directly to chromatography on Sepharose 4B or to electrophoresis in agarose/polyacrylamide gels after dialysis against water.

Cetylpyridinium chloride precipitation of the papain digest

A portion of the solution containing the papain-digested D1 preparation was subjected to precipitation in 1% cetylpyridinium chloride (Roughley & Barrett, 1977). The precipitate was washed twice with 1% cetylpyridinium chloride, redissolved in a minimal volume of propan-2-ol/water (3:2, v/v), and then reprecipitated by the addition of 2vol. of a saturated solution of potassium acetate in ethanol. The dried (vacuum desiccator) precipitate was dissolved in water and analysed for hexosamine content.

Reduction and alkylation

The proteoglycan (D1) was dissolved in 4M-guanidinium chloride/0.05M-Tris/HCl, pH7.35, containing 5mM-dithiothreitol at a concentration of 2mg/ml (Heingegård, 1977). The mixture was incubated at 40°C for 4h, and then iodoacetic acid was added to a concentration of 15mM and the mixture left at 25°C for a further 18h. After conversion to its potassium salt, the solution was exhaustively dialysed against water and freeze-dried. This sample was redissolved in 0.2M-sodium acetate, pH5.5, for determination of viscosity and chromatography on Sepharose 2B.

Hyaluronic acid content of the D1 preparation

Proteoglycan (D1) was dissolved in 20mM-NaCl to a concentration of 2mg/ml. Sulphated proteoglycans were precipitated under acid conditions by the addition of cetylpyridinium chloride as described by Cleland & Sherblom (1976). Neutralization of the supernatant then resulted in the precipitation of hyaluronic acid. The precipitate was dissolved in a minimal volume of propan-2-ol/water (3:2, v/v), and then reprecipitated by the addition of 2vol. of ethanol saturated with potassium acetate. The dried precipitate was redissolved in water and uronic acid content was determined.

Chondroitinase degradation

The proteoglycan (D1) was dissolved in 0.1M-sodium acetate/0.1M-Tris/HCl, pH7.3, at a concentration of 8mg/ml (Hascall & Heinegård, 1974). Chondroitinase ABC (5units/ml) was added at a concentration of 10μl/mg of D1 and the mixture was incubated at 40°C for 2h. The samples were then heated at 100°C for 10min before being analysed by t.l.c. (Wasserman *et al.*, 1977) on cellulose plates (250μm thickness) with zinc silicate incorporated as a fluorescent agent. A portion (25μl) of the digestion mixture was applied, and elution was first performed with butanol/ethanol/water (13:8:4, by vol.), and then with butanol/acetic acid/aq. 1M-NH_3 (2:3:1, by vol.). The degradation products were identified as areas of fluorescent quenching under u.v. irradiation; they were then removed and eluted from the powdered cellulose by incubation in 0.5ml of water at 40°C for 16h. A portion (0.2ml) of the resulting solution was assayed for disaccharide content by the method of Koseki *et al.* (1978). A mixture of unsaturated disaccharides (non-sulphated, 4-sulphated and 6-sulphated) was used for reference standards in the above procedure. In other experiments chondroitinase ABC or AC (5units/ml) was added to both D1 and D2 preparations, at a concentration of 10μl/mg of D1 or D2, and incubated for 4h. A second identical addition of enzyme was then made and the samples were incubated for a further 16h. The resulting solution was assayed for disaccharide content.

Viscometry

The proteoglycan (D1) was dissolved in 0.2M-sodium acetate, pH5.5, at 2mg/ml. The flow time of this solution (1ml) was measured at 25°C in a Cannon–Manning semi-microviscometer. The specific viscosity of the proteoglycan was calculated relative to the flow time of an equal volume of buffer. Hyaluronic acid (2mg/ml in the same buffer) was then added to give a weight ratio of 100:1 (proteoglycan/hyaluronic acid). After allowance of 30min for equilibrium to be obtained, the flow time was again recorded.

Sepharose chromatography

The proteoglycan (D1) was dissolved in 0.2M-sodium acetate, pH5.5, at a concentration of 2mg/ml, and chromatographed on Sepharose 2B (Pharmacia). Proteolytic degradation products of D1 were chromatographed on Sepharose 4B. In each case 1ml samples were applied to the column (53cm × 1.0cm) and chromatography was by downward elution at 4°C with a flow rate of 8ml/h. Fractions (1ml) were collected and assayed for their uronic acid content. Column void volumes were determined by the elution of a proteoglycan aggregate preparation from bovine nasal cartilage, and the column total volumes by the elution of glucuronolactone. In all cases the equilibration and elution buffer was 0.2M-sodium acetate, pH5.5.

Agarose/polyacrylamide-gel electrophoresis

The proteoglycan (D1) and its proteolytic degradation products were electrophoresed in agarose/polyacrylamide gels composed of 0.6% agarose and 1.2% acrylamide (McDevitt & Muir, 1971). The sample (20μl) for electrophoresis consisted of the proteoglycan (2mg/ml in water), chondroitin sulphate (1mg/ml in water) and 50% sucrose containing 0.05% Bromophenol Blue in the proportions 2:1:1 (by vol.). Chondroitin sulphate was prepared by degradation of the proteoglycan from bovine nasal cartilage with 0.5M-NaOH (Roughley & Barrett, 1977). After electrophoresis the gels were stained with 0.02% Toluidine Blue in 0.1M-acetic acid at 25°C, and then destained with 3% (v/v) acetic acid at 35°C. The mobility of the stained bands was calculated relative to the mobility of the chondroitin sulphate (R_{CS}).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis was performed in a slab gel containing 10% (w/v) acrylamide. A stacking gel and discontinuous buffer system were employed as described by Laemmli & Favre (1973). The D1 preparations were dissolved to 2mg/ml in water and the D3 preparations were dissolved to 2mg/ml in 1% sodium dodecyl sulphate to ensure complete solubilization. Equal volumes of the sample solutions and sample buffer were mixed and boiled for 3min. Samples of volume 20μl were applied to the gel. The gel was stained with 0.1% Coomassie Brilliant Blue G250 in methanol/acetic acid/water (10:4:5, by vol.) at 25°C. Destaining was performed at 55°C with methanol/water/formic acid (30:69:40, by vol.).

Analytical techniques

Uronic acid was determined by the carbazole reaction (Bitter & Muir, 1962). Amino acids and hexosamines were determined by using a Durrum

amino acid analyser. Hydrolysis for amino acid analysis was performed on a 2 mg/ml solution of D1 in 6 M-HCl at 105°C for 20 h under vacuum. Hydrolysis for hexosamine analysis was performed on a 1 mg/ml solution of D1 in 4 M-HCl at 100°C for 8 h. Protein content was determined by summation of the amino acid residues, excluding cystine/cysteine.

Results

Extraction of proteoglycan

Examination of the yields of the D1 preparations showed that, in general, slightly more material was extracted from the adult tissues than from those of the child. The D1 preparations ranged from about 1 mg/g to 2.8 mg/g of wet tissue. The yield from the adult was about one-eighth of the yield from the mature human articular cartilage (Table 1). The yield of D2 remained relatively constant for all ages, averaging 4.4 mg/g of wet tissue. The percentage uronic acid content of the D1 preparations increased from about 9% in the foetal/newborn sample to about 15% in the adult samples. This is opposite to the trend observed in articular cartilage (Roughley & White, 1979), although both adult preparations are of comparable uronic acid content. The D2 preparations showed no age-related trend with respect to uronic acid content, and the content was always lower than that found in the equivalent D1 preparation (Table 2). The yield of D3 showed a decrease with age, ranging from about 31 mg/g of tissue in the foetal/newborn age group to 14 mg/g of tissue in the 61–70 year-age group. Amino acid analyses of the D3 preparations demonstrated the presence of hydroxyproline only in the foetal/newborn and the 4 year-old material.

Analysis of the tissue residues after extraction showed that there was an increase in uronic acid retention with age, ranging from about 0.4 mg/g of wet tissue in the foetal/newborn group to 1.2 mg/g in the 61–70 year-age group (Table 2). The uronic acid content of D1, expressed as a percentage of the total uronic acid content of the tissue, showed an increase with age, which is the reverse of the situation in articular cartilage (Roughley & White, 1979). The susceptibility of the meniscus to degradation by collagenase and papain showed considerable variation with age, with resistance to proteolysis increasing with age. At all ages the meniscus was much more resistant to degradation than an age-matched cartilage sample.

Table 1. *Yields of D1-, D2- and D3 preparations after extraction*

For experimental details see the text.

Age (years)	Yield (mg/g of wet tissue) D1	D2	D3
Miniscus			
Foetal/newborn	1.0	4.6	30.9
4	2.6	4.5	37.2
10	1.4	3.0	29.5
22	1.4	3.7	29.1
23	2.6	4.3	16.8
37	2.8	5.0	18.0
41–50	2.6	5.4	17.1
51–60	2.7	4.8	16.2
61–70	2.6	3.2	14.1
Cartilage			
50–60	20.0	14.0	24.6

Table 2. *Uronic acid content of the tissue extract and residue*

For details see the text.

Age (years)	Uronic acid content: D1 preparation (% of dry wt.)	D2 preparation (% of dry wt.)	Tissue residue (mg/g of wet tissue)	D1 preparation (% of total uronic acid)
Meniscus				
Foetal/newborn	9.4	6.9	0.44	10.6
4	8.9	8.4	0.66	18.8
10	10.6	7.5	0.73	14.7
22	15.2	8.3	0.93	15.0
23	12.8	8.6	0.74	24.8
37	14.7	6.7	0.90	24.4
41–50	13.1	5.3	0.87	23.0
51–60	15.7	7.6	0.74	27.8
61–70	15.2	7.7	1.17	21.1
Cartilage				
50–60	15.5	7.6	3.4	45.0

Size of the D1 proteoglycan

Viscometric examination of the D1 preparations revealed no definite age-related trend. Addition of hyaluronic acid to the D1 preparations produced only minor increases in viscosity, in contrast with the pronounced interaction seen between the articular-cartilage D1 preparation and hyaluronic acid (Table 3). On Sepharose 2B chromatography, the adult D1 preparations (Fig. 1) were eluted mainly at the void volume, suggesting the presence of large macromolecular components. None of the elution profiles were altered significantly by the addition of hyaluronic acid. To determine whether the void-volume component was derived from the interaction of smaller components, the D1 preparations were subjected to reduction with dithiothreitol and subsequent alkylation with iodoacetic acid. The resulting profiles on Sepharose 2B (Fig. 1) showed decreased elution at the void volume, with much of the uronic acid-containing material now being eluted in the included volume. These preparations appeared to be very heterogeneous in size, having a continuous spectrum of $K_{av.}$ values between 0 and 0.75. This was similar to the profile obtained for the articular-cartilage proteoglycan after reduction and alkylation. The decrease in size after reduction and alkylation was also reflected by a decrease in viscosity (Table 3).

It is possible that interaction within the D1 preparation was due to the presence of hyaluronic acid. Therefore to determine if hyaluronic acid was present, the D1 preparation was subjected to cetylpyridinium chloride precipitation under acid conditions, as described by Cleland & Sherblom (1976). The occurrence of uronic acid in the supernatant indicated that hyaluronic acid was present, but accounted for no more than 2.3% of the total weight of the D1 preparation.

Electrophoresis in agarose/polyacrylamide gels demonstrated that, irrespective of age, there were two major components present in the D1 preparations, having average R_{CS} values of 0.61 and 0.70. In the meniscus D1 preparations from younger age groups, a more mobile minor component was also seen with R_{CS} about 0.79. The major components were of similar mobility to those present in the adult human articular cartilage, where R_{CS} values of 0.59, 0.64 and 0.69 were observed. Examination of the D2 preparation revealed that the three components seen on electrophoresis of the D1 preparations were present, although the intensity of the more mobile component was often greater.

Glycosaminoglycan composition of the D1 preparations

A decrease in the galactosamine/glucosamine ratio was observed (Table 4) with age, from 6.8 in the foetal/newborn D1 preparation to 2.7 in the adult D1 preparation. The addition of cetylpyridinium chloride to the glycosaminoglycans obtained after papain digestion of a D1 preparation from the 51–60-year age group precipitated material with a galactosamine/glucosamine molar ratio of about 60:1. This indicated that the precipitate contained predominantly chondroitin sulphate and/or dermatan sulphate and little if any heparan sulphate. The small amount of glucosamine is probably attributable to traces of hyaluronic acid present in the preparation. As keratan sulphate is soluble under the conditions of precipitation, it would appear that this glycosaminoglycan is responsible for most of the glucosamine content of the D1 preparation. Degradation of the D1 preparations from older adults with chondroitinases ABC and AC indicated that dermatan sulphate contributed about 20% of the galactosaminoglycans. However, there was an increased content of dermatan sulphate in the D2 fraction (about 50%), indicating a greater abundance of this glycosaminoglycan in the low-density protein-rich proteoglycans.

Table 3. *Specific viscosity of D1 preparations*

The viscosities of the D1 preparations were measured relative to that of 0.2 M-sodium acetate, pH 5.5, in the absence and presence of hyaluronic acid, and after reduction/alkylation.

Age (years)	Specific viscosity: D1 preparation	D1 with 1% (w/w) hyaluronic acid	D1 after reduction/alkylation
Meniscus			
23	0.66	0.88	—
41–50	0.58	0.72	—
51–60	0.71	0.82	0.36
61–70	0.59	0.83	0.30
Cartilage			
50–60	0.52	1.66	0.44

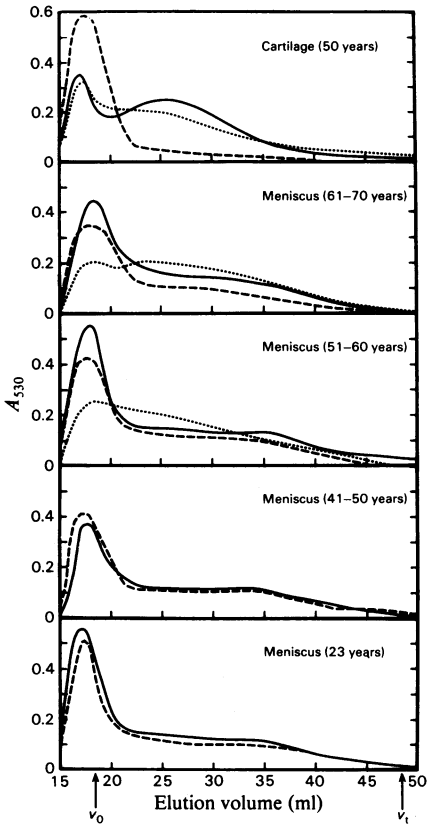

Fig. 1. *Gel chromatography on Sepharose 2B of meniscus and cartilage D1 preparations in the absence and presence of hyaluronic acid, and after reduction/alkylation*

Samples were eluted with 0.2 M-sodium acetate, pH 5.5, and the elution profiles were determined by measurement of uronic acid content. The void volume (V_0) and total volume (V_t) of the column were determined by the elution position of proteoglycan aggregate from bovine nasal cartilage and glucuronolactone respectively. The profiles represent the D1 preparations (——), the D1 preparations after addition of 1% (w/w) hyaluronic acid (----) and the D1 preparations after reduction/alkylation (••••).

Chondroitinase ABC was employed to determine the position of sulphation along the chondroitin sulphate or dermatan sulphate chains (Seno *et al.*, 1975). In no sample was the disulphated disaccharide detected, and the non-sulphated disaccharide was always found to be a minor component comprising 5% or less of the disaccharides. At least 95% of the disaccharides were sulphated in the 4 or 6 position, with 6-sulphation predominating in the older specimens (Table 4). The amount of 6-sulphation ranged from about 50% in the D1 preparation from the 10-year-age group to about 94% in the D1 preparation from the 61–70-year-age group. The non-sulphated disaccharide, characteristic of hyaluronic acid (Wasserman *et al.*, 1977), was not detected on t.l.c., and therefore, if present, was only a minor component of the D1 preparations.

Table 4. *Hexosamine content and sulphation of galactosamine residues in D1 preparations*

For details see the text.

Age (years)	Molar ratio: Galactosamine/glucosamine	Molar ratio: 6-Sulphation/4-sulphation
Meniscus		
Foetal/newborn	6.78	—
4	5.21	1.6
10	4.63	1.0
22	3.48	4.6
23	3.24	5.1
37	2.62	3.8
41–50	2.91	6.6
51–60	2.89	7.1
61–70	2.67	14.8
Cartilage		
50–60	2.30	21.0

Protein composition of the D1 preparation

Amino acid analysis of the meniscus D1 preparation from the 51–60-year-age group was similar to that of age-matched articular-cartilage proteoglycan, with serine, glutamate, proline and glycine being the predominant amino acids (Table 5). Relative to articular cartilage of an equivalent age, there was an increased abundance of leucine, aspartate, lysine, arginine, isoleucine, histidine, and methionine and a decreased abundance of valine, alanine, threonine, phenylalanine and tyrosine. No hydroxyproline, characteristic of collagen, was detected in the D1 preparation.

The protein content of the D1 preparation, calculated by summation of the amino acid residues, indicated that the proteoglycan from the 51–60-year age group contained 16.7% protein. This compares with a value of 11.8% of an age-matched articular-cartilage D1 preparation. The presence of extraneous proteins in the D1 preparations was assessed by electrophoresis in a 10% (w/v) polyacrylamide gel. In the preparations from the youngest groups only minute traces of protein entered the gels, indicating that the preparations were essentially free from protein contamination. The older preparations showed no evidence of extraneous protein.

Table 5. *Amino acid analyses of meniscus and cartilage D1 preparations from the 50–60-year age group*

For details see the text.

Amino acid	Content (residues/1000 residues) Meniscus	Cartilage
Asp	73	65
Thr	65	75
Ser	89	91
Glu	116	133
Pro	93	105
Gly	96	112
Ala	68	82
Val	78	81
Met	9	6
Ile	44	32
Leu	92	75
Tyr	17	31
Phe	27	41
His	25	14
Lys	58	18
Arg	51	39

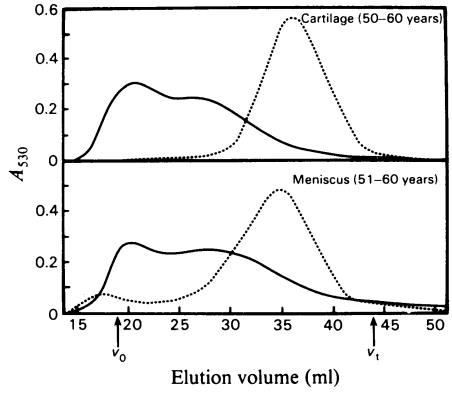

Fig. 2. *Gel chromatography on Sepharose 4B of D1 preparations of cartilage (a) and meniscus (b) from 50–60-year-old adults after proteolytic degradation*

Samples were eluted with 0.2 M-sodium acetate, pH 5.5, and the elution profiles were determined by measurement of uronic acid content. The void volume (V_0) and total volume (V_t) of the column were determined by the elution position of proteoglycan aggregate from bovine nasal cartilage and glucuronolactone respectively. The profiles represent the D1 preparations after proteolytic degradation by papain (····) and pepsin (——).

Proteolytic degradation of the D1 preparation

Human meniscus proteoglycan from the 51–60-year age group, subjected to degradation by papain, produced a single band on agarose/polyacrylamide-gel electrophoresis which was indistinguishable in mobility from single-chain chondroitin sulphate-linked peptides produced from bovine nasal cartilage by the same enzyme. Human articular-cartilage proteoglycan behaved in a similar manner (Roughley & White, 1979). On Sepharose 4B chromatography (Fig. 2), the uronic acid-elution profile after papain degradation exhibited a single peak of $K_{av.}$ 0.66, which is similar to that obtained from age-matched human articular cartilage. In addition, traces of material were eluted at the void volume, which may represent the small amounts of hyaluronic acid present in the preparation. No material was eluted in the vicinity of the total column volume.

When the meniscus D1 preparation from the 51–60-year-age group was subjected to pepsin degradation and then examined by Sepharose 4B chromatography (Fig. 2), the uronic acid-elution pattern showed a bi-modal distribution. Material was eluted from the void volume into the included volume, with the uronic acid maxima having $K_{av.}$ values of 0.06 and 0.38. No material was eluted near the total column volume. Fragments produced by pepsin are therefore larger than single-chain chondroitin sulphate-linked peptides. The degradation products from age-matched articular-cartilage proteoglycan have a similar elution profile, indicating a similar range in sizes. Electrophoresis of the degradation products on agarose/polyacrylamide gels showed two zones of staining with R_{CS} values of 0.74 and 0.89 respectively. This pattern was different from that obtained with age-matched articular-cartilage proteoglycans, where two major zones of staining were seen wtih R_{CS} values of 0.68 and 0.74.

Discussion

There has been no reported study of the isolation and structural characterization of the intact meniscus proteoglycan, although analyses of the chemical composition of meniscus have been made. Solheim (1965) first described the presence of glycosaminoglycans in the meniscus, and demonstrated that chondroitin sulphate in the 6-sulphated form and small amounts of hyaluronic acid were present. Lehtonen *et al.* (1966) extended these observations by showing the presence of keratan sulphate. Peters & Smillie (1972) made the observation that there was an increase in the hexosamine and uronic acid content with age. Habuchi *et al.* (1973) subsequently demonstrated that chondroitin sulphate and dermatan sulphate were in the form of a co-polymer. Ingman *et al.* (1974) demonstrated that the collagen and hexosamine contents increased with age, and that the non-collagenous protein content de-

creased with age. They also observed that, in a preliminary experiment in which the dissociative extraction technique described by Sajdera & Hascall (1969) was used, proteoglycan could be released from the tissue.

In the present study, the yield of proteoglycan extracted under dissociative conditions (D1 preparation) appeared to increase slightly with age up to maturity and thereafter remained relatively constant. The reverse is true of articular cartilage where extractable proteoglycan is high in the young and decreases steadily with age (Inerot *et al.*, 1978; Roughley & White, 1979). Examination of the tissue residues showed an increased uronic acid retention with age. This indicates an overall increase in the total proteoglycan content of the tissue with age, but also suggests that more of the uronic acid-containing material may interact strongly with collagen in the older specimens. In contrast with the yields of the D1 preparations, the yields of the D2 preparations showed little variation with age. This may suggest that the increased content of tissue proteoglycan is due to the preferential synthesis of a high-density glycosaminoglycan-rich proteoglycan. The yields of the D3 preparation showed a significant decrease with age. Analysis of this preparation for hydroxyproline showed that soluble collagen was only found in the youngest tissues. These observations are consistent with additional and/or more stable collagen cross-linking with age and therefore lower extractability (Bornstein, 1976).

Examination of the D1 preparations on Sepharose 2B chromatography showed that much of the proteoglycan was eluted near the void volume, and raised the question as to whether the proteoglycan was already present in an aggregated form. The profile obtained on Sepharose 2B was certainly modified after reduction/alkylation, suggesting the presence of aggregation which is dependent on disulphide bonds. If aggregation does occur by a mechanism similar to that described for cartilage (Hardingham & Muir, 1972, 1974), the apparent lack of significant interaction between additional hyaluronic acid and the D1 preparation may indicate that the proteoglycan is already bound to hyaluronic acid, and the presence of small amounts of hyaluronic acid has been demonstrated in the preparations. Crossed immunoelectrophoresis (Clarke & Freeman, 1967) in the absence and presence of hyaluronic acid (D. McNicol & A. R. Poole, unpublished work) has demonstrated that the meniscus D1 preparation may be in a partly aggregated form similar to that of the cartilage proteoglycan–hyaluronic acid complex.

After reduction/alkylation, the proteoglycan present in the meniscus D1 preparation showed a similar elution profile on Sepharose 2B to that of the articular-cartilage proteoglycans. This suggests that they are of comparable size and are also very heterogeneous. Additionally, electrophoresis in agarose/polyacrylamide gels demonstrates that both proteoglycan preparations are of similar mobility. The difference in the observed band patterns, however, indicates that structural variations are present.

Evidence from the digestion of the D1 preparations with chondroitinases ABC and AC indicates the presence of chondroitin sulphate and dermatan sulphate. Whether all proteoglycan contains both chondroitin sulphate and dermatan sulphate on a common core is open to question, and the possibility of different populations of proteoglycans existing between the D1 and D2 preparations cannot be discounted. The third more mobile band seen on agarose/polyacrylamide-gel electrophoresis appeared more intense in the D2 preparations, and these preparations also possessed a much higher dermatan sulphate content. It may be postulated that the D2 preparations contain a protein-rich dermatan sulphate-rich proteoglycan as the major component, whereas chondroitin sulphate-rich proteoglycans are more abundant in the D1 preparations. However, a continuous spectrum of changes cannot be discounted.

The fall in the galactosamine/glucosamine ratio with age resembles that seen in articular cartilage (Bayliss & Ali, 1978; Roughley & White, 1979). This suggests that the age changes in chondroitin sulphate/dermatan sulphate to keratan sulphate are similar for both tissues, with keratan sulphate being more prevalent in older tissues. Likewise the position of sulphation along the chondroitin sulphate/dermatan sulphate chains shows that 6-sulphation dominates with age, and in age-matched articular cartilage a similar trend is seen (Roughley & White, 1979). Thus, although the age-related changes in the abundance and structure of the meniscus proteoglycans may differ from articular cartilage, the synthetic enzyme systems responsible for glycosaminoglycan structure are subject to the same age-related trends in both tissues.

Those amino acids that have been suggested to be involved in the protein-binding site of chondroitin sulphate in cartilage proteoglycan (Isemura & Ikenaka, 1975) also predominate in the meniscus proteoglycan. This therefore suggests that similar chondroitin sulphate-binding sites may be present in both tissues. However, the overall dissimilarity in the amino acid analyses suggests differences in the core-protein sequences in the proteoglycan of the two tissues. Likewise, the protein contents of proteoglycan from age-matched articular cartilage and meniscus differ, with that from meniscus having a higher protein content. This implies less glycosylation of the core protein in the meniscus proteoglycan, and may contribute to the lower viscosity of the meniscus proteoglycan after reduction and alkylation.

Papain degradation of the meniscus D1 preparation revealed that the chondroitin sulphate/dermatan sulphate chains were of a similar length to those found in an age-matched articular cartilage. Further, only single-chain chondroitin sulphates were produced by this enzyme from either the cartilage or the meniscus. This is to be expected, however, if the linkage peptides for chondroitin sulphate are common to both proteoglycans and if papain cleaves within these regions (Roughley & Barrett, 1977). In contrast, the products after pepsin degradation of the meniscus D1 preparations differ in mobility on electrophoresis from those obtained from cartilage. This suggests that the structure of the proteoglycan remote from the chondroitin sulphate chains may be different for the two tissues.

If the human meniscus is involved in a significant weight-bearing role, it would seem advantageous to contain a cartilage-like proteoglycan, to convey elasticity to the tissue enabling it to tolerate weight-bearing more readily. This would be particularly important in the adult, and in this age range the meniscus proteoglycan appears to have similar size and compositional properties to that from articular cartilage. However, in the adult meniscus the D1 preparation accounted for only 2.8mg/g wet wt. of tissue compared with 20mg/g wet wt. in adult cartilage. The large difference in tissue concentrations suggests that, if the proteoglycan is distributed evenly throughout the meniscus, any elastic properties imparted to the tissue may not be significant. However, histochemical examination (D. McNicol, unpublished work) with Safranin O staining (Rosenberg, 1971) has demonstrated that the proteoglycan of the adult meniscus is localized in a circumscribed central area and as such may help elastic properties.

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