Biosynthesis of proteoglycan *in vitro* by cartilage from human osteochondrophytic spurs

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Proteoglycan biosynthesis by human osteochondrophytic spurs (osteophytes) obtained from osteoarthritic femoral heads at the time of surgical joint replacement was studied under defined culture conditions *in vitro*. Osteophytes were primarily present in two anatomic locations, marginal and epi-articular. Minced tissue slices were incubated in the presence of [35 S]sulphate or [14 C]glucosamine. Osteophytes incorporated both labelled precursors into proteoglycan, which was subsequently characterized by CsCl-isopycnic-density-gradient ultracentrifugation and chromatography on Sepharose CL-2B. The material extracted with 0.5 M-guanidinium chloride showed 78.1% of [35 S]sulphate in the A1 fraction after centrifugation. Only 23.0% of the [35 S]sulphate in this A1 fraction was eluted in the void volume of Sepharose CL-2B under associative conditions. About 60–80% of the [35 S]sulphate in the tissue 4 M-guanidinium chloride extract was associated with monomeric proteoglycan (fraction D1). The average partition coefficient (K_{av}) of the proteoglycan monomer on Sepharose CL-2B was 0.28–0.33. Approx. 12.4% of this monomer formed stable aggregates with high-molecular-weight hyaluronic acid *in vitro*. Sepharose CL-2B chromatography of fractions with lower buoyant densities (fractions D2–D4) demonstrated elution profiles on Sepharose CL-2B substantially different than that of fraction D1, indicative of the polydisperse nature of the newly synthesized proteoglycan. Analysis of the composition and chain size of the glycosaminoglycans showed the following: (1) preferential elution of both [35 S]sulphate and [14 C]glucosamine in the 0.5 M-LiCl fraction on DEAE-cellulose; (2) the predominant sulphated glycosaminoglycan was chondroitin 6-sulphate (60–70%), with 9–11% keratan sulphate in the monomer proteoglycan; (3) K_{av} values of 0.38 on Sephadex G-200 and 0.48 on Sepharose CL-6B were obtained with papain-digested and NaBH₄-treated D1 monomer respectively. A comparison of the synthetic with endogenous glycosaminoglycans indicated similar types. These studies indicated that human osteophytes synthesized *in vitro* sulphated proteoglycans with some characteristics similar to those of mature human articular cartilage, notably in the size of their proteoglycan monomer and predominance of chondroitin 6-sulphate. They differed from articular cartilage primarily in the lack of substantial quantities of keratan sulphate and aggregation properties associated with monomer interaction with hyaluronic acid.

The progression of human (Howell *et al.*, 1976) and experimentally induced osteoarthritis in animals (Moskowitz *et al.*, 1973; Gilbertson, 1975) is often characterized by the development of osteochondrophytic spurs (osteophytes) in the affected joint. Most investigations concerning the pathogenesis of osteoarthritis have been directed primarily towards an understanding of the erosive lesions of articular

cartilage, with less attention paid to the proliferative osteophytic response of the disease. At present, the role of the osteophyte in the development of osteoarthritis is unknown, but pathophysiologically its histogenesis may result in alterations in the biomechanics of the joint by interfering with joint movement (Sokoloff, 1980). Indeed, the question as to whether osteophytes are part of or are apart from

osteoarthritic cartilage lesions is a subject of current controversy (Danielsson & Hernborg, 1970a,b; Hernborg & Nilsson, 1973; Jeffery, 1975).

The proliferative nature of the osteophyte as evidenced by histopathology has been confirmed in organ-explant culture (Malemud *et al.*, 1979, 1980). The findings indicated that osteophytes incorporated more [^3H]thymidine into DNA than did contiguous osteoarthritic cartilage, with an elevated [^3H]thymidine-labelling index. Migration of cells from human osteophytes in explant culture and the establishment of a monolayer occurred sooner when compared with the remaining osteoarthritic cartilage obtained from the joint surface. The active cell growth of the osteophyte suggested the possibility that the phenotype of this tissue with respect to proteoglycan might resemble an embryonic cartilage. Indeed, in osteoarthritic cartilage a relative increase in chondroitin 4-sulphate and a decrease in keratan sulphate were noted, in a pattern similar to that of immature cartilage, suggesting the reversion of the chondrocytes to a 'blastic' phase to accomplish repair (Mankin, 1974). Alternatives to this interpretation have been presented (Hjertquist & Lemperg, 1972; Lemperg *et al.*, 1974).

The purpose of the present investigation was to assess the phenotype of human osteophyte *in vitro* as pertains to synthesis of proteoglycan and glycosaminoglycans. These results are compared with the characteristics of the endogenous osteophyte proteoglycans.

Experimental

Materials

Cartilaginous portions of osteophytes were sampled from human femoral heads from patients undergoing total joint replacement for disabling osteoarthritis. The mean age of patients was 60.7 ± 7.8 years (mean $\pm$ S.D., $n = 10$). The osteophytes were usually present in two anatomical zones: (1) at the margin of the femoral neck and (2) in an epi-articular topography (Jeffery, 1975). The tissues were clearly distinguishable grossly from remaining articular cartilage. Osteophytes were removed aseptically with a scalpel blade tangential to the joint surface, with care being taken to avoid subchondral bone, and placed in Gey's balanced salt solution (Grand Island Biological Co., Grand Island, NY, U.S.A.) containing 1% penicillin/streptomycin (10000 units/ml and 10000 μg /ml respectively), 1% fungizone and 0.1% mycostatin. Random minced pieces were placed in neutral buffered formalin containing 1% hexadecyltrimethylammonium chloride and processed for histology (Sachs *et al.*, 1982).

Sephacrose gels and Sephadex G-200 were obtained from Pharmacia Fine Chemicals, Piscataway,

NJ, U.S.A. DEAE-cellulose (Whatman DE-52) was from H. Reeve Angel and Co., Clifton, NJ, U.S.A. Chondroitinase AC-II, chondroitinase ABC and keratanase (*Pseudomonas* sp. IFO-13309) (Seikagaku) were from Miles Laboratories, Elkhart, IN, U.S.A.; papain (2 $\times$ crystallized) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Guanidinium chloride was purchased from Bethesda Research Laboratories, Rockville, MD, U.S.A., and CsCl from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. All other chemicals used were of analytical grade.

Organ culture

Osteophytic tissues were treated briefly with bovine testicular hyaluronidase (0.05%) (Worthington Biochemicals, Freehold, NJ, U.S.A.) at room temperature to remove synovial mucins and contaminating erythrocytes. The tissue (15–20 minced slices, approx. 2 mm²) was then placed in 60 mm $\times$ 15 mm culture dishes (Falcon Plastics, Oxnard, CA, U.S.A.) containing sulphate-free Dulbecco's Modified Eagle's medium (Grand Island Biological Co.) containing 10% (v/v) non-heat-inactivated foetal bovine serum (Associated Biomedic Systems, Buffalo, NY, U.S.A., or KC Biologicals, Lanexa, KS, U.S.A.), antibiotics, fungizone and mycostatin and [^{35}S]sulphate (600–800 mCi/mmol; New England Nuclear, Boston, MA, U.S.A.) (5–10 μCi /ml) or [^{14}C]glucosamine (297 mCi/mmol; New England Nuclear) (0.5 μCi /ml). Tissues were cultured for 4, 22, 42, 48 or 71 h at 37°C without change of the medium. Radioactivity incorporated into sulphated and non-sulphated macromolecules was measured by liquid-scintillation spectrometry in Aquasol (New England Nuclear) in a Packard model 3255 liquid-scintillation system (see below).

Since some studies (Keiser & Hatcher, 1977) have indicated that testicular hyaluronidase preparations have significant proteinase activity, we compared the qualitative synthesis of proteoglycan with or without hyaluronidase pretreatment. No apparent differences were seen after such treatment when compared with untreated specimens, and the results reported do not distinguish between tissues treated in either manner.

Preparative procedure

Osteophyte tissue was suspended in either 0.5 M-guanidinium chloride/0.05 M-sodium acetate buffer, pH 5.8, or 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors 6-aminohexanoic acid (200 mM), disodium EDTA (20 mM) and benzamidine hydrochloride (10 mM) (Oegema *et al.*, 1975). Tissues were stirred for 20–24 h at 4°C. The extract was dialysed in 6000–8000 MCO membranes against

distilled water overnight at 4°C and freeze-dried. In general, 75 mg wet wt. of tissue was extracted with 20 ml of guanidine/acetate buffer. The residual tissue was frozen at -20°C until analysed.

Determination of hexuronic acid, hexosamine and protein

Hexuronic acid content in column effluents of osteophyte guanidine extracts or CsCl-density-gradient fractions was determined by the carbazole procedure (Bitter & Muir, 1962). Glucosamine and galactosamine content was assayed by the method of Wagner (1979). Protein content was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (globulin-free) as standard.

Separation of glycosaminoglycans

The types of glycosaminoglycans synthesized by osteophyte organ explants were determined after papain digestion of tissue and medium in 0.1 M-sodium acetate buffer, pH 5.5, containing 5 mM-cysteine hydrochloride and disodium EDTA for 24 h at 56°C (Srivastava *et al.*, 1974a). This was followed by incubation with 0.2 M-NaOH for 6 h at 37°C and dialysis overnight against running cold tap water. LiCl was added to a final concentration of 4 M, and glycosaminoglycans were precipitated with 4 vol. of ethanol. To facilitate precipitation of glycosaminoglycans in the medium, carrier chondroitin sulphate (mixed isomers A/C; Sigma Chemical Co.) (0.5–1.0 mg/ml) was added. No carrier was added to tissue preparations. After standing overnight at 4°C, the precipitate was collected by centrifugation at 1000 g for 3 min at 4°C. The precipitate was suspended in 50 mM-Tris/HCl buffer, pH 7.2, centrifuged (if required) and applied by gravity to a DEAE-cellulose column (2 cm × 12 cm). Glycosaminoglycans were separated by stepwise elution with increasing molarities (0.2–0.6 M) of LiCl in 50 mM-Tris/HCl buffer, pH 7.2.

Characterization of glycosaminoglycans

Dermatan sulphate and chondroitin 4,6-sulphate were identified in eluted DEAE-cellulose fractions after digestion with chondroitinase ABC or chondroitinase AC-II as described by Srivastava *et al.* (1974a,b). Glycosaminoglycans in 4 M-guanidine extracts of non-cultured osteophytes, 4 M-guanidine extracts of [³⁵S]sulphate-labelled osteophytes, Sepharose CL-2B fractions and D1 monomer fractions were determined after chondroitinase digestion (Saito *et al.*, 1968) by high-pressure liquid chromatography (Varian model 5000 chromatograph, equipped with a Spectra Physics model 4100 integrator) by the method of Lee & Tieckelmann (1979). Varian MicroPak amino-substituted resin (10 µm particle size) was utilized in the analysis. [³⁵S]Sulphate incorporation into keratan

sulphate specifically was determined by comparing radioactivity co-migrating with authentic keratan sulphate in DEAE-cellulose chromatography. Additionally, the [³⁵S]sulphate in this fraction was incubated with keratanase in enriched Tris buffer (Saito *et al.*, 1968), pH 8.0, for 24 h at 37°C under toluene followed by Sepharose CL-6B chromatography.

Gel chromatography

Sepharose CL-2B columns (0.5 cm × 80 cm or 0.8 cm × 105 cm) were eluted with 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 5.8 (dissociative buffer). Alternatively, a Sepharose CL-2B column (0.4 cm × 110 cm) was eluted with 0.5 M-sodium acetate buffer, pH 5.8 (associative buffer). Fractions of volume 1.0 ml were collected from the column eluted with the dissociative buffer, and 0.5 ml fractions were collected from the column eluted with the associative buffer. Void volume was determined with Blue Dextran 2000 and bovine nasal-septum proteoglycan aggregate, and included volume with Na₂³⁵SO₄. The size of the glycosaminoglycan chains released by β-elimination after papain digestion (Srivastava *et al.*, 1974a) or by reduction with alkaline NaBH₄ (Yanagashita *et al.*, 1979) was measured by chromatography on a Sephadex G-200 column (1.5 cm × 97 cm) eluted with 0.5 M-guanidinium chloride/50 mM-Tris/HCl buffer, pH 7.2, or a Sepharose CL-6B column (0.4 cm × 115 cm) eluted with 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 5.8. The average partition coefficient (*K*_{av}) was determined as described by Wasteson (1971).

CsCl-density-gradient ultracentrifugation

Samples of pooled column fractions (Sepharose CL-2B), 0.5 M-guanidine extracts of tissue and medium or 4 M-guanidine extracts of tissue and medium were dialysed against distilled water and freeze-dried. The freeze-dried samples were dissolved in 3.7 ml of 0.5 M-guanidinium chloride/0.05 M-sodium acetate buffer (associative gradient) or 4 M-guanidinium chloride/0.1 M-sodium acetate buffer (dissociative gradient) containing proteinase inhibitors (see above). Solid CsCl was added to each sample to an initial density of 1.6 g/ml (associative gradient) and 1.5 g/ml (dissociative gradient). Samples were centrifuged for 48 h at 35 000 rev./min (81 900 *g*_{av}) at 10°C in a Beckman model L5-50 ultracentrifuge equipped with an SW 50.1 rotor. Four equal fractions of the tube were cut with a gel-tube slicer and densities were re-measured in each fraction. Final densities of associative gradients were: A4 (top), 1.50; A3, 1.58; A2, 1.65; A1 (bottom), 1.74. Those for dissociative gradients were: D4 (top), 1.38; D3, 1.44; D2, 1.51; D1 (bottom), 1.61. Reduction and alkylation of D1

monomer fractions were performed by the method of Heinegård (1977).

Aggregation of D1 fractions with hyaluronic acid

CsCl-density-gradient tissue D1 fraction containing [35 S]sulphate was mixed with high-molecular-weight hyaluronic acid (Healon; courtesy of Dr. A. Balazs) in a ratio of 50:1 by uronic acid content of the D1 fraction and hyaluronate (Wiebkin & Muir, 1977). The mixture was incubated for 2 h at room temperature and chromatographed over Sepharose CL-2B, eluted with 0.5 M-sodium acetate buffer, pH 7.2. Under these conditions, the hyaluronate-uronic acid aggregate was eluted in the void volume of this column and the D1 fraction was eluted in the included volume with a K_{av} value of 0.30.

Histology

After fixation, tissues were processed in an Autotechnicon (Tarrytown, NY, U.S.A.) with graded ethanol concentrations, cleared in xylene, embedded in paraffin wax and sectioned at a thickness of 7 μ m. All sections were stained with Haematoxylin and Eosin. Some representative sections were stained with Safranin O counterstained with Fast Green. The sections were examined for specific cellular morphology and orthochromatic and metachromatic staining characteristics of the tissue matrix (Sachs *et al.*, 1982).

Results

Tissue specimens

Most of the osteophytic specimens used in the present study were obtained from the margins of the femoral neck of patients undergoing total joint replacement. The histological appearance of the specimens indicated that it was heterogeneous in character. One component of this tissue, fibrocartilaginous in appearance, contained densely packed small stellate cells. Safranin O staining revealed an irregular, often inconsistent, uptake of the dye. By contrast, a second component of the specimens consisted of larger, more evenly dispersed, cells consistent with hyaline cartilage chondrocytes. Despite this appearance, the matrix of this component also stained irregularly with Safranin O. A more detailed description of the histopathological character of the osteophytic specimens used in these studies is presented elsewhere (Sachs *et al.*, 1982).

Gel-chromatography profile of synthetic sulphated proteoglycans

We typically extracted 90–95% of the incorporated [35 S]sulphate from osteophytes with 4 M-guanidinium chloride. Chromatography on Sepharose CL-2B of 4 M-guanidine extracts of tissue and medium from osteophytes labelled for 22, 42 and 72 h with [35 S]sulphate showed that most of the

radioactivity was eluted in a polydisperse profile (Fig. 1). Fraction I (K_{av} 0.07) was not prominent in the culture medium until 42 h, when it was eluted with the same K_{av} , as the corresponding tissue radioactivity. In addition to the large predominant peak, a smaller one with K_{av} 0.63 (fraction II) was found in the tissue extract. This peak was less prominent in the fraction from the medium. Fraction II was completely absent from the tissue when the tissue was incubated with [35 S]sulphate for 4 h (Fig. 2). A third peak (III) appeared coincidental with the column total volume. Peak III was not unincorporated [35 S]sulphate. It was retarded during chromatography on Sepharose CL-6B (Fig. 3). Fraction II (component 1) radioactivity was eluted in the void volume of this column, whereas most of fraction III showed a polydisperse pattern with molecular weights corresponding to 17000 (component 2) and 4500 (component 3) (Wasteson, 1971). This radioactivity may be sulphated glycosaminoglycan chains or fragments thereof released into the medium after prolonged time in culture. A direct comparison of the profile of synthetic proteoglycan and endogenous proteoglycan is shown in Fig. 4. The results indicated that most of the radioactivity was eluted from Sepharose CL-2B with a lower K_{av} than that of the endogenous material containing uronic acid.

CsCl-density-gradient ultracentrifugation of Sepharose CL-2B fractions

The distribution of radioactivity and the uronic acid/protein, uronic acid/hexosamine and galactosamine/glucosamine ratios of Sepharose CL-2B fractions I and II were determined on CsCl density gradients centrifuged in dissociative buffer. After 42 and 72 h, 70.4–91% of the radioactivity was found in the D1 fraction of the gradient (when expressed per μ g of protein) (Table 1), known to contain monomeric proteoglycan (Hascall & Heinegård, 1974a,b; Hardingham & Muir, 1972, 1973a,b). The uronic acid/hexosamine ratio of the osteophyte tissue D1 monomer was 1.12. The galactosamine/glucosamine ratio was 7.3. At 71 h of [35 S]sulphate labelling, more radioactivity was associated with the D1 monomer in Sepharose CL-2B fraction I than with fraction II. Furthermore, the uronic acid/protein ratio of the D1 fraction of fractions I and II differed substantially (Table 1).

When the D1 fraction from Sepharose CL-2B fraction I was rechromatographed on Sepharose CL-2B and eluted with 4 M-guanidine, the elution profile showed K_{av} 0.33, which differed from the K_{av} value obtained with the crude guanidine extract (Fig. 1). These results were confirmed by direct CsCl-density-gradient centrifugation of 4 M-guanidine extracts without prior Sepharose CL-2B chromatography. The elution profile over Sepharose

Fig. 1. *Synthesis and secretion of sulphated proteoglycan by osteophyte organ explants*

Sepharose CL-2B columns were eluted with dissociative buffer (0.9 ml/fraction). Tissue and medium were separately extracted with 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 5.8, containing proteinase inhibitors (Oegema *et al.*, 1975), dialysed against water and freeze-dried. (a)–(c) Elution profiles of (a) 22 h, (b) 42 h and (c) 71 h [^{35}S]sulphate-labelled tissue. (d)–(f) Elution profiles of (d) 22 h, (e) 43 h and (f) 71 h [^{35}S]sulphate label in the culture medium. For full experimental details see the text. In this and subsequent Figures V_0 and V_t represent the void volume and the total volume respectively.

Fig. 2. *Synthesis of sulphated proteoglycan by osteophyte tissue after short-term (4 h) incubation with [^{35}S]sulphate*

Sepharose CL-2B chromatography of 4 M-guanidine extract eluted with dissociative buffer (0.9 ml/fraction) is shown. For full experimental details see the text.

CL-2B showed a peak eluted with K_{av} 0.28, which was not altered after reduction and alkylation (Heinegård, 1977) (Fig. 5). Taken together, these results indicated that the low K_{av} value of fraction I seen on direct Sepharose CL-2B chromatography of 4 M-guanidine extracts may have resulted from interactions between proteoglycan monomers.

The polydispersity of the synthetic proteoglycans was studied further by analysis of the hydrodynamic volume of each of the CsCl-density-gradient fractions (D1–D4) on Sepharose CL-2B under dissociative conditions (Fig. 6). These results indicated that the monomeric proteoglycan (fraction D1) was distinct in size from other proteoglycan species (fractions D2–D4). There were, however, overlaps in the K_{av} values of some of these latter fractions.

Fig. 3. *Sepharose CL-6B chromatography of fractions II and III from Sepharose CL-2B chromatography of osteophyte 4 M-guanidine extract*

Fractions II and III (Fig. 1f) were dialysed and rechromatographed on Sepharose CL-6B eluted with dissociative buffer (0.5 ml/fraction). For full experimental details see the text.

Fig. 4. *Sepharose CL-2B chromatography of [35S]sulphate-labelled osteophyte 4 M-guanidine extracts (—) and endogenous 4 M-guanidine-extractable uronic acid (----)*

Osteophyte organ explants were labelled with [³⁵S]sulphate. The tissue was extracted with 4 M-guanidine and chromatographed on Sepharose CL-2B eluted with dissociative buffer (0.9 ml/fraction). For full experimental details see the text.

Osteophyte D1 proteoglycan interaction with hyaluronic acid

Only 12.5% of D1 proteoglycan monomer radioactivity was shifted from the included volume of a Sepharose CL-2B column eluted under associative conditions to the void volume after incubation with hyaluronic acid (Fig. 7). Because this result might have arisen from several factors governing aggregation of proteoglycan to hyaluronic acid *in vitro* (Oegema, 1981), 0.5 M-guanidine extracts of osteo-

Fig. 5. *Sepharose CL-2B chromatography of osteophyte tissue synthetic monomeric D1 sulphated proteoglycan* Osteophyte explants were labelled for 48 h with [³⁵S]sulphate and extracted with 4 M-guanidinium chloride. The extract was subjected to CsCl-density-gradient ultracentrifugation under dissociative conditions. The bottom fraction of the gradient (D1) was dialysed against water and chromatographed on Sepharose CL-2B eluted with dissociative buffer (0.9 ml/fraction). For full experimental details see the text.

phyte labelled with [³⁵S]sulphate were subjected to CsCl-density-gradient ultracentrifugation under 'associative' conditions. The A1 fraction of this gradient was found to contain 78% of the [³⁵S]sulphate label when expressed per µg of protein of the extract A1 fraction. A 4 M-guanidine extract dialysed against water and centrifuged in an identical manner showed that 63% of the radioactivity was in the A1 fraction. When the osteophyte A1 fraction was analysed on Sepharose CL-2B under associative conditions, approx. 20% of the [³⁵S]sulphate label was eluted in the void-volume peak, where a well-characterized bovine nasal-septum-cartilage A1 proteoglycan preparation was also eluted (Fig. 8). The results thus indicate (1) that only a small percentage of newly synthesized proteoglycan of human osteophyte is present as proteoglycan aggregate, and (2) that most of the A1 fraction is comprised of proteoglycan that is eluted from Sepharose CL-2B with a K_{av} value identical with that of the monomeric D1 proteoglycan.

Osteophyte glycosaminoglycans

DEAE-cellulose chromatography of [³⁵S]-sulphate- and [¹⁴C]glucosamine-labelled papain-digested osteophyte tissue and culture medium indicated that 73% of the [³⁵S]sulphate label was eluted in the chondroitin sulphate-rich fractions, i.e. with 0.4–0.6 M-LiCl (Srivastava *et al.*, 1974a) (Table 2). The tissue appeared to synthesize little hyaluronic acid, since the percentages of radioactivity in the

Fig. 6. Polydispersity of osteophyte synthetic proteoglycans

Fractions [(a) D1, (b) D2, (c) D3 and (d) D4] from dissociative CsCl-density-gradient ultracentrifugation were chromatographed over Sepharose CL-2B eluted with 4 M-guanidinium chloride (0.9 ml/fraction). Uronic acid content (----) of the endogenous D1 proteoglycan was measured in addition to the radioactivity elution profile (—). For full experimental details see the text.

0.3 M-LiCl fraction were similar when $[^{35}\text{S}]$ sulphate and $[^{14}\text{C}]$ glucosamine were compared. A standard preparation of hyaluronic acid was found to be eluted solely in the 0.3 M-LiCl fraction (Srivastava *et al.*, 1974a,b).

The glycosaminoglycan composition of 4 M-guanidine extracts and D1 proteoglycan monomer were also examined (Table 3). The predominant sulphated glycosaminoglycan synthesized by osteophytes in organ-explant culture was chondroitin 6-sulphate. These results compare favourably with the ratio of chondroitin 6-sulphate to chondroitin 4-sulphate of endogenous D1 proteoglycan monomer from osteophytes (Fig. 9). Furthermore, an analysis of the composition of the endogenous chondroitin sulphates from Sepharose CL-2B fractions I and II showed that fraction I was devoid of chondroitin 4-sulphate. To test that the high-pressure liquid-chromatography system was not excluding chondroitin 4-sulphate by virtue of possible overlaps with other unidentified glycosaminoglycans, we also assayed the glycosaminoglycan composition of bovine nasal-septum cartilage proteoglycan monomer for comparison. This fraction contained more chondroitin 4-sulphate than chondroitin 6-sulphate (Table 3). Dermatan sulphate and chondroitin 4,6-sulphate represented 6.5 and 2.9% respectively of the newly synthesized sulphated glycosaminoglycans. The K_{av} of chondroitin sulphate chains labelled with $[^{35}\text{S}]$ sulphate was 0.38 on Sephadex G-200 and 0.48 on Sepharose CL-6B.

$[^{35}\text{S}]$ Sulphate label found in keratan sulphate was analysed by co-elution of radioactivity with authentic keratan sulphate by DEAE-cellulose chromatography. The standard was eluted in a single peak at a molarity of 0.6 M-LiCl. Monomeric D1 proteoglycan reduced with NaBH_4 was digested with chondroitinase ABC. The undigested residue was analysed by DEAE-cellulose chromatography. Approx. 8.7% of the radioactivity associated with this undigested residue was found in the 0.6 M-LiCl fraction. In addition, NaBH_4 -reduced $[^{35}\text{S}]$ sulphate-labelled D1 monomer was incubated with keratanase and then chromatographed on Sepharose CL-6B (Fig. 10). Approx. 11% of the radioactivity was shifted from a K_{av} value of 0.47 before digestion to 0.89 after digestion, indicative of the effect of keratanase on keratan sulphate containing $[^{35}\text{S}]$ sulphate label.

Discussion

Despite its characteristic appearance in most osteoarthritic femoral heads at the time of surgical intervention, osteophyte pathophysiology is poorly understood. The role of osteophytes in the response of articular cartilage to injury (Mankin, 1974) and in the development of osteoarthritis in man (Byers *et al.*, 1977) and in experimental models in dogs (Gilbertson, 1975) and rabbits (Moskowitz *et al.*, 1973, 1981) has been the subject of continuing controversy. Several features of osteophyte histogenesis, however, are apparent. (1) In man, osteo-

Table 1. *Distribution of [^{35}S]sulphate label in CsCl density gradients from human osteophyte explants*

Organ explants of human osteophytes were incubated in sulphate-free medium containing 10% foetal bovine serum for various periods of time in the presence of [^{35}S]sulphate (10 $\mu\text{Ci}/\text{ml}$). The tissue was extracted with 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 5.8, containing proteinase inhibitors (see the Experimental section) for 20–24 h at 4°C. The medium was mixed with an equal volume of 8 M-guanidinium chloride/0.2 M-sodium acetate buffer, pH 5.8, containing twice the concentration of proteinase inhibitors to give the same final concentration of guanidinium chloride and proteinase inhibitors as the tissue extract. N.D., Not determined. The results given for Expt. 1 were obtained after chromatography of 4 M-guanidine extracts on Sepharose CL-2B eluted with 4 M-guanidinium chloride. Fractions from radioactive peaks (Fig. 1) were pooled, dialysed and freeze-dried. The freeze-dried material was dissolved in 4 M-guanidinium chloride containing proteinase inhibitors, and solid CsCl was added to give an initial density of 1.5 g/ml. This solution was subjected to CsCl-density-gradient ultracentrifugation as described in the Experimental section. The results given for Expt. 2 were obtained after direct CsCl-gradient-density ultracentrifugation of 4 M-guanidine extracts under dissociative conditions.

Expt. no.	Sample	Distribution of [^{35}S]sulphate label (%)				Uronic acid/protein ratio of D1 fraction
		Fraction D4	Fraction D3	Fraction D2	Fraction D1	
1	Tissue					
	42 h, fraction I	1.3	16.6	11.6	70.4	2.34
	71 h, fraction I	4.2	0.4	4.4	91.0	2.81
	71 h, fraction II	4.5	7.7	12.9	74.8	1.73
	Medium					
	71 h, fraction I	2.9	2.0	5.4	89.7	N.D.
2	Tissue					
	48 h	4.4	12.3	22.5	60.6	2.49
	Medium					
	48 h	0.5	3.1	15.5	80.9	N.D.

Fig. 7. *Interaction of osteophyte monomeric proteoglycan with hyaluronic acid*

The D1 fraction from CsCl-density-gradient ultracentrifugation was incubated with high-molecular-weight hyaluronic acid (see the Experimental section for details). Monomeric D1 fraction alone (a) and monomeric D1 fraction and hyaluronic acid (b) were chromatographed on Sepharose CL-2B eluted with associative buffer (0.5 ml/fraction). For full experimental details see the text.

Fig. 8. *Elution profile of synthetic A1 fraction*

The A1 proteoglycan was chromatographed over Sepharose CL-2B eluted with 0.5 M-sodium acetate buffer, pH 5.8 (0.5 ml/fraction). For full experimental details see the text.

phytes can develop during the course of bone remodelling in the absence of frank osteoarthritic lesions of the articular surface (Sokoloff, 1980). This response was similarly observed, although very infrequently, in New Zealand White rabbits (Moskowitz *et al.*, 1973). (2) Osteophytes often are found in the marginal zone where synovium merges with fibrocartilage (Gilbertson, 1975), suggesting that osteophytes may possess a phenotype resembling fibrocartilage rather than articular cartilage, or features of both. (3) New bone formation at the site of osteophyte formation is thought to proceed through endochondral ossification (Jeffery, 1975), with a prior vascularization believed to be brought about by the failure of normally occurring pro-

Table 2. Comparison of DEAE-cellulose elution profiles of [^{35}S]sulphate-labelled and [^{14}C]glucosamine-labelled osteophyte explants

Osteophyte organ explants were labelled with either [^{35}S]sulphate ($5\mu\text{Ci/ml}$) or [^{14}C]glucosamine ($0.5\mu\text{Ci/ml}$) in sulphate-free medium for 20h on the first day of culture. The tissue and medium were treated separately with papain as described in the Experimental section, and were chromatographed on DEAE-cellulose eluted stepwise with LiCl.

Labelled precursor	Sample	Distribution of radioactivity (%)				
		0.2 M-LiCl fraction	0.3 M-LiCl fraction	0.4 M-LiCl fraction	0.5 M-LiCl fraction	0.6 M-LiCl fraction
[^{35}S]Sulphate	Tissue	8.3	12.1	15.8	46.7	12.9
[^{14}C]Glucosamine	Tissue	13.5	15.3	15.1	31.0	16.8
[^{35}S]Sulphate	Medium	14.4	15.4	19.8	22.0	19.5
[^{14}C]Glucosamine	Medium	19.2	13.9	12.8	34.1	15.6

Fig. 9. High-pressure liquid chromatography of papain-digested alkali-treated osteophyte monomeric D1 proteoglycan after digestion with chondroitinase AC and chondroitinase ABC

(a) Disaccharide standards (—) (key: DiOS, non-sulphated disaccharide; Di4S, disaccharide 4-sulphate; Di6S, disaccharide 6-sulphate). (b) D1 monomer + chondroitinase AC: ----, endogenous disaccharides liberated after enzymic digestion; —, elution profile of [^{35}S]sulphate label. (c) D1 monomer + chondroitinase ABC: ----, endogenous disaccharides liberated after enzymic digestion; —, elution profile of [^{35}S]sulphate label. A $10\mu\text{l}$ volume of each sample was injected on to a $0.4\text{ cm} \times 30\text{ cm}$ column packed with amino-substituted resin and eluted with 65% 0.5 M-ammonium formate buffer (pH 5.8)/35% methanol (0.2 ml/fraction). For full experimental details see text.

Table 3. *Comparative analysis of synthetic and endogenous chondroitin sulphates by human osteophytes*

Chondroitinase ABC digestion of the indicated samples was performed as described in the Experimental section. After incubation for 48 h at 37°C, undigested glycosaminoglycans and chondroitinase ABC were precipitated with 3 vol. of ethanol for 72 h at 4°C. The precipitate was removed by centrifugation at 20000g (10000 rev./min) in a Sorvall model RC-5B centrifuge for 15 min. The supernatant containing digestion products was evaporated to dryness and redissolved in buffer before analysis by high-pressure liquid chromatography. For the two types of synthetic human osteophyte chondroitin sulphates osteophyte organ explants were labelled with [³⁵S]sulphate (10 µCi/ml) for 48 h. The 4M-guanidine extract or monomeric D1 proteoglycan fraction was subjected to chondroitinase ABC digestion. The endogenous chondroitin sulphate content of the D1 monomer preparation was analysed simultaneously along with the radioactivity (Fig. 9). The percentages of chondroitin 6-sulphate and chondroitin 4-sulphate were 65.5% and less than 5% respectively. For the three fractions of endogenous human osteophyte chondroitin sulphates fresh osteophyte tissue proteoglycan was subjected to chondroitinase ABC digestion after papain digestion of the indicated fractions. Endogenous bovine nasal-septum-cartilage D1 monomer was a gift from Dr. Harold D. Keiser.

Type	Source	Composition (% of total digestible glycosaminoglycans after chondroitinase ABC)	
		Chondroitin 6-sulphate	Chondroitin 4-sulphate
Synthetic	4M-Guanidine extract	69.7	16.7
Synthetic	D1 monomer	60.8	2.6
Endogenous	4M-Guanidine extract	56.8	18.9
	Sephacose CL-2B fraction I	69.8	Trace
	Sephacose CL-2B fraction II	65.2	9.7
Endogenous	Bovine nasal-septum D1 monomer	30.9	52.5

Fig. 10. *Sepharose CL-6B chromatography of the product of digestion of NaBH₄-reduced synthetic D1 monomer with keratanase*

—, Reduced D1 monomer after keratanase treatment; ····, reduced D1 monomer incubated with enriched Tris buffer, pH 8.0. For full experimental details see the text. The arrow at fraction 33 indicates the digestion product (K_{av} 0.89).

teinase inhibitors to control the development of vascular channels (Stephens *et al.*, 1979) in a manner similar to the calcification event in the hypotrophic region of the articular cartilage (Blumenthal *et al.*, 1979).

The present investigation sought to establish whether osteophytic spurs removed from human

osteoarthritic femoral heads synthesized proteoglycans that were similar in character or were different from data reported for articular cartilage. The glycosaminoglycan types of osteophytes resembled those of mature cartilage in that they were composed primarily of chondroitin 6-sulphate with normal chain size (M_r 15000–22500). These findings are characteristic of adult articular-cartilage glycosaminoglycans from a variety of species (Mathews & Glasgow, 1966; Choi & Meyer, 1975; Murata & Bjelle, 1980). Noteworthy, however, in the present study was the fact that osteophytes synthesized smaller amounts of keratan sulphate (9–11% of the total [³⁵S]sulphate in D1 monomer) than might be expected in a normal mature hyaline cartilage. Owing to the variability in the molecular weights of various keratan sulphates (M_r 3000–10000) (Muir, 1980; Hascall & Riolo, 1972; Hascall, 1977), we may be considerably underestimating the abundance of keratan sulphate, since some preparations were dialysed after papain digestion against membranes with molecular-weight cut-off 6000–8000. We have been unable to detect endogenous keratan sulphate in these preparations. The high galactosamine/glucosamine ratio may be related to this.

Proteoglycan biosynthesis was studied in osteophyte organ-explant culture. A 4 h labelling with [³⁵S]sulphate of tissue explants demonstrated the synthesis of a single peak of radioactive proteoglycan (K_{av} 0.07) after chromatography on Sepharose CL-2B. By 22 h of labelling a second peak of radioactivity (K_{av} 0.63) was apparent in the

4M-guanidine tissue extract in addition to the larger peak of radioactivity seen at the 4h labelling time (Fig. 1). The appearance of this second peak suggested the possibility that at least two different proteoglycan species were synthesized. This possibility was examined further, and the results indicated that (1) both radioactive peaks were composed predominantly of proteoglycan whose buoyant density in CsCl under dissociative conditions was interpreted as intact proteoglycan subunit (D1 monomer), (2) the glycosaminoglycan content of fraction I consisted mainly of chondroitin 6-sulphate, whereas fraction II was composed of chondroitin 6-sulphate and chondroitin 4-sulphate, and (3) the appearance of fractions I and II in the culture medium occurred at different times. The presence of several species of proteoglycan monomer rather than a single species has been suggested previously for human femoral heads (Bouvet *et al.*, 1978), human processus xiphoid cartilage in culture (Triphaus *et al.*, 1980) and rat chondrosarcoma in the presence of foetal bovine serum (Stevens & Hascall, 1981). The possibility that fraction II with the lower K_{av} value was a degradation product seems less likely, since the cultures contained foetal bovine serum, which inhibits the chondrocyte neutral proteoglycanase (Sapolsky *et al.*, 1976, 1981). A more likely possibility is that fraction II D1 monomer differs from fraction I in having fewer serine residues substituted with chondroitin sulphate, thus accounting for the lower uronic acid/protein ratio (Table 1). Furthermore, the difference in hydrodynamic size of fractions I and II on Sepharose CL-2B cannot be accounted for by increases or decreases in glycosaminoglycan chain size, since analysis of papain-digested D1 proteoglycan fractions I and II showed identical K_{av} values on Sepharose CL-6B (0.48). Contamination of [35 S]sulphate-labelled proteoglycan D1 with other more polydisperse proteoglycan species was also ruled out (Fig. 6).

The low K_{av} value of osteophyte fraction I on chromatography of 4M-guanidine extracts on Sepharose CL-2B under dissociative conditions may have come about by interactions between proteoglycan monomers. Self-association of proteoglycan subunits under physiological conditions has been reported (Sheehan *et al.*, 1978). This view was substantiated by CsCl-density-gradient ultracentrifugation of fraction I under dissociative conditions. The D1 fraction so generated when rechromatographed over Sepharose CL-2B (Fig. 5) gave a K_{av} value of 0.28. Thus the D1 monomer size of fraction I was compatible with those reported for young human hip cartilage (Roughley & White, 1980), human osteoarthritic chondrocytes (Oegema & Thompson, 1981), rat chondrosarcoma monolayers in the presence of horse serum (Kimura *et al.*, 1979)

and 8-day-chick limb-bud chondrocytes (De Luca *et al.*, 1978). By contrast, the osteophyte D1 synthetic monomer of fraction I is hydrodynamically larger than the endogenous D1 monomer derived from the same tissue (Figs. 4 and 6) and from articular cartilage of elderly individuals (Perricone *et al.*, 1977). The significance of this finding remains uncertain at the present time, but may reflect post-synthetic processing by the cells (Upholt *et al.*, 1979).

Aggregation of osteophyte D1 monomer to high-molecular-weight hyaluronic acid was found to occur. However, a substantial proportion of monomer failed to form aggregates, as evidenced by the failure of the radioactivity in the retarded volume of the Sepharose CL-2B column (Fig. 7) to move entirely to the void volume after incubation with hyaluronic acid. A similar small percentage of [35 S]sulphate label appeared to be excluded in A1 fractions (Fig. 8). Recent evidence has indicated that aggregation of proteoglycan monomer to exogenous hyaluronic acid is dependent on several factors, among which may be a functional hyaluronate-binding region (Brandt *et al.*, 1976), time and conditions of incubation of monomer and hyaluronate *in vitro* (Oegema, 1981) and stabilization of the aggregated proteoglycan by link glycoproteins (Hardingham & Muir, 1972, 1973a; Hascall & Heinegård, 1974a,b, 1975; Caterson & Baker, 1978; Hardingham, 1979). The link proteins apparently serve a facilitatory rather than an obligatory role in proteoglycan aggregation (Swann *et al.*, 1976). Because we have not yet established whether osteophyte organ explants synthesize link proteins, we cannot state unequivocally that two populations (one that aggregates and one that does not) of proteoglycans are synthesized. The fact that human osteophytes, unlike rabbit (Srivastava *et al.*, 1974a; Gillard *et al.*, 1975), human (Srivastava *et al.*, 1974b) and sheep chondrocytes (Gillard *et al.*, 1975), appear to synthesize little hyaluronic acid (Table 3) may also be important in this regard.

The sulphated glycosaminoglycans synthesized by osteophyte organ explants and those found endogenously were similar (Fig. 9). The relative abundances of chondroitin 6-sulphate and chondroitin 4-sulphate were consistent with those found in normal human (Mourao *et al.*, 1976) and adult steer (Murata & Bjelle, 1979) articular cartilage and human chondrosarcoma (Sweet *et al.*, 1976; Thonar *et al.*, 1979). As was reported previously with rabbit (Srivastava *et al.*, 1974a) and human cartilage slices (Oegema & Thompson, 1981), human osteophytes synthesized little dermatan sulphate. The size of the glycosaminoglycan chains in the chondroitin sulphate-rich DEAE-cellulose 0.5M-LiCl fraction was similar in size to that reported from cartilage of adult steer (K_{av} 0.48 on Sepharose CL-6B, 0.38 on

Sephadex G-200) (Sweet *et al.*, 1979). Of interest was the finding that the uronic acid/protein ratio and galactosamine/glucosamine ratio were substantially different from those of immature articular cartilage (Sweet *et al.*, 1978). The latter ratio was in keeping with the predominance of chondroitin sulphate as both the major endogenous and synthetic product of the tissue. Thus the present studies support the contention that, although human osteophytes are capable of rapid proliferation *in vitro* (Malemud *et al.*, 1979), and therefore resemble embryonic cartilage in that respect, they appear to synthesize and contain glycosaminoglycans of mature cartilage.

Finally, the present studies provide initial evidence for the potential of osteophytes to synthesize human articular-cartilage-like proteoglycans. Since osteophyte proliferation fails to re-establish a normal articular cartilaginous surface during the course of osteoarthritic pathogenesis, its histogenesis must be mediated by environmental, nutritional and biomechanical events in the joint that prevent their growth over eburnated articular surfaces. Our osteophyte samples were obtained from individuals in an advanced stage of osteoarthritis, so their biochemical properties may reflect those acquired during years of development. The serial study of osteophyte histogenesis and matrix biochemistry in a model of osteoarthritis in the rabbit (Moskowitz *et al.*, 1973) seems a logical step in pursuing our understanding of the role that osteophytes play in the pathophysiology of aging and osteoarthritis.

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