Glycosaminoglycan Sulfation in Human Osteoarthritis

DISEASE-RELATED ALTERATIONS AT THE NON-REDUCING TERMINI OF CHONDROITIN AND DERMATAN SULFATE

Anna H. K. Plaa‡§, Leigh A. West‡, Shirley Wong-Palm‡, and Fred R. T. Nelson∥
From the ‡Shriners Hospital for Children, Tampa Unit, and the §Department of Biochemistry and Molecular Biology, University of South Florida Medical School, Tampa, Florida 33612 and the ∥Henry Ford Hospital, Bone and Joint Center, Detroit, Michigan 48202

Chondroitin lyase products of aggrecan and small proteoglycans from normal and osteoarthritic cartilages were analyzed for chain internal disaccharides and terminal mono- or disaccharides. Chondroitin and dermatan sulfate chains from arthritic cartilages were of essentially normal size and internal sulfation but had significantly altered sulfation of the terminal residues. Whereas in normal cartilage, ~60% of terminal GalNAc4S was 4,6-disulfated, it was reduced to ~30% in osteoarthritic cartilage. This is most likely due to a lower terminal GalNAc4,6S-disulfotransferase activity and reveals that metabolic changes in osteoarthritis can affect this distinct sulfation step during chondroitin and dermatan sulfate synthesis. GlcAβ1,3GalNAc6S, the mimotope for antibody 3B3(−), was present on ~8 and ~10% of chains from normal and osteoarthritic cartilages, respectively. 3B3(−) assayed by immunodot blot was within the normal range for most osteoarthritic samples, with only 5 of 24 displaying elevated reactivity. This resulted not from a higher content of mimotope, but possibly from other structural changes in the proteoglycan that increase mimotope reactivity. In summary, chemical determination of sulfation isomers at the non-reducing termini of chondroitin and dermatan sulfate provides a reliable assay for monitoring proteoglycan metabolism not only during normal growth of cartilage but also during remodeling of cartilage in osteoarthritis.

The function of normal adult joint cartilage resides in its ability to provide resistance to compressive, tensile, and shear forces that occur during normal joint motion. The specialized extracellular matrix consists of a fibrillar collagen network (1), highly organized and stabilized through inter- and intramolecular cross-links that restrain the swelling pressure exerted by the high concentration of negatively charged aggrecan aggregates (2). The metabolic activity of chondrocytes in adult cartilage is adapted to maintain the composition of a functional extracellular matrix. The collagen network is relatively metabolically inert (3), whereas other constituents, such as aggrecan aggregates, undergo a distinct turnover process (4, 5) in which catabolic cleavage (6, 7) and removal of molecules from the extracellular matrix (8, 9) are in balance with synthesis and deposition of new molecules. However, at the onset and throughout progression of degenerative joint disease, such as OA, this metabolic balance is perturbed, and episodes of increased catabolism (10, 11) and increased synthesis (12–16) of matrix components by the chondrocyte take place.

It has been proposed that the increased anabolic activity in OA is related to a change in the differentiated state of the chondrocyte (17) with expression of pathways that are characteristic of cells in fetal or postnatal growth cartilages. This hypothesis has been particularly supported by published reports (18–21) that the GAG composition of aggrecan synthesized during the early stages of cartilage degeneration in animal models of OA or accumulated in human OA cartilages was altered. Changes included decreased keratan sulfate/CS ratio, increased 4-sulfation of CS, and alterations in hydrodynamic size of CS chains. In addition, the use of monoclonal antibodies to CS epitopes that were considered abundant on fetal, but not on normal mature adult aggrecan, indicated that aggrecan from OA cartilages displayed elevated immunoreactivity compared with normal adult cartilages (22–26). However, the use of the immunological data to describe CS fine structures remains problematic, because the structure of the carbohydrate epitopes recognized by the antibodies has not been established in many cases. Furthermore, immunoreactivity of CS epitopes is likely influenced by assay conditions that affect chain presentation and epitope concentration much as was seen in solid phase immunonasays with monoclonal antibody 3B3(−) (27). Assays with this antibody have been very widely interpreted as a quantitative measure of the non-reducing terminal sequence GlcAβ1,3GalNAc6S on CS chains (23–26, 28–30).

Despite the large number of reported changes in composition of CS in OA cartilages as well as of CS in synovial fluids (30–32), methods have not been available to determine if such disease-related alterations in structure are randomly distributed within the GAG chain or confined to specific domains. Because of this limitation it has not been possible to establish the importance of changes in the process of GAG biosynthesis as opposed to alterations in matrix catabolism to the observed changes in GAG composition of tissue and synovial fluid proteoglycans.

Our previous work on the fine structure of the non-reducing terminal residues on aggrecan CS (33) showed that such struc-

∥To whom correspondence should be addressed: Cell Biology Laboratory, Shriners Hospital for Children, 12502 North Pine Dr., Tampa, FL 33612. Tel.: 813-972-2250; Fax: 813-975-7127; E-mail: annaplaa@delphi.com.

‡This work was supported by a grant from the Shriners of North America (to A. H. K. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: OA, osteoarthritis; PG, proteoglycan; GAG, glycosaminoglycan; CS, chondroitin sulfate; HPLC, high performance liquid chromatography; DS, dermatan sulfate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; the structures of the unsaturated disaccharides ΔDi0S ΔDi-4S, ΔDi-6S, ΔDiB, ΔDiD and ΔDiS are given (33); GalNAc4S, N-acetylgalactosamine 4-sulfate; GalNAc4,6S, N-acetylgalactosamine 4,6-disulfate.

1 The abbreviations used are: OA, osteoarthritis; PG, proteoglycan; GAG, glycosaminoglycan; CS, chondroitin sulfate; HPLC, high performance liquid chromatography; DS, dermatan sulfate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; the structures of the unsaturated disaccharides ΔDi0S ΔDi-4S, ΔDi-6S, ΔDiB, ΔDiD and ΔDiS are given (33); GalNAc4S, N-acetylgalactosamine 4-sulfate; GalNAc4,6S, N-acetylgalactosamine 4,6-disulfate.
Glycosaminoglycan Sulfation in Osteoarthritis

Cartilages were also taken from right knees of patients with unicompartmental late stage OA, within 12–18 h of a total knee arthroplasty. Tibial plateaus were obtained as an intact surface and femoral condyles separated into medial and lateral compartments (Fig. 1B, donor I). For donors III–V, cartilage plugs (~5 mm diameter) were harvested from the same 20 individual locations as for normal knees. Donors I–II had insufficient cartilage at the select locations of the medial surfaces, as illustrated in Fig. 1. An approximately 1-mm² full thickness slice was removed from the middle of each plug of normal cartilage and stored separately for total GAG preparation, and the remainder was pooled for PG preparation (see below). An additional set of plugs was taken from donors I–IV for total GAG preparation, and these were removed adjacent to the first 20 sites, with the exact location chosen to match closely the macroscopic appearance. All tissues were frozen at −70 °C until processed as described below.

Preparation of Aggrecan and Low Buoyant Density PGs from Human Knee Cartilages—For the preparation of purified PGs, cartilages were pooled as follows: from normal donor V and OA donors I–III, plugs from the medial femoral condyle, plugs from the lateral femoral condyle, plugs from the medial tibial plateau, or plugs from the lateral tibial plateau were pooled separately; from normal donors I–IV, the plugs from both medial and lateral cartilage condyles (FC) or from medial and lateral tibial plateaus were pooled. The tissues were rinsed with ice-cold phosphate-buffered saline containing protease inhibitors (5 mM EDTA, 1 mM aminobenzalenosulfonate fluoride, 1 μl/ml pepstatin, 1 μm N-ethylmaleimide, 10 μm aminohexanoic acid), minced finely on ice, and then extracted for 48 h at 4 °C in 15 volumes of 4% guanidine HCl, 50 mM sodium acetate, pH 7.0, 1% (v/v) CHAPS, and the protease inhibitor mixture described above. The extracts were centrifuged at 10,000 × g for 10 min and assayed for total GAG by dimethylmethylene blue (34). About 84 and 89% of total GAG was solubilized from normal and OA cartilage samples, respectively. The extracts were adjusted to a final density of 1.48 g/ml with CsCl and subjected to centrifugation at 38,000 rpm for 48 h in a Beckman 50Ti rotor. The tubes were divided into four equal parts (D1–D4). For both normal and OA preparations, ~8% of the total GAG was recovered in the D1 fraction, ~6% in each of the D2 and D3 fractions, and ~1% in the D4 fractions. Each fraction was dialyzed consecutively against water, 1 M NaCl, and water and then stored at final GAG concentrations of 2 mg/ml at −20 °C.

10-μg portions (as dimethylmethylene blue reactivity) of the D1 and pooled D2/D3 gradient fractions were incubated at 37 °C for 2 h with 0.2 milliunits protease-free chondroitinase ABC and an additional 2 h with 0.2 milliunits each of keratanase II and endo-β-D-galactosidase, in 20 μl of 50 mM sodium acetate, 50 mM Tris-HCl, 10 mM EDTA, pH 7.0, containing 7 μl each of aminobenzalenosulfonate fluoride and N-ethylmaleimide. The samples were electrophoresed on 4–12% polyacrylamide gels under reducing conditions; separated core proteins were transferred to nitrocellulose (at 75 V for 1.5 h), and membranes were developed with anti-ATEG or anti-LEC peptide-antiserum (1:5000) or 28B6 monoclonal ascites fluid (1:1000) as described (26). Reactive core proteins were visualized by exposure to preflashed Hyperfilm for 15–30 s after incubation with ECL detection reagent.

In the D1 fractions from both normal and OA cartilages, four G1-containing core protein species were seen, and these migrated with apparent molecular sizes of ~350, 250, 180, and 115 kDa. Western blots of identical samples using the G1-specific anti-LEC antiserum showed that only the 350-kDa species was reactive representing the full-length core protein containing both the N-terminal G1 and the C-terminal G3 globular domains. The faster migrating, G1-negative, core proteins represented aggrecan species that had undergone proteolytic processing in the CS-1 or CS-2 domains (26, 36). The relative abundance of the core protein species from normal and OA cartilages, estimated by densitometric scanning, showed that in OA samples the 180-kDa species was markedly lower than in normals (reduced to 25–50%), with the 350-kDa species correspondingly increased (40–70%). There was little or no change in the 250- and 115-kDa species. There was no distinction in the degree of change between normal and OA D1 aggrecan for the different anatomical sites or between patients. PGs from both normal and OA cartilages, purified into the D2 and D3 fractions, were composed of a ~250-kDa core protein (aggrecan and its proteolytic fragments) based on determination of the core protein sizes by SDS-polyacrylamide gel electrophoresis and reactivity in Western blotting with anti-ATEG and 2B6 monoclonal antibody (40).

Preparation of GAGs from Normal and OA Knee Cartilages—Cartilage plugs from the individual sampling locations (see Fig. 1) were solubilized with 167 μg of papain in 250 μl of 50 mM sodium acetate, pH 7.0. Cartridges were also taken from right knees of patients with unicompartmental late stage OA, within 12–18 h of a total knee arthroplasty. Tibial plateaus were obtained as an intact surface and femoral condyles separated into medial and lateral compartments (Fig. 1B, donor I). For donors III–V, cartilage plugs (~5 mm diameter) were harvested from the same 20 individual locations as for normal knees. Donors I–II had insufficient cartilage at the select locations of the medial surfaces, as illustrated in Fig. 1.

An approximately 1-mm² full thickness slice was removed from the middle of each plug of normal cartilage and stored separately for total GAG preparation, and the remainder was pooled for PG preparation (see below). An additional set of plugs was taken from donors I–IV for total GAG preparation, and these were removed adjacent to the first 20 sites, with the exact location chosen to match closely the macroscopic appearance. All tissues were frozen at −70 °C until processed as described below.

Preparation of Aggrecan and Low Buoyant Density PGs from Human Knee Cartilages—For the preparation of purified PGs, cartilages were pooled as follows: from normal donor V and OA donors I–III, plugs from the medial femoral condyle, plugs from the lateral femoral condyle, plugs from the medial tibial plateau, or plugs from the lateral tibial plateau were pooled separately; from normal donors I–IV, the plugs from both medial and lateral cartilage condyles (FC) or from medial and lateral tibial plateaus were pooled. The tissues were rinsed with ice-cold phosphate-buffered saline containing protease inhibitors (5 mM EDTA, 1 mM aminobenzalenosulfonate fluoride, 1 μl/ml pepstatin, 1 μm N-ethylmaleimide, 10 μm aminohexanoic acid), minced finely on ice, and then extracted for 48 h at 4 °C in 15 volumes of 4% guanidine HCl, 50 mM sodium acetate, pH 7.0, 1% (v/v) CHAPS, and the protease inhibitor mixture described above. The extracts were centrifuged at 10,000 × g for 10 min and assayed for total GAG by dimethylmethylene blue (34). About 84 and 89% of total GAG was solubilized from normal and OA cartilage samples, respectively. The extracts were adjusted to a final density of 1.48 g/ml with CsCl and subjected to centrifugation at 38,000 rpm for 48 h in a Beckman 50Ti rotor. The tubes were divided into four equal parts (D1–D4). For both normal and OA preparations, ~8% of the total GAG was recovered in the D1 fraction, ~6% in each of the D2 and D3 fractions, and ~1% in the D4 fractions. Each fraction was dialyzed consecutively against water, 1 M NaCl, and water and then stored at final GAG concentrations of 2 mg/ml at −20 °C.

10-μg portions (as dimethylmethylene blue reactivity) of the D1 and pooled D2/D3 gradient fractions were incubated at 37 °C for 2 h with 0.2 milliunits protease-free chondroitinase ABC and an additional 2 h with 0.2 milliunits each of keratanase II and endo-β-D-galactosidase, in 20 μl of 50 mM sodium acetate, 50 mM Tris-HCl, 10 mM EDTA, pH 7.0, containing 7 μl each of aminobenzalenosulfonate fluoride and N-ethylmaleimide. The samples were electrophoresed on 4–12% polyacrylamide gels under reducing conditions; separated core proteins were transferred to nitrocellulose (at 75 V for 1.5 h), and membranes were developed with anti-ATEG or anti-LEC peptide-antiserum (1:5000) or 28B6 monoclonal ascites fluid (1:1000) as described (26). Reactive core proteins were visualized by exposure to preflashed Hyperfilm for 15–30 s after incubation with ECL detection reagent.

In the D1 fractions from both normal and OA cartilages, four G1-containing core protein species were seen, and these migrated with apparent molecular sizes of ~350, 250, 180, and 115 kDa. Western blots of identical samples using the G1-specific anti-LEC antiserum showed that only the 350-kDa species was reactive representing the full-length core protein containing both the N-terminal G1 and the C-terminal G3 globular domains. The faster migrating, G1-negative, core proteins represented aggrecan species that had undergone proteolytic processing in the CS-1 or CS-2 domains (26, 36). The relative abundance of the core protein species from normal and OA cartilages, estimated by densitometric scanning, showed that in OA samples the 180-kDa species was markedly lower than in normals (reduced to 25–50%), with the 350-kDa species correspondingly increased (40–70%). There was little or no change in the 250- and 115-kDa species. There was no distinction in the degree of change between normal and OA D1 aggrecan for the different anatomical sites or between patients. PGs from both normal and OA cartilages, purified into the D2 and D3 fractions, were composed of a ~250-kDa core protein (aggrecan and its proteolytic fragments) based on determination of the core protein sizes by SDS-polyacrylamide gel electrophoresis and reactivity in Western blotting with anti-ATEG and 2B6 monoclonal antibody (40).
5.5, containing 1 μM cysteine HCl, overnight at 60 °C. The digests were clarified by centrifugation at 10,000 × g for 10 min. Three volumes of 95% ethanol were added to the supernatants, and GAGs were precipitated at 4 °C for 18 h. Precipitates were washed once with 95% ethanol, and GAGs were quantitated as described (27, 33). Additional mercuric acetate treatment prior to HPLC analyses was essential to quantitate accurately the sulfated saturated disaccharides Galβ1,3GalNAc4S- and Galβ1,3GalNAc6S from the non-reducing termini.

The efficiency of CS/DS digestion by the chondroitin lyases was checked in each sample. Digests were spun through MicroCon 3 filtration units, and both filtrate and retentate were assayed for uronic acid content (35). Thus, for all samples >92% of the uronic acid was recovered in the filtrate consistent with an effective digestion of the CS/DS chains under the conditions used.

RESULTS

ΔDisaccharide Sulfation Isomers of High and Low Buoyant Density PGs from Normal and OA Cartilages—Sulfated Δdisaccharide composition of the D1 and D2/3 PGs was examined by fluorescence-based anion exchange HPLC (Fig. 2). In all D1 digests, D6S was predominant accounting for ~95% of the chain internal disaccharide in normal samples and ~93% in OA samples (Fig. 2, top panels). D4S was low in all samples, and accounted for ~1 in 20 disaccharides in the normal. This was also seen in most osteoarthritic samples, and only two were slightly higher with ~1/15 disaccharides 4-sulfated. The sulfated disaccharides, D6S and D4S, were detected in all samples whether methylated (data not shown), but only minor components (~0.07 and ~1.2%, respectively), and no significant difference was observed between the normal and OA samples. Interestingly, D6S, previously shown to be present only in fetal but not postnatal human aggrecan CS (27), was not detected in any of the normal or OA samples.

Chondroitin lyase digestion products from D2/D3 PGs in normal cartilages and present on D1s, respectively. Of those products, sulfated GalNAc residues were not significantly different from each other. D6S was more abundant (~30%) in the low buoyant density fractions and was only quantitatively obtained with chondroitinase ABC, but not chondroitinase ACII, and therefore is most likely derived from digests of DS of the decorin and biglycan present in these fractions.

Fig. 2. Separation of Δdisaccharide products by fluorescence-based ion exchange chromatography. Typical elution profiles of 500 μg portions of derivatized chondroitinase products of D1 or D2/3 PGs are shown. The data show the analyses of PGs prepared from medial femoral condyles of normal donor IV and OA donor IV.
Glycosaminoglycan Sulfation in Osteoarthritis

Separation of non-reducing terminal mono- and disaccharides by AS4A ion exchange chromatography. 5-μg portions of derivatized chondroitin lyase products from samples shown in Fig. 2 were chromatographed before (A) and after (B) merccuric acetate treatment. The elution positions of the non-reducing terminal residues are indicated and are as follows: GalNAc4S (peak 1); GalNAc4,6S (peak 2); GlcAβ1,3GalNAc4S, Di-4S (peak 3); GlcAβ1,3GalNAc6S, Di-6S (peak 4). Peaks in chromatographic runs of D2/3 PGs indicated by * were tentatively identified as ΔDi-HA, based on their detection in such samples by capillary zone electrophoresis and the sensitivity to mercuric acetate treatment.

Table I

| Sample | % lyase products as NRT residues | No average ΔDiS per NRT | Mn* | % chains terminating with GalNAcβ | GlcAβ |
|--------|---------------------------------|--------------------------|-----|----------------------------------|-------|
| Normal |                                 |                          |     |                                  |       |
| I'     | 5.5 ± 0.5                       | 18.2                     | 10.0| 90.5 ± 1.8                       | 9.5 ± 0.8 |
| II'    | 5.1 ± 0.3                       | 19.6                     | 10.8| 90.8 ± 1.8                       | 9.2 ± 0.8 |
| III'   | 5.7 ± 1.0                       | 17.5                     | 9.6 | 90.5 ± 1.5                       | 9.5 ± 1.0 |
| IV'    | 5.3 ± 0.8                       | 18.9                     | 10.4| 90.5 ± 1.0                       | 9.5 ± 0.9 |
| V'     | 5.2 ± 0.5                       | 19.2                     | 10.6| 91.5 ± 1.5                       | 8.5 ± 1.3 |
| Mean ± S.D. | 5.3 ± 0.6                     | 18.7                     | 10.3| 90.8 ± 1.5                       | 9.2 ± 1.0 |
| OA     |                                 |                          |     |                                  |       |
| I      | 5.9 ± 1.1                       | 16.9                     | 9.6 | 89.8 ± 1.6                       | 10.2 ± 1.5 |
| II     | 6.1 ± 0.9                       | 16.4                     | 9.3 | 87.1 ± 1.8                       | 12.9 ± 1.7 |
| III    | 6.2 ± 0.4                       | 16.1                     | 9.1 | 88.7 ± 1.5                       | 11.4 ± 1.7 |
| Mean ± S.D. | 6.1 ± 1.13                    | 16.5                     | 9.3 | 88.5 ± 1.6                       | 11.5 ± 1.6 |

* Number of averaged molecular masses, calculated from the No average repeating ΔDiS, corrected for molecular weight of sulfated ΔDiS (476 Da) plus the molecular weight of the non-reducing termini and the molecular weight of the linkage region (1136 Da), as described (27).

† Represents the sum of GalNAc4S and GalNAc4,6S (Fig. 3A, peaks 1 and 2, respectively).

‡ Represents the sum of Di4S and Di6S (Fig. 3B, peaks 3 and 4, respectively).

§ Data represent the means ± S.D. (n = 4) of duplicate analyses of the D1 preparations from the pooled medial and lateral tibial plateau cartilage preparations.

¶ Data represent the means ± S.D. (n = 8) of duplicate analyses of the D1 preparations from the medial and lateral tibial plateau cartilage and the medial and lateral femoral condyle preparations.

(Fig. 4A, left-hand panel). Significantly, PGs from OA cartilages revealed striking differences in the abundance of GalNAc4,6S as termini on both D1 and D2/3 PGs (Fig. 4A, right-hand panels). Thus in OA, terminal 4,6-sulfation was present only on ~30% of chains, whereas GalNAc4S was increased to about 60% of all chains.

GalNAc6S, as a non-reducing terminus could not be accurately quantitated by HPLC in the current study, since a small proportion (5–10%) of the ΔDi-6S undergoes decomposition to a fluorescent product which co-elutes with GalNAc6S during HPLC (Fig. 3 and Ref. 33). However, the amount of this residue, if present at the non-reducing terminus, must be very small, as calculations of the molecular weight of the CS chains based on the sum of all quantifiable termini gave a size range (~10 kDa) that is consistent with that expected for adult cartilage CS (Table I).

Both the 4- and 6-sulfation isomers of the saturated disaccharides were on all PGs from normal cartilages. Di-6S was predominant and terminated ~7% of chains in D1 and ~5% of chains in D2/3, compared with Di-4S which was present on only ~2% of chains in either D1 or D2/3 PGs (Fig. 4B, left-hand panel). The total number of chains with terminal GlcA was not significantly altered in OA samples, and furthermore, there was no change in the relative abundance of Di4S or Di-6S termini in either the D1 or the D2/3 PGs (Fig. 4B, right-hand panel). This result was of particular interest since chain terminal Di-6S is the mimotope recognized by monoclonal antibody 3B3(−) (27), and previous reports suggested that PGs carrying immunoreactive chains were apparently absent in normal adult human cartilages but present in OA cartilages. The chemical determination of this structure (Fig. 4B) does not, however, support the previous widely accepted quantitative interpretations of the 3B3(−) immunochemical data.

3B3(−) Immunochemical Analyses of D1 and D2/3 PGs from Normal and OA Cartilages—We showed in a recent study, using aggrecan from normal fetal and adult cartilages, that concentration and presentation of CS chains containing terminal Di-6S can either enhance or lower 3B3(−) reactivity of PGs immobilized to membranes (27). PGs from normal and OA cartilages were therefore assayed for 3B3(−) by quantitative...
immunodot blotting (see “Experimental Procedures”) to examine if the reported immunochemical distinction between normal and OA PGs may be due to differential mimotope reactivities. With this dot blot method, 3B3(−) reactivity was readily detectable in all D1 PG samples from normal cartilages (Fig. 5, top panel). Indeed, equivalent reactivities of 100 integrated pixel density units were obtained with ~0.32 and ~0.22 µg of GAG on nitrocellulose or nylon membranes, respectively, in keeping with our previous results with normal adult human aggrecan (27). PGs in D2/3 fractions from normal cartilages were also reactive, and 100 integrated pixel density units were generated with ~0.20 µg of GAG assayed on nitrocellulose and with ~0.10 µg assayed on nylon (Fig. 5, bottom panel).

Under identical assay conditions, the majority of the D1 and D2/3 PGs from OA cartilages gave 3B3(−) reactivities much like those seen with normal PG samples (Fig. 5), and this is in agreement with the chemical determination of Di-6S (Fig. 4B). Interestingly, however, three D1 preparations from OA cartilage donor I and two D2/3 preparations from OA donor III were significantly more immunoreactive than the normals (Fig. 5). Since these samples did not contain a greater abundance of 3B3(−) mimotope, the most likely explanation for this finding is that reactivity per mol of terminal mimotope is increased, presumably due to structural alterations in the proteoglycan (27).

This was indeed supported when reactivities were expressed per ng of Di-6S and compared for the different sample groups (Table II). The threshold for mimotope detection on nitrocellulose, for the highly reactive samples, was significantly lowered to 20–30 ng of GAG compared with 50–100 ng for the normal and most OA PGs. Thus, an elevated 3B3(−) immunoreactivity due to a higher reactivity per mol of mimotope would therefore be more readily demonstrated at very low concentrations of immobilized PGs (<0.1 µg of GAG), where reactivity of normal and most OA PGs is barely detectable (Fig. 5 and Table II). In the linear range of the assay (100–500 ng of GAG) for normal PGs, reactivity of such samples remained ~5-fold greater, but at the GAG concentrations (>1 µg of GAG) a distinction between 3B3(−) reactivities was no longer possible, since pixel densities generated by all samples were outside the linear range for detection with the film used, and mimotope reactivities become apparently equal in all samples.

Non-reducing Terminal GalNAc Sulfation Isomers on GAGs from Distinct Sites on Normal and OA Knee Joint Cartilages—The data in Fig. 4A clearly indicate that the marked decrease of 4,6-disulfation of non-reducing terminal GalNAc residues on CS and DS could represent a readily quantifiable, specific alteration in the fine structure of GAGs in cartilages from OA knee joints. We therefore examined the effect of anatomical site on the proportion of chains with this terminal structure in normal cartilages. Then we determined if the decreased content in OA cartilages could be related to any variations in the gross macroscopic appearance of the OA cartilages. Total tissue GAGs were prepared from the individual sampling sites (see “Experimental Procedures.” Immunoreactivity was quantitated by densitometric scanning of individual dots and expressed per µg of GAG loaded. Results for normal PGs (●) are given as the mean ± S.D. of the 12 D1 and D2/3 preparations. Results for OA PGs (○) are shown as individual data points. The broken lines connect the data points for the D1s from the medial and lateral femoral condyle and the medial tibial plateau cartilage of OA donor I (top panel) or the D2/3 from the medial and lateral femoral condyles of OA donor III (bottom panel).
mean contents of GalNAc4,6S terminating chains in medial tibial, lateral tibial, medial femoral, and lateral femoral joint cartilages from normal donors were 52.1 ± 6.4%, 52.0 ± 8.0%, 50.6 ± 10.2%, and 50.3 ± 9.3%, respectively, indicative of no marked site-variation in content. Furthermore, this was significantly reduced to 33.5 ± 14.2%, 28.1 ± 11.9%, 38.4 ± 12.6%, and 31.9 ± 9.8%, respectively, for the corresponding cartilages from OA patients (Fig. 6).

The individual data points from the 20 sites of each donor that were used to calculate the mean content of chains with GalNAc4,6S termini are also shown in Fig. 6. The GalNAc4,6S contents in these ranged from 30 to 68% in normal cartilages and from 9 to 60% in OA cartilages. In each of the OA donor knees examined, there were several apparently randomly distributed sites, in which the GalNAc4,6S terminating chains were as abundant as in the normal cartilages. However, the majority of locations from diseased joints had a significantly reduced content, and in some cases this was as low as that seen in pooled juvenile cartilages (27). Moreover, the degree of change in the content of these chains in OA joints was not apparently correlated with the macroscopic appearance of the tissue (normal or fibrillated surface), nor the degree of cartilage erosion, nor the anatomical location of the sampling site. In addition, GAG analyses (data not shown) on four cartilage plugs from OA patients that had been divided into superficial, middle, and deep zones showed that chain internal 6-sulfation generally increased with depth (from ~75 to ~92% of internal disaccharides), in keeping with previous reports for normal human cartilages (41), but the proportion of terminal GalNAc4,6S, whether high or low, remained constant throughout the tissue depth. Taken together, the data support the idea that a widespread metabolic change in OA cartilages results in the accumulation of CS/DS PGs with a low level of disulfated chain terminal GalNAc residues.

**DISCUSSION**

We report here the analyses of the GAG fine structure of CS/DS PGs from femoral condyles and tibial plateau cartilages from healthy adults and patients with late stage OA. Our particular interest was in the separation and quantitation of sulfation isomers of both internal disaccharides and non-reducing terminal residues of the GAG chains (27, 33) in order to identify distinct structural alterations of GAGs in cartilages from OA patients.

Minor increases (1–2%) in 4-sulfation of D1 CS was seen in 2 of 12 OA samples, which had previously been observed using either chemical (20) or immunochromatographic (26, 42) analyses of CS PGs prepared from OA femoral head cartilages. However, for all other CS and DS PG preparations from the diseased cartilages, the degree of sulfation and the abundance of sulfation

**Table II**

| Nanogram of GAG assayed | Pixel density per ng of Di6S<sup>a</sup> | (D1 PGs) | (D2/3 PGs) |
|-------------------------|----------------------------------------|----------|------------|
|                         | Normal<sup>b</sup> | OA<sup>c</sup> | OA<sup>d</sup> | Normal<sup>b</sup> | OA<sup>c</sup> | OA<sup>d</sup> |
| 20                      | 0 | 0 | 0 | 0 | 0 | 0 |
| 40                      | 109 ± 22 | 87 ± 35 | 180 ± 35 | 165 ± 33 | 115 ± 41 | 270 ± 45 |
| 160                     | 111 ± 37 | 115 ± 28 | 375 ± 81 | 192 ± 28 | 179 ± 36 | 340 ± 65 |
| 320                     | 93 ± 10 | 96 ± 15 | 112 ± 11 | 95 ± 17 | 87 ± 19 | 91 ± 12 |
| 1250                    |                            |          |            |            |            |            |

<sup>a</sup> Calculated from the area units of pixel densities measured in 3B3<sup>c</sup> immunodot blot assays (as shown in Fig. 5) and the ng of Di6S per μg of GAG determined by fluorescence HPLC (as shown in Fig. 2).

<sup>b</sup> Data represent the mean ± S.D. of 12 samples from normal donors I–V.

<sup>c</sup> Data represent the mean ± S.D. of 3 samples from OA donor I.

<sup>d</sup> Data represent the mean ± S.D. of 8 samples from OA donors II–V and 1 sample from OA donor II.

<sup>e</sup> Data represent the mean ± S.D. of 2 samples from OA donor III.

<sup>f</sup> Data represent the mean ± S.D. of 8 samples from OA donors II–V and 1 sample from OA donor II.

**Fig. 6.** Non-reducing terminal GalNAc sulfation isomers on GAGs from distinct sites on normal and OA knee joint cartilages. GAGs were prepared from papain-digested cartilage plugs harvested from the individual sites on the medial (MF) and lateral femoral condyle (LF) or medial (MT) and lateral tibial plateau (LT) (see Fig. 1). The percentage of CS/DS chains terminating with GalNAc4,6S in each sample was determined by HPLC analyses of chondroitinase ABC/ACII digestion products (see Fig. 3). Each data point represents the assay for an individual plug from the four joint surfaces from five normal (●) and five OA donors (○), and those from an individual donor are vertically aligned. The mean content of GalNAc4,6S termini for each normal and OA cartilage surface is shown by the horizontal line in each sample group.

**TABLE II**

| Nanogram of GAG assayed | Pixel density per ng of Di6S<sup>a</sup> | (D1 PGs) | (D2/3 PGs) |
|-------------------------|----------------------------------------|----------|------------|
|                         | Normal<sup>b</sup> | OA<sup>c</sup> | OA<sup>d</sup> | Normal<sup>b</sup> | OA<sup>c</sup> | OA<sup>d</sup> |
| 20                      | 0 | 0 | 0 | 0 | 0 | 0 |
| 40                      | 109 ± 22 | 87 ± 35 | 180 ± 35 | 165 ± 33 | 115 ± 41 | 270 ± 45 |
| 160                     | 111 ± 37 | 115 ± 28 | 375 ± 81 | 192 ± 28 | 179 ± 36 | 340 ± 65 |
| 320                     | 93 ± 10 | 96 ± 15 | 112 ± 11 | 95 ± 17 | 87 ± 19 | 91 ± 12 |
| 1250                    |                            |          |            |            |            |            |

<sup>a</sup> Calculated from the area units of pixel densities measured in 3B3<sup>c</sup> immunodot blot assays (as shown in Fig. 5) and the ng of Di6S per μg of GAG determined by fluorescence HPLC (as shown in Fig. 2).

<sup>b</sup> Data represent the mean ± S.D. of 12 samples from normal donors I–V.

<sup>c</sup> Data represent the mean ± S.D. of 3 samples from OA donor I.

<sup>d</sup> Data represent the mean ± S.D. of 8 samples from OA donors II–V and 1 sample from OA donor II.

<sup>e</sup> Data represent the mean ± S.D. of 2 samples from OA donor III.

<sup>f</sup> Data represent the mean ± S.D. of 8 samples from OA donors II–V and 1 sample from OA donor II.

<sup>g</sup> Data represent the mean ± S.D. of 2 samples from OA donor III.

**FIG. 6.** Non-reducing terminal GalNAc sulfation isomers on GAGs from distinct sites on normal and OA knee joint cartilages. GAGs were prepared from papain-digested cartilage plugs harvested from the individual sites on the medial (MF) and lateral femoral condyle (LF) or medial (MT) and lateral tibial plateau (LT) (see Fig. 1). The percentage of CS/DS chains terminating with GalNAc4,6S in each sample was determined by HPLC analyses of chondroitinase ABC/ACII digestion products (see Fig. 3). Each data point represents the assay for an individual plug from the four joint surfaces from five normal (●) and five OA donors (○), and those from an individual donor are vertically aligned. The mean content of GalNAc4,6S termini for each normal and OA cartilage surface is shown by the horizontal line in each sample group.

**DISCUSSION**

We report here the analyses of the GAG fine structure of CS/DS PGs from femoral condyles and tibial plateau cartilages from healthy adults and patients with late stage OA. Our particular interest was in the separation and quantitation of sulfation isomers of both internal disaccharides and non-reducing terminal residues of the GAG chains (27, 33) in order to identify distinct structural alterations of GAGs in cartilages from OA patients.

Minor increases (1–2%) in 4-sulfation of D1 CS was seen in 2 of 12 OA samples, which had previously been observed using either chemical (20) or immunochromatographic (26, 42) analyses of CS PGs prepared from OA femoral head cartilages. However, for all other CS and DS PG preparations from the diseased cartilages, the degree of sulfation and the abundance of sulfation

isomers in the chain interior was the same as that in healthy adult cartilages (27). Our analyses indicated that the mean size of CS chains on aggrecan in OA cartilages may be slightly smaller than in normal cartilages (Table I). Thus chains from the normal group had on average ~19 repeating disaccharide units, compared with ~16 repeating disaccharide units in the diseased group. This observation is in general agreement with earlier reports describing unchanged (22) or decreased (43) hydrodynamic sizes of GAG chains in OA cartilages as determined by gel filtration chromatography.

Of particular interest were the quantitative chemical data for the non-reducing terminal sequence GlcAβ1,3GalNAc6S (Di-6S), and these were present on ~6–8% of chains from PGs from both normal and OA cartilages. This chain terminal structure represents the mimotope recognized by the monoclonal antibody 3B3<sup>c</sup> (27) and all PGs prepared for this study exhibited easily detectable reactivity in immunodot blot assays (Fig. 5). These findings are in apparent contrast to conclusions drawn from reports by others (24, 30) that 3B3<sup>c</sup>-reactive PGs were apparently absent in normal cartilages but could be demonstrated in OA cartilages. This discrepancy might, however, in part be explained by observations made here as follows. First, several PG preparations from OA cartilages displayed a significantly higher 3B3<sup>c</sup>-reactivity than normal PGs (Fig. 5); however, this was not due to differences in mimotope con-
tent (Fig. 4B). On the other hand, the mimotopes were more reactive in these samples (Table II), and as we had previously shown for membrane-immobilized PGs, this can be influenced by the sulfation or length of the chains ending in Di-6S. Second, the threshold for detection of 3B3(−) in the solid phase assay is very steep (27) and was also seen in the present study (Fig. 5). With the differences for individual PGs (Ref. 27, and Table II), it is evident that reactivities in solid phase immunassays at low PG concentrations (i.e. <100 ng of GAG, see Table II) could be easily interpreted as the presence or absence of mimotope. These data further support our previous conclusion that 3B3(−) reactivity detected by Western blots or immunohistochemistry cannot be used alone to quantitate changes in the fine structure of CS chains during cartilage development (27) or pathologies (Figs. 4 and 5).

On the other hand, CS and DS PGs in all OA cartilages analyzed here could be distinguished from those in normal adult cartilages by the sulfation of the chain terminal GalNAc residue. PGs from all normal adult human cartilages showed a high content of GalNAc4,6S chain termini (Fig. 5 and Ref. 27), but in OA cartilages, these were 40–50% less, with proportionally more GalNAc4S termini. This sulfation change was seen in both femoral condyle and tibial plateau cartilages, and it was not confined to a particular site on these surfaces, nor was it related to the gross morphological appearance or the degree of erosion of the tissues.

The distinctive compositional change of PGs in OA cartilages could well be the result of changes in both the catabolic and anabolic pathways of chondrocytes during the progression of the disease. Thus, during phases of increased matrix degradation in the OA cartilage, normal adult PGs, abundantly substituted with chains terminating with GalNAc4,6S, might be removed from the cartilage into the synovial fluid for clearance. In this respect it is interesting to note that Hazell et al. (30) detected an increase in 3B3(−)-reactive PG fragments in human synovial fluid after traumatic knee injuries, by competitive enzyme-linked immunosorbent assay. Based on our findings that 3B3(−)-reactive CS is abundant in the normal adult cartilage matrix, the immunosassay might have detected release of degraded aggrecan that is resident in the healthy adult articular cartilage, rather than release of degraded aggrecan synthesized in response to the injury. Future work aimed at quantitation of CS chains with GalNAc4,6S in synovial fluids of such a patient group, as an alternative measure for release of normal resident aggrecan, should yield additional information on the origin of PG fragments released from cartilages after joint trauma.

The terminal GalNAc 4,6-disulfation reaction occurs as a final step in CS/DS biosynthesis, is carried out by a distinct 6-sulfotransferase with specificity for GalNAc4S, and is modulated independently of the chain interior 6-sulfation (27, 44). Alterations in the GAG biosynthesis pathways in OA cartilages may lower their capacity for 4,6-disulfation of the non-reducing termini, thus resulting in the synthesis and deposition of CS/DS with predominantly monosulfated termini. It should be noted that terminal 4,6-disulfation of aggrecan CS is also low or absent in normal fetal and postnatal growth human knee cartilages (27). However, the change in terminal GalNAc sulfation in OA cannot simply be explained by the often quoted suggestion (18–21) that chondrocytes in human OA cartilages synthesize aggrecan substituted with CS typical of fetal or growth chondrocytes. CS chains in fetal and postnatal growth cartilages are typically large (containing ~40 disaccharide repeats) and have a high content (~50%) of 4-sulfated internal disaccharides (27). If the proportion of normal adult aggrecan CS which remains in the OA cartilages, estimated from the abundance of chains with GalNAc4,6S termini, ranged from 50 to 80% of the total, then 20–50% of chains present on aggrecan would have been synthesized during the progression of the disease. If this newly synthesized CS was in the fetal or growth structure, both the ΔDi-4S content and the average chain lengths would be expected to be significantly increased in OA (by 10–20%), but this was not detected in the current analyses (Fig. 2 and Table I). However, based on chain size and both internal and non-reducing terminal sulfation patterns, it is tempting to speculate that newly synthesized CS in OA cartilages may be similar to that in cartilages taken from adolescents (15–17 years of age), just before reaching skeletal maturity (27).

In conclusion, the data described here provide for the first time quantitative chemical evidence to support the contention that metabolic changes in osteoarthritic cartilages may affect distinct sulfation steps in chondroitin and dermatan sulfate synthesis. Furthermore, the detection and quantitation of such alterations from assays of sulfation isomers of non-reducing terminal GalNAc residues, using the methods described here, should provide a powerful new approach to monitoring proteoglycan metabolism during cartilage remodeling in osteoarthritis (46).

Acknowledgments—We thank J. Boyer and V. Thompson (Shriners Hospital, Tampa Unit) for performing the Western blot analyses. We are grateful to Dr. W. E. Kilgore and the Operating Room Staff of Morton Plant Hospital, St. Petersburg, FL, as well as the Organ Procurement Agency of Michigan for their invaluable cooperation in the collection of the cartilage specimens.

REFERENCES

1. Poole, A. R. (1996) in Arthritis and Allied Conditions (Koopman, W. J. ed) pp. 279–333, Williams & Wilkins, Baltimore
2. Sandy, J. D., Plaas, A. H. K., and Rosenberg, L. C. (1996) in Arthritis and Allied Conditions (Koopman, W. J. ed) pp. 252–279, Williams & Wilkins, Baltimore
3. Eyre, D. R., McDevitt, C. A., and Muir, H. (1975) Ann. Rheum. Dis. 34, 138–148
4. Sandy, J. D., and Plaas, A. H. K. (1986) J. Orthop. Res. 4, 263–272
5. Morales, T. I., and Hascall, V. C. (1988) J. Biol. Chem. 263, 3632–3638
6. Ilie, M. Z., Handley, C. J., Robinson, H. C., and Mok, M. T. (1992) Arch. Biochem. Biophys. 294, 155–162
7. Lark, M. W., Gordy, J. T., Weidner, J. R., Ayala, J., Kimura, J. N., Williams, H. R., Manfred, R. A., Flannery, C. R., Carlson, S. S., Iwata, M., and Sandy, J. D. (1995) J. Biol. Chem. 270, 2550–2556
8. Ilie, M. Z., Haynes, R. H., Winter, G. M., and Handley, C. J. (1996) Acta Orthop. Scand. 66, 33–35
9. Sandy, J. D., Flannery, C. R., Neame, P. J., and Lohmander, L. S. (1992) J. Clin. Invest. 89, 1512–1516
10. Lohmander, L. S., Neame, P. J., and Sandy, J. D. (1993) Arthritis & Rheum. 36, 1214–1222
11. Sandy, J. D., Plaas, A. H. K., and Koob, T. J. (1996) Acta Orthop. Scand. 66, 26–32
12. Cs-Szabo, G., Melching, L. I., Roughley, P. J., and Glant, T. T. (1997) Arthritis & Rheum. 40, 1037–1045
13. Sandy, J. D., Adams, M. E., Billingham, M. E. J., Plaas, A. H. K., and Muir, H. (1984) Arthritis & Rheum. 27, 388–397
14. Matyas, J. R., Adams, M. E., Huang, D., and Sandell, L. J. (1995) Arthritis & Rheum. 38, 420–425
15. Adams, M. E., Matyas, J. R., Huang, D., and Dourado, G. S. (1995) J. Rheumatol. 22, 138–148
16. Carney, S. L., Billingham, M. E., Muir, H., and Sandy, J. D. (1995) J. Orthop. Res. 3, 140–147
17. Cox, M. J., McDevitt, C. A., Aronczeck, S. P., and Warren, R. F. (1985) Biochim. Biophys. Acta 795, 228–234
18. Micheliacci, Y. M., Mourou, P. A. S., Laredo, J., and Dietrich, C. P. (1987) Connect. Tissue Res. 7, 29–36
19. Sweet, B. M. E., Thonar, E. J. M., Immelman, A. R., and Soloman, L. (1977) Ann. Rheum. Dis. 36, 347–358
20. Rizzikall, G., Reigner, A., Bogoch, E., and Poole, A. R. (1992) J. Clin. Invest. 90, 2268–2277
21. Viseo, D. M., Johnstone, B., Hill, M. A., Jolly, G. A., and Caterson, B. (1993) Arthritis & Rheum. 12, 1718–1725
22. Slater, R. R., Bayliss, M. T., Lachiewicz, P. F., Viseo, D. M., and Caterson, B. (1995) Arthritis & Rheum. 38, 655–659
23. Carlsson, C. S., Loeiser, R. F., Johnston, B., Tulli, H. M., Dobson, D. B., and Caterson, B. (1995) J. Orthop. Res. 13, 399–409
24. Cs-Szabo, G., Roughley, P. J., Plaas, A. H. K., and Glant, T. T. (1995) Arthritis & Rheum. 38, 660–668
27. Plaas, A. H. K., Wong-Palms, S., Roughley, P. J., Midura, R. J., and Hascall, V. C. (1997) *J. Biol. Chem.* **272**, 20603–20610
28. Caterson, B., Mahmoodian, F., Sorrell, J. M., Hardingham, T. E., Bayliss, M. T., Carney, S. L., Ratcliffe, A., and Muir, H. (1990) *J. Cell Sci.* **97**, 411–417
29. Ratcliffe, A., Shurety, W., and Caterson, B. (1993) *Arthritis & Rheum.* **36**, 543–551
30. Hazell, P. K., Dent, C., Fairclough, J. A., Bayliss, M. T., and Hardingham, T. E. (1995) *Arthritis & Rheum.* **38**, 953–959
31. Shinmei, M., Miuachy, S., and Machida, A. (1992) *Arthritis & Rheum.* **35**, 1304–1308
32. Bitter, T., and Muir, H. (1962) *Anal. Biochem.* **4**, 330–335
33. Vilim, V., and Fosang, A. J. (1994) *Biochem. J.* **304**, 887–889
34. Cheng, F., Heinegard, D., Fransson, L.-A., Bayliss, M., Bielicki, J., Hopwood, J., and Yoshida, K. (1996) *J. Biol. Chem.* **271**, 28572–28580
35. Hamai, A., Hashimoto, N., Mochizuki, H., Kato, F., Makiguchi, Y., Horie, K., and Suzuki, S. (1997) *J. Biol. Chem.* **272**, 9123–9130
36. Holmblad, L. S., and Felson, D. T. (1997) *J. Rheumatol.* **24**, 782–785
37. Hollander, A. P., Heathfield, T. F., Webber, C., Iwata, Y., Bourne, R., Roraback, C., and Poole, A. R. (1994) *J. Clin. Invest.* **93**, 1722–1732