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The presence of a cartilage-like proteoglycan in the adult human meniscus

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Proteoglycans were extracted from the adult human meniscus under dissociative conditions and purified by CsCl-density-gradient centrifugation. The preparations of highest density contained proteoglycan that possessed the ability to interact with hyaluronic acid, was of large subunit size and was composed of chondroitin sulphate, keratan sulphate and sialic acid-containing oligosaccharides. This 'cartilage-like' proteoglycan also exhibited subunit and aggregate structures analogous to those of hyaline-cartilage proteoglycans when examined by electron microscopy. However, the composition of this proteoglycan was more comparable with proteoglycans from immature cartilage than from age-matched cartilage. The preparations from lower density, which were enriched in dermatan sulphate, contained smaller proteoglycan that was not able to interact with hyaluronic acid. This non-aggregating proteoglycan may be structurally distinct from the 'cartilage-like' proteoglycan, which does not contain dermatan sulphate.

The menisci of the human knee joint are located between the femoral condyles and the tibial plateau. Such a localization results in the menisci being subjected to compressive forces during weight-bearing. Shrive (1974) and Krause *et al.* (1976) have shown that between 30 and 65% of the total load across the joint is transmitted through the menisci, and they concluded that this load distribution lessens the compressive forces on the articular cartilage and subchondral bone. If the menisci are to be effective in this weight-bearing role, it would be advantageous if they contained proteoglycan of a similar structure and functional capacity to that of articular cartilage, as it is the proteoglycan content of articular cartilage that provides the tissue with much of its resilience to compressive loading (Kempson *et al.*, 1976).

In a previous paper (McNicol & Roughley, 1980) we demonstrated that high-density preparations (D1) obtained by CsCl-density-gradient centrifugation of extracts from the adult human meniscus contained proteoglycan molecules of a similar size and glycosaminoglycan content to those present in articular cartilage, though the tissue concentrations were considerably lower. Moreover, these proteoglycans appeared to be less prevalent in the younger meniscus. Differences were observed in the properties of equivalent preparations from meniscus and cartilage, particularly with respect to the presence of dermatan sulphate in the former preparation and the inability of this preparation to interact with hyaluronic acid. This latter observation was thought to be due to hyaluronic acid already present in the meniscus proteoglycan preparation. Further, it was not clear whether the dermatan sulphate was an integral part of the 'cartilage-like' proteoglycans.

The purpose of the present study was (1) to purify further the 'cartilage-like' proteoglycans from the D1 preparation of adult human meniscus in order to demonstrate conclusively their interaction with hyaluronic acid and to compare in detail their structure with that of an equivalent preparation from age-matched articular cartilage, and (2) to characterize any other classes of proteoglycan that may be present, with particular reference to their ability to aggregate with hyaluronic acid and to their content of dermatan sulphate.

Experimental

The materials employed in the present study were obtained from the same sources as those described by McNicol & Roughley (1980). This previous paper also gives experimental details for (1) degradation of proteoglycan by papain, pepsin, chondroitinase ABC and chondroitinase AC, (2) electrophoresis of proteoglycan and its proteolytic degradation products in agarose/polyacrylamide gels, (3) viscometric determinations of the proteoglycan

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in the presence and in the absence of hyaluronic acid, and (4) chromatography of the proteoglycan on Sepharose 2B.

Source of tissue

Human menisci, from both the lateral and medial compartments of the knee joint, were removed at autopsy, within 18h of death, from patients aged 62–81 years. Joints were chosen that appeared macroscopically normal. Tissue was carefully trimmed to remove all synovium and capsular material. Age-matched articular cartilage was taken from the tibial plateau of the same cadaver knees, directly beneath the meniscus, and in all cases it was of normal macroscopic appearance. The meniscus and cartilage samples were stored at −20°C before extraction.

Extraction of proteoglycan

Diced menisci were extracted at 4°C with 10 vol. of 4M-guanidinium chloride, containing proteinase inhibitors, as described by McNicol & Roughley (1980). The extract was filtered through glass-wool and its density was then adjusted to 1.51g/ml by the addition of solid CsCl (0.76g/ml of extract). Guanidinium chloride (0.22g/ml of extract) was also added to maintain its concentration at 4M. This solution was then subjected to density-gradient centrifugation at 100000 $g_{av.}$ ($r_{av.}$ 6.3cm) for 48h at 10°C, and the resulting gradients were fractionated into three preparations of decreasing density: D1 (density greater than 1.55g/ml), D2 (density between 1.45 and 1.55g/ml) and D3 (density less than 1.45g/ml). The D1 preparation was mixed with 4M-guanidinium chloride so that its density was lowered to 1.53g/ml, and this solution was subjected to a second density-gradient centrifugation under the conditions described above. The gradients were fractionated to yield three further preparations: D1D1 (density greater than 1.57g/ml), D1D2 (density between 1.47 and 1.57g/ml) and D1D3 (density less than 1.47g/ml). All preparations were converted into their potassium salts by dialysis against potassium acetate, then dialysed exhaustively against water and freeze-dried. Equivalent preparations were obtained from the articular cartilage in a similar manner.

Alkaline degradation

The D1D1 preparations were dissolved at 5mg/ml in 50mM-NaOH containing 1M-$NaBH_4$, and incubated at 45°C for 48h (Carlson, 1968). The solution was then neutralized by the addition of acetic acid, and chromatographed immediately on Sephacryl S200. Alternatively, samples were stored at −20°C before chromatography (DeLuca *et al.*, 1980).

Sephacryl S200 chromatography

The proteolytic and alkaline-degradation products of D1D1 preparations were chromatographed at 4mg/ml on a Sephacryl S200 column (57cm × 0.9cm). A sample volume of 0.8ml was applied and 0.8ml fractions were collected, elution being with 0.5M-NaCl/0.1M-sodium acetate buffer, pH5.5, at 8ml/h. Fractions were assayed for their uronic acid and sialic acid contents. The column void volume was determined by the elution position of the cartilage D1 proteoglycan and the total volume by the elution of glucuronolactone.

Analytical methods

Uronic acid was determined by the carbazole reaction (Bitter & Muir, 1962) and sialic acid by the periodate/resorcinol method (Jourdian *et al.*, 1971). Unsaturated disaccharides obtained after chondroitinase degradation of the proteoglycan were separated by t.l.c. on cellulose plates (Wasserman *et al.*, 1977) and then quantified by the periodate/thiobarbiturate method (Koseki *et al.*, 1978). Hexosamines and amino acids were determined by using a Durrum amino acid analyser. Hydrolysis for hexosamine analysis was performed on a 1mg/ml solution of the proteoglycan preparation in 4M-HCl at 100°C for 8h. Hydrolysis for amino acid analysis was performed on a 2mg/ml solution of the proteoglycan preparation in 6M-HCl at 105°C for 20h under an N_2 atmosphere. Protein content was estimated by summation of the amino acid residues.

Electron microscopy

Proteoglycans were mixed with cytochrome *c* and spread on the surface of a 0.3M-ammonium acetate solution (pH5). The resulting film was picked up on grids coated with parlodion or carbon-reinforced parlodion (Rosenberg *et al.*, 1970, 1975; Buckwalter, 1979). Specimens were stained with uranyl acetate, shadowed with platinum/palladium (4:1) and examined by electron microscopy. Molecular dimensions were measured with a MOPP 3 graphics analyser (Carl Zeiss).

Results

Fractionation of meniscus components

Each 1g wet wt. of meniscus yielded 3.1mg, 6.3mg and 20.7mg of the D1, D2 and D3 preparations respectively, and further fractionation of the D1 preparation yielded 2.1mg, 0.7mg and 0.3mg of the D1D1, D1D2 and D1D3 preparations respectively.

Size and aggregation of meniscus and cartilage proteoglycans

The proteoglycan in the meniscus D1D1 preparation was fully retarded on Sepharose 2B, with

$K_{av.} = 0.13$ (Fig. 1). This is lower than that observed for age-matched articular cartilage ($K_{av.} = 0.23$), suggesting an increased average hydrodynamic size. On the addition of hyaluronic acid much of the proteoglycan was now eluted at the void volume and the solution exhibited a marked increase in specific viscosity (Fig. 1), compatible with the specific aggregation phenomenon described for hyaline-cartilage proteoglycans (Hardingham & Muir, 1973; Hascall & Heinegård, 1974). However, the increased amount of uronic acid still retarded and the lower specific viscosity after the addition of hyaluronic acid suggest that the meniscus preparation may also contain a proportion of molecules that are unable to aggregate with hyaluronic acid.

The proteoglycan in the meniscus D1D2 preparation was eluted with a bimodal distribution of uronic acid on Sepharose 2B (Fig. 2). The first peak was located at the void volume and the second was included, with $K_{av.} = 0.50$, and therefore contained

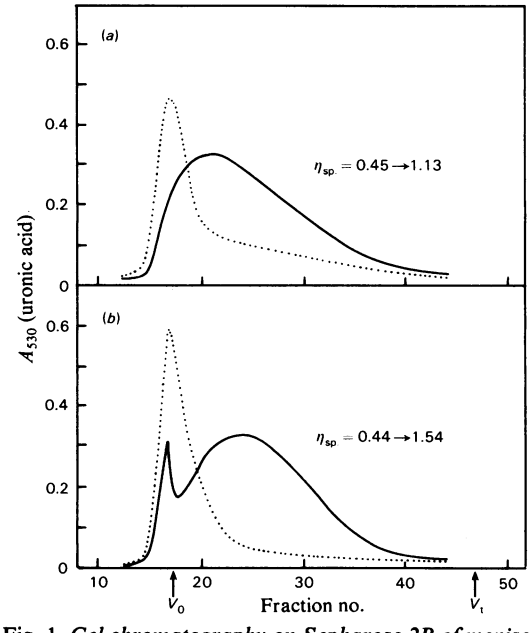

Fig. 1. *Gel chromatography on Sepharose 2B of meniscus (a) and cartilage (b) D1D1 preparations in the absence and in the presence of hyaluronic acid*

Samples were dissolved at 2 mg/ml in 0.2 M-sodium acetate buffer, pH 5.5, and eluted with the same buffer. Elution profiles were determined by the measurement of uronic acid. The profiles represent the D1D1 preparations (——) and the D1D1 preparations after the addition of 1% (w/w) hyaluronic acid (·····). The specific viscosity ($\eta_{sp.}$) of the D1D1 preparations before and after the addition of hyaluronic acid is also given. Measurements were made at 25°C on 1 ml samples in the above buffer.

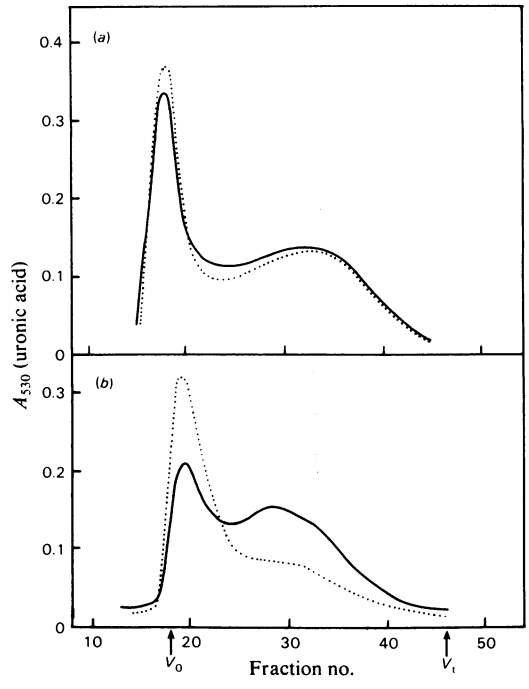

Fig. 2. *Gel chromatography on Sepharose 2B of meniscus (a) and cartilage (b) D1D2 preparations in the absence and in the presence of hyaluronic acid*

Samples were dissolved at 2 mg/ml in 0.2 M-sodium acetate buffer, pH 5.5, and eluted with the same buffer. Elution profiles were determined by the measurement of uronic acid. The profiles represent the D1D2 preparations (——) and the D1D2 preparations after the addition of 1% (w/w) hyaluronic acid (·····).

molecules of smaller size than those present in the D1D1 preparation. On the addition of hyaluronic acid the profile was unaltered, indicating a population of proteoglycans that is unable to aggregate with hyaluronic acid. A similar preparation from age-matched articular cartilage also showed uronic acid being eluted at the void volume and in the included volume, though on the addition of hyaluronic acid much of the included material appeared to be capable of aggregation.

On agarose/polyacrylamide-gel electrophoresis the meniscus D1D1 preparation appeared as three bands ($R_{cs} = 0.61$, 0.67 and 0.73), with that of intermediate mobility being most prevalent (Fig. 3). In contrast, the D1D2 preparation showed two major bands ($R_{cs} = 0.72$ and 0.82), with the less-mobile band being most prevalent and corresponding in mobility to the band of fastest mobility seen in the D1D1 preparation. The D1D1 preparation of age-matched articular cartilage exhibited two major bands ($R_{cs} = 0.61$ and 0.67), corres-

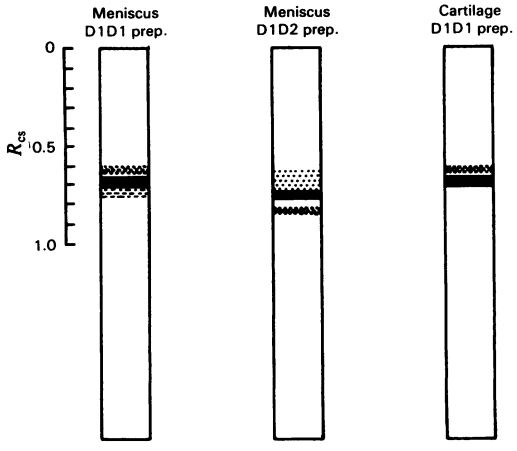

Fig. 3. *Agarose/polyacrylamide-gel electrophoresis of meniscus D1D1 and D1D2 preparations and cartilage D1D1 preparation*

The bands represent material that stained intensely (■) and diffusely (▨) with Toluidine Blue. Electrophoretic mobilities (R_{cs}) are indicated relative to chondroitin sulphate (R_{cs} = 1.0) prepared by alkaline degradation of the proteoglycan from bovine nasal cartilage.

ponding in mobility and prevalence to the less-mobile bands observed in the equivalent meniscus preparation.

Chemical composition of meniscus and cartilage proteoglycans

The meniscus D1D1 preparation has a chemical analysis compatible with the presence of a proteoglycan rich in chondroitin sulphate chains and containing smaller amounts of keratan sulphate and oligosaccharides (Table 1). In comparison with age-matched articular cartilage, the meniscus proteoglycan contained increased amounts of uronic acid and galactosamine and decreased amounts of sialic acid and glucosamine. This would be compatible with an increase in the presence of chondroitin sulphate (either more chains or longer chains) and a decrease in the presence of keratan sulphate and oligosaccharides. In addition, the proteoglycan from the meniscus possessed a lower protein content, compatible with a proteoglycan that is more glycosylated. Neither the meniscus D1D1 preparation nor the cartilage D1D1 preparation was found to contain dermatan sulphate when subjected to comparative degradation by chondroitinase AC and chondroitinase ABC, though the meniscus

Table 1. *Chemical composition of D1D1 and D1D2 preparations from meniscus and cartilage*

Chemical composition (% of dry wt.)	Meniscus D1D1	Meniscus D1D2	Cartilage D1D1
Uronic acid	19.5	14.5	18.3
Galactosamine	21.3	16.5	19.4
Glucosamine	5.7	8.6	7.3
Sialic acid	1.5	1.9	2.4
Protein	8.0	19.0	11.7
Chondroitin 6-sulphate/ chondroitin 4-sulphate (molar ratio)	13.0	2.9	22.8
Amino acid composition (residues/1000 residues)			
Asp	85	102	72
Thr	77	66	77
Ser	114	86	97
Glu	140	122	135
Pro	87	80	102
Gly	124	93	119
Ala	72	62	83
Val	68	62	76
Met	6	15	7
Ile	35	42	36
Leu	77	94	82
Tyr	17	26	20
Phe	26	34	27
His	25	28	22
Lys	20	48	15
Arg	27	41	31

preparation had slightly more 4-sulphation along its chondroitin sulphate chains.

The amino acid analysis for the meniscus D1D1 preparation is typical of those obtained for proteoglycans isolated from hyaline cartilage (Hardingham & Muir, 1974; Rosenberg *et al.*, 1976; Heinegård, 1977; Dickson & Roughley, 1978), with serine, glutamate, proline and glycine being the predominant residues. In comparison with the age-matched cartilage preparation, there is an increased abundance of serine, glycine and glutamate, and a decreased abundance of proline. Such changes are unlikely to be due to the presence of small amounts of the non-aggregating proteoglycan, as serine, glycine and glutamate are all of decreased abundance in the meniscus D1D2 preparation (Table 1). The D1D2 preparation also possessed an increased abundance of keratan sulphate relative to oligosaccharides and protein, and a decreased abundance of chondroitin sulphate. 4-Sulphation of the chondroitin sulphate chains is also much more prominent than in the D1D1 preparation.

Degradation of meniscus and cartilage proteoglycans

The meniscus D1D1 preparation was degraded by papain, a proteinase that is capable of releasing single-chain chondroitin sulphate-peptides from hyaline-cartilage proteoglycans (Roughley, 1978; Roughley & Dickson, 1980; Roughley & White, 1980). Uronic acid in the degradation products was eluted as a single included peak, with $K_{av.} = 0.15$, on Sephacryl S200 (Fig. 4). This was less retarded than an equivalent preparation from age-matched articular cartilage ($K_{av.} = 0.25$), suggesting a larger average hydrodynamic size for the chondroitin sulphate chains. In contrast, the sialic acid distribution, which represents the presence of keratan sulphate and smaller oligosaccharide chains (Thonar & Sweet, 1979; Lohmander *et al.*, 1980), gave a bimodal profile for both the meniscus and cartilage preparations, with maxima at $K_{av.} = 0.35$ and 0.85 and $K_{av.} = 0.35$ and 0.80 respectively (Fig. 4).

The D1D1 preparations were also treated with alkaline borohydride, a procedure that cleaves the *O*-glycoside linkage between glycosaminoglycans or oligosaccharide chains and serine or threonine (Kieras, 1975). Uronic acid in the degradation products from the meniscus preparation was eluted as a single peak on Sephacryl S200 (Fig. 5), with a $K_{av.}$ value identical with that for the degradation products produced by papain. However, the sialic

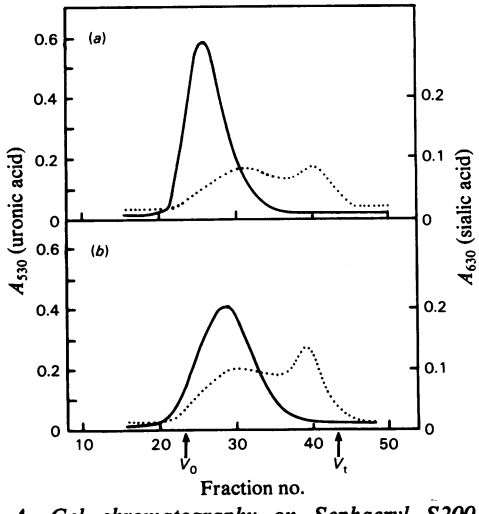

Fig. 4. *Gel chromatography on Sephacryl S200 of meniscus (a) and cartilage (b) D1D1 preparations after degradation by papain*

Samples were dissolved at 4 mg/ml in 0.2 M-sodium acetate buffer, pH 5.0, containing 5 mM-cysteine and 5 mM-EDTA, and subjected to degradation by papain. The degradation products were chromatographed on Sephacryl S200, with elution by 0.1 M-sodium acetate buffer, pH 5,5, containing 0.5 M-NaCl. Elution profiles were determined by measurement of uronic acid (——) and sialic acid (·····).

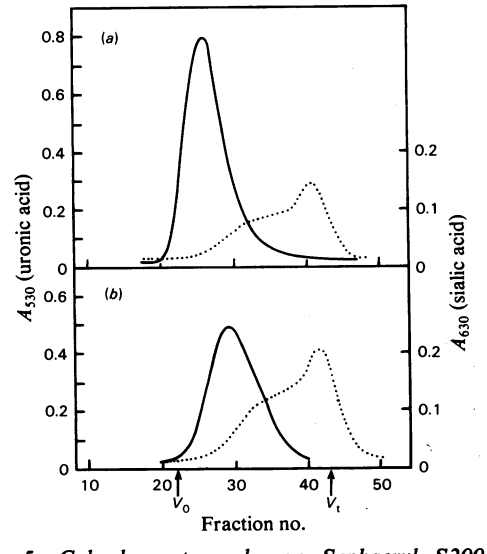

Fig. 5. *Gel chromatography on Sephacryl S200 of meniscus (a) and cartilage (b) D1D1 preparations after treatment with alkaline borohydride*

Samples were dissolved at 5 mg/ml in 50 mM-NaOH containing 1 M-$NaBH_4$. After neutralization of the solutions with acetic acid, the degradation products were chromatographed on Sephacryl S200, with elution by 0.1 M-sodium acetate buffer, pH 5.5, containing 0.5 M-NaCl. Elution profiles were determined by measurement of uronic acid (——) and sialic acid (·····).

Table 2. *Electron-microscopic measurement of proteoglycan subunits*

Source	Subunit length (nm)		Length of side chains (nm)		Mean distance between side chains (nm)	
	n	$\bar{x}$	n	$\bar{x}$	n	$\bar{x}$
Human meniscus (age range 62 to 81 years)	25	290 ± 106	797	61 ± 12	25	10 ± 2
Mature bovine nasal cartilage* (3-year-old cow)	50	321 ± 127	1823	50 ± 13‡	25	9 ± 1
Immature bovine nasal cartilage* (2-month-old calf)	75	341 ± 109‡	2936	64 ± 13‡	75	10 ± 1
Human chondrosarcomas†	200	290 ± 72	6879	73 ± 19‡	200	9 ± 1

* Data from Buckwalter & Rosenberg (1980).
† Data on four human chondrosarcomas from Buckwalter (1979).
‡ $P < 0.05$ when compared with human meniscus subunits.

acid elution profile was different between the papain and alkaline treatments, with the latter procedure resulting in greater retention. The profile now exhibited a shoulder adjacent to a more-retarded peak, of $K_{av.} = 0.87$ (Fig. 5). A similar sialic acid elution profile was obtained for the age-matched articular cartilage preparation. Thus, although papain treatment of the meniscus and cartilage D1D1 preparations gave rise to single-chain chondroitin sulphate–peptides, it may produce fragments containing multiple keratan sulphate and oligosaccharide chains on the same peptide.

Electron microscopy of meniscus proteoglycans

Monolayers of the meniscus D1 and D1D1 preparations were studied by electron microscopy. The D1 preparation contained both proteoglycan subunits and aggregates. The aggregates resembled those seen in preparations from hyaline cartilage, having numerous subunits arranged around a single central filament, which presumably represents hyaluronic acid. In contrast, the D1D1 preparation was composed entirely of subunits consisting of a core filament with side chains that presumably represent clusters of glycosaminoglycan chains.

The meniscus proteoglycan subunits were shorter than subunits from either mature or immature bovine nasal cartilage (Buckwalter & Rosenberg, 1980), but were of similar length to subunits from human chondrosarcomas (Buckwalter, 1979) (Table 2). The average length of the meniscus subunit side chains was closest to that of the immature bovine cartilage, though still shorter than that from the human chondrosarcomas. A side-chain length longer than that present in the mature bovine cartilage is in agreement with the results of biochemical analysis, which suggested that the meniscus proteoglycan subunit may be of an immature nature compared with that from autologous articular cartilage. For all the subunits the mean distance between side chains was remarkably consistent.

Discussion

The proteoglycans extracted from the adult human meniscus appeared to be of two types. The preparations isolated at high buoyant density on CsCl-density-gradient centrifugation contained molecules of large hydrodynamic size that were composed of protein, chondroitin sulphate, keratan sulphate and sialic acid-containing oligosaccharides. These proteoglycans were able to aggregate with hyaluronic acid, and possessed subunit and aggregate structures comparable with those of hyaline-cartilage proteoglycans when examined by electron microscopy. In addition to their similar structural and functional properties, the meniscus and cartilage proteoglycans also behaved in a similar manner towards degradation by either papain or alkali. Thus these aggregating meniscus proteoglycans, which do not contain dermatan sulphate, may be termed 'cartilage-like'.

In contrast, many of the molecules present in preparations isolated from lower buoyant density were incapable of aggregation with hyaluronic acid. The non-aggregating meniscus proteoglycans were of smaller size than the 'cartilage-like' proteoglycans and possessed an increased protein content relative to carbohydrate. The low-density preparations also contain dermatan sulphate (McNicol & Roughley, 1980), and it is possible that some of the non-aggregating molecules represent a genetically distinct population of proteoglycans that are the exclusive source of dermatan sulphate in the meniscus. However, one cannot exclude the possibility that some non-aggregating proteoglycans, containing only chondroitin sulphate, may be derived from the 'cartilage-like' molecules by proteolytic modification of their core proteins (Hardingham *et al.*, 1976). Unique non-aggregating proteoglycans have previously been isolated from cartilage (Swann *et al.*, 1979) and dermis (Damle *et al.*, 1979), and some of those isolated from the dermis were shown to contain dermatan sulphate.

The 'cartilage-like' proteoglycans isolated from the adult human meniscus are similar to but not identical with the proteoglycans present in an equivalent preparation from age-matched human articular cartilage. The meniscus proteoglycan possesses a larger subunit size, longer chondroitin sulphate chains with more 4-sulphation and an increased abundance of chondroitin sulphate relative to keratan sulphate and oligosaccharides. Such parameters are more directly comparable with those of the proteoglycans isolated from a more immature human articular cartilage (Roughley & White, 1980). Moreover, the amino acid composition of the core protein is also more analogous to that of an immature cartilage proteoglycan. It would therefore appear that the cells present in the adult meniscus that are responsible for the production of the 'cartilage-like' proteoglycans are behaving more as immature chondrocytes than mature chondrocytes. The production of immature 'cartilage-like' proteoglycans in the adult has also been described in chondrosarcomas, where there seemed to be a relationship between the immature nature of the proteoglycan and the degree of malignancy of the tumour (Pal *et al.*, 1978).

The 'cartilage-like' proteoglycan appears to be more prevalent in the adult meniscus than in the younger meniscus (McNicol & Roughley, 1980). As body mass increases during growth, it is possible that the meniscus is involved in an increased weight-bearing role and responds by producing a 'cartilage-like' proteoglycan in an attempt to counteract compressive loading in a manner similar to that shown by articular cartilage. Certain of the meniscus cells may then be expected to present a chondrocytic morphology, and indeed such large round cells, rich in endoplasmic reticulum, were observed by electron microscopy (D. McNicol, unpublished work) in the central region of the meniscus, where the proteoglycan concentration is greatest (McNicol & Roughley, 1980). One might also expect that such a process may be more prevalent in a joint where the articular cartilage is subject to degenerative changes and the involvement of the meniscus in a weight-bearing role would be even more important. It has been reported that the hexosamine concentration of the meniscus in the arthritic knee is increased (Ghosh *et al.*, 1975), and such an observation would be consistent with an increased proteoglycan production.

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