The glycosaminoglycans of canine menisci

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The semilunar menisci of the knee have an important mechanical function and are commonly involved in joint degeneration. However, previously published analyses of the compositions of normal and degenerate human menisci vary widely. In the present study the glycosaminoglycan content and composition of selected areas of the menisci of eight normal knees of working foxhounds were determined. The menisci contained 10% less water and about 8-fold less glycosaminoglycan than did the articular cartilage of these animals. Although the glycosaminoglycan composition was the same in different regions of the menisci, the total amounts varied considerably. Of the chondroitinase-digestible material, approx. 60% was chondroitin 6-sulphate, 25% chondroitin 4-sulphate, 10% chondroitin and 5% dermatan sulphate. Hyaluronic acid accounted for about 6% of the total uronic acid.

The knee joints of most vertebrates contain two weight-bearing wedge-shaped fibrocartilaginous menisci, one medial and one lateral, insinuated between and conforming to the shape of the femoral condyles and tibial plateaux. Studies with human and canine menisci suggest that they bear between 40 and 70% of the total load across the joint (Shrive, 1974; Seedhom *et al.*, 1974; Walker & Erkman, 1975; Krause *et al.*, 1976). The menisci are frequently damaged in otherwise healthy individuals, and the torn or loose fragments may sufficiently impair knee function that a partial or total meniscectomy is necessary. However, experimental evidence suggests that removal of even normal menisci can lead to degenerative arthritis (osteoarthrosis) in the knee (King, 1936; Shapiro & Glimcher, 1980), and clinical evidence suggests that total or partial meniscectomy or meniscal tears can lead to osteoarthritis in the knee (reviewed by Johnson *et al.*, 1974).

The glycosaminoglycans and proteoglycans provide cartilage with compressive stiffness by holding water in the tissue (Kempson *et al.*, 1970). They control the penetration of solutes into the cartilage (Maroudas, 1980), and by interacting with collagen probably influence its properties *in vivo* (Lindahl & Höök, 1978). In addition, they appear to play a role in tissue development, differentiation and regeneration (Linsenmayer & Toole, 1977). Thus the present

study was undertaken in order to determine the content and composition of glycosaminoglycans in various regions of normal canine menisci as a basis for future studies of the changes in glycosaminoglycans in experimental osteoarthritis.

Experimental

Materials

Papain (EC 3.4.22.2) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4), chondroitinase AC (chondroitin AC lyase, EC 4.2.2.5), hyaluronidase (*Streptomyces hyalurolyticus* hyaluronoglucosaminidase, EC 3.2.1.35) and the disaccharide degradation products of chondroitin (Δ -Di-0-S), chondroitin 4-sulphate (Δ -Di-4-S) and chondroitin 6-sulphate (Δ -Di-6-S) were obtained from Miles Biochemicals, Slough, Berks., U.K. Umbilical-cord hyaluronic acid was obtained from BDH Chemicals, Poole, Dorset, U.K. Cellulose t.l.c. plates were obtained from Anachem Manufacturing, Luton, Beds., U.K. All other chemicals were reagent grade or the best commercial grade available. Cetylpyridinium chloride was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Tissue

Five foxhounds aged 7–9 years of both sexes were used. Four came from the Percy Hunt, one of which had severe osteoarthritis in one knee, and the fifth, which came from the Bedale Hunt, had mild osteoarthritis in one knee. Both osteoarthritic joints

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were excluded from the present study, so that the menisci from a total of eight normal knees were analysed. Intact joints were removed within a few minutes of death and kept frozen at -20°C until dissection. The joints were thawed, opened and inspected. The menisci were removed and dissected free of their synovial and ligamentous attachments. On dissection eight joints were found to be macroscopically normal and showed no fibrillation by Indian-ink staining (Meachim, 1972).

Since the horns of the meniscus (Fig. 1) resemble ligaments and contain blood vessels (O'Connor, 1976), they were not included in this study. Each meniscus was arbitrarily divided into three portions, anterior, middle and posterior, and each portion was sampled for histology (Fig. 2). In the medial menisci, the anterior and posterior portions were thick and triangular in cross-section, whereas the central portion was thinner and flatter. In the lateral meniscus, although all portions were triangular in

Fig. 1. Diagram of the menisci of the canine stifle (knee) joint

Fig. 2. Diagram of the tissue dissection and sampling used in this study

Key: LA, lateral anterior; MA, medial anterior; LC, lateral central; MC, medial central; LP, lateral posterior; MP, medial posterior. The diagram is approx. $1\frac{1}{2}$ times actual size.

cross-section, the central portion was very thick, and, although the anterior and posterior portions were thinner in comparison, they were about the same thickness as the anterior and posterior portions of the medial meniscus. The anterior and posterior portions of each meniscus were therefore pooled as one sample (A + P) and the central portion was kept separate (Fig. 2).

Histology

The tissues taken for histology, as in Fig. 2, were embedded in paraffin and sections were stained with Haematoxylin and Eosin, Safranin O with Fast Green counterstain or Verhoeff stain with Van Gieson counterstain.

Chemical analysis

To determine the water content, each specimen was weighed, dried *in vacuo* over P_2O_5 at 80°C for 48 h and re-weighed. The glycosaminoglycans were analysed for uronic acid by an automated (Heinegård, 1973) version of the Bitter & Muir (1962) procedure. For hexosamine analysis, the tissues were hydrolysed with 8 M-HCl at 95°C for 3 h under N_2 (Swann & Balazs, 1966). The acid was removed by evaporation at reduced pressure and the residues were analysed for total hexosamine by either the manual or the automated version of the method of Blumenkrantz & Asboe-Hansen (1976), with galactosamine as a standard.

Papain digestion and isolation of glycosaminoglycans

Dried specimens were digested with papain at 60°C for 48 h in 0.1 M-sodium acetate/10 mM-cysteine hydrochloride/50 mM-disodium EDTA buffer, pH 6.0, at which time the menisci were solubilized, except for a fine particulate suspension, which was removed by centrifugation at $15000g$ (r_{av} , 18 cm) for 30 min. The supernatants were mixed with an equal volume of distilled water, and 10% (w/v) cetylpyridinium chloride was added dropwise to obtain a flocculent precipitate. This was kept at 40°C for 2 h, then centrifuged at $15000g$ for 30 min, washed twice with 0.05% cetylpyridinium chloride and redissolved in a small volume of 80% (v/v) propan-1-ol. A small amount of saturated sodium acetate was added, and the glycosaminoglycans were precipitated with a large volume of 90% (v/v) ethanol, left for 16 h at 4°C and then centrifuged at $15000g$ for 30 min at 4°C . The clear supernatants were discarded and the precipitates were dried and redissolved in water for subsequent analysis.

Characterization and analysis of glucosaminoglycans

The amount of hyaluronic acid in the glycosaminoglycans was determined by digesting a sample

of the glycosaminoglycans in 40 mM-sodium acetate buffer, pH 5.0, with 5 turbidity-reducing units of hyaluronidase (Jourdan *et al.*, 1979). After incubation for 16 h, the digests were analysed in duplicate by an automated version of the periodate/thiobarbituric acid assay (Adams & Muir, 1980). For measurement of chondroitin sulphates, samples containing 50–300 μ g of uronic acid were digested for 16 h with 0.02 unit (μ mol/min) of chondroitinase ABC or AC in 50 μ l of 50 mM-Tris/50 mM-sodium acetate buffer, pH 8.0, containing 100 mg of bovine serum albumin/l (Cöster *et al.*, 1979). The activity of the chondroitinase was assayed before use, and NaF (10 mM) was added to the reaction mixtures to inhibit any contaminating chondrosulphatases. The resulting unsaturated uronic acid disaccharides in the digests were applied to MN 300 cellulose t.l.c. plates, desalted with butan-1-ol/acetic acid/water (13:8:2, by vol.) and developed with butan-1-ol/acetic acid/2M-NH₃ (2:3:1, by vol.). The plates were dried and the separated disaccharides were located under u.v. light, scraped from the plates, suspended in 0.5 ml of water, mixed vigorously and left at 4°C for 16 h. The cellulose was removed by centrifugation at

15 000 g for 30 min and the supernatants were analysed by the automated periodate/thiobarbituric acid assay.

Results

Composition of the menisci

The water content of the normal foxhound menisci was about 63%, with little local variation (Table 1), which was significantly lower than the value of 70% for normal foxhound articular cartilage. There was, however, quite pronounced regional variation in the concentration of uronic acid and hexosamine in these menisci (Table 1). Thus the uronic acid concentration of the central portion of the lateral meniscus was almost twice ($20.0 \pm 3.2 \mu$ g of uronic acid/mg dry wt.) that of the central portion of the medial meniscus ($10.2 \pm 2.0 \mu$ g of uronic acid/mg dry wt.).

Characterization of the glycosaminoglycans

Despite regional differences in concentration of uronic acid, there was little regional difference in the composition of the chondroitins (Table 2). Chondroitin 6-sulphate comprised 55–60%, chondroitin

Table 1. Analysis of canine meniscus

All chemical analyses were performed on four regions (see Fig. 2) of menisci from eight normal joints. The values for water content (% of wet weight) are the means $\pm$ s.d. of single measurements made on each region from the eight joints. The values for hexuronic acid and hexosamine are the means $\pm$ s.d. of measurements made in duplicate on glycosaminoglycans isolated by cetylpyridinium chloride precipitation of papain digests of the same samples (see the text for details).

Sample	Water content (% of wet wt.)	Uronic acid (μ g/mg dry wt.)	Hexosamine (μ g/mg dry wt.)
Lateral central	63.1 ± 1.2	20.0 ± 3.2	17.6 ± 2.2
Lateral A + P	63.8 ± 0.9	18.2 ± 3.5	16.0 ± 3.2
Medial central	62.8 ± 1.2	10.2 ± 2.0	7.7 ± 1.9
Medial A + P	63.5 ± 1.4	15.3 ± 3.0	12.5 ± 2.6

Table 2. Glycosaminoglycan composition of canine meniscus

Portions of the glycosaminoglycans isolated by cetylpyridinium chloride precipitation (see Table 1 legend) were analysed as follows. The values for chondroitin and chondroitin 6-sulphate are calculated from the μ mol of Δ -Di-0-S and Δ -Di-6-S recovered from the t.l.c. plate divided by the total recovery (μ mol) of unsaturated disaccharides. The value for chondroitin 4-sulphate is calculated from the μ mol of Δ -Di-4-S recovered from the plate after digestion with chondroitinase AC divided by the total unsaturated disaccharides recovered after digestion with chondroitinase ABC. The amount of dermatan sulphate is calculated by subtracting the amount of Δ -Di-4-S released by the chondroitinase AC from that released by chondroitinase ABC, divided by the total unsaturated disaccharides released by the latter. Single complete analyses were carried out on each of the eight glycosaminoglycan preparations. The values are the means $\pm$ s.d. for these determinations.

Sample	Composition (% of chondroitinase-digestible glycosaminoglycans)				Δ Di-4S/ Δ Di-6S molar ratio
	Chondroitin 6-sulphate	Chondroitin 4-sulphate	Chondroitin	Dermatan sulphate	
Lateral central	60.9 ± 3.5	26.2 ± 3.7	8.8 ± 1.7	4.1 ± 4.1	0.43
Lateral A + P	62.0 ± 3.9	25.4 ± 3.8	9.8 ± 3.8	$2.8 (0-13.9)$	0.41
Medial central	55.6 ± 2.5	25.3 ± 4.4	13.3 ± 3.2	5.8 ± 3.9	0.46
Medial A + P	59.4 ± 2.4	27.1 ± 2.9	10.7 ± 2.0	3.0 ± 2.5	0.46

Table 3. *Hyaluronic acid in canine meniscus*

Portions of the glycosaminoglycans isolated by cetylpyridinium chloride precipitation (see Table 1 legend) were analysed in duplicate by the *Streptomyces* hyaluronidase method. The values for each region are the means $\pm$ S.D. for these determinations. The amino sugar composition of the hyaluronic acid standard was determined after hydrolysis by using an amino acid analyser (see the text). The proportion of glucosamine was 72.8% of the total hexosamine, and when assayed after chondroitinase digestion by a t.l.c. thiobarbituric acid procedure (Adams & Muir, 1980) hyaluronic acid comprised only 73.9%. Adjustments were therefore made in the standard to account for this impurity.

Sample	Hyaluronic acid	
	(μ g/mg dry wt.)	(% of total uronic acid)
Lateral central	2.4 ± 0.5	6.0 ± 1.4
Lateral A + P	2.6 ± 0.6	7.0 ± 1.4
Medial central	1.4 ± 0.3	6.0 ± 1.8
Medial A + P	2.2 ± 0.5	6.9 ± 1.1

4-sulphate 25–27% and non-sulphated chondroitin 9–13%. Although there was an average of only 3.6% dermatan sulphate present in these menisci by this method, there was a wide dispersion in the measurements, with the individual values ranging from 0% to 13.9%. Disulphated chondroitin sulphate, however, was not detected in any of the samples.

Hyaluronic acid comprised about 6–7% of the total uronic acid of the tissue (Table 3) and, although the latter showed regional variation, the relative proportion of the hyaluronic acid to the chondroitin sulphates remained constant (Table 1).

Microscopic findings

Overall, the tissue of the meniscus was far from homogeneous microscopically. Although largely composed of dense fibres with a spare Safranin O-positive interfibrillar matrix, there were distinct small foci resembling hyaline cartilage. These were fewer in the central portion of the medial meniscus. Towards the surface there was less Safranin staining and the cells were more elongated or spindle-shaped, whereas in deeper regions Safranin staining was more intense and cells were more rounded.

Discussion

In most previous studies human menisci have been used. Peters & Smillie (1972) examined normal human menisci and those with horizontal and vertical tears, and found the water content to be 73.6%, which is markedly greater than the 63%

found here for foxhound menisci (Table 1). On the other hand, dry human menisci contained only 0.9% uronic acid and 1.1% hexosamine, which are less than the values found here for canine menisci in all regions other than the medial central region (Table 1). Ghosh *et al.* (1975) also found that hexosamine comprises only 1% of the dry weight of human menisci.

Analyses of the glycosaminoglycan composition of human menisci have given widely variable results. Earlier analyses of a few specimens by Solheim (1965) and Lehtonen *et al.* (1967) indicated that chondroitin 6-sulphate and chondroitin 4-sulphate were present, together with small amounts of hyaluronic acid, but dermatan sulphate was not noted as being present. However, Habuchi *et al.* (1973) found considerable amounts of dermatan sulphate in pooled normal human menisci from individuals aged 60–75 years. Iduronic acid, indicative of dermatan sulphate, comprised 26.8% of the hexuronic acid of the glycosaminoglycans isolated after Pronase digestion of the tissue. Proteoglycans extracted by 4M-guanidinium chloride, which accounted for less than one-third to one-tenth of the total uronic acid in the tissue, were also said to contain about 20% dermatan sulphate (McNicol & Roughley, 1980). In contrast, in the menisci of adult foxhounds dermatan sulphate accounted for only a few per cent of the total glycosaminoglycan. This difference could be attributable either to a difference in the load-bearing role of menisci in the knees of bipeds and quadrupeds, or to the facts that dermatan sulphate is the predominant glycosaminoglycan in tendons (Gillard *et al.*, 1977), and in the present study the tendon-like horns of the menisci (O'Connor, 1976) were excluded from the tissue taken for analysis (see Fig. 1). Precise anatomical details of samples used for analysis are not given in other studies. However, in both mature human (Habuchi *et al.*, 1973) and in the canine menisci examined here, chondroitin 6-sulphate was the predominant glycosaminoglycan and, despite variation in the microscopical appearance and in the total glycosaminoglycan content of different regions of the foxhound menisci, the relative proportions of the different glycosaminoglycans did not vary in the different regions.

The present analyses of normal mature canine menisci provide a basis for the study of the meniscus in natural and experimentally induced canine osteoarthritis. As the time of onset of the disease in the latter is known, it has been used to discern the initial biochemical changes in articular cartilage before lesions develop (Muir, 1977). A comparable study of the meniscus is needed. Working foxhounds that were being culled at the end of the hunting season were chosen because they were mature animals and were fit and in hard training, unlike

animals confined in cages, and would therefore be a more representative normal group.

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