Sulfation of Chondroitin Sulfate in Human Articular Cartilage
THE EFFECT OF AGE, TOPOGRAPHICAL POSITION, AND ZONE OF CARTILAGE ON TISSUE COMPOSITION

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The chondroitin ABC lyase digestion products of normal human femoral condyle articular cartilage and of purified aggrecan were analyzed for their mono- and nonsulfated disaccharide composition. Changes in the total tissue chemistry were most pronounced during the period from birth to 20 years of age, when the [GlcAβ3GalNAcβ] disaccharide content increased from approximately 50% to 85% of the total disaccharide content and there was a concomitant decrease in the content of the 4-sulfated disaccharide. In general, the disaccharide content of the deeper layers of immature cartilage were richer in the 4-sulfated residue than the upper regions of the tissue. As the tissue aged and decreased in thickness, the disaccharide composition became more evenly 6-sulfated. The newly synthesized chondroitin sulfate chains had a similar composition to the endogenous chains and also underwent the same age and zonal changes. The monoclonal antisera 3B3(1) and 2B6(2) were used to immunolocalize the unsaturated 6- and 4-sulfated disaccharides in joint fluids are now being published as 6- and 4-sulfated residues generated at the reducing termini of the chondroitin sulfate chains by digestion with chondroitin ABC lyase, and these analyses indicated that the sulfation pattern at this position did not necessarily reflect the internal disaccharide composition of the chains. In summary, the sulfation pattern of chondroitin sulfate disaccharides from normal human articular cartilage varies with the age of the specimen, the position (topography) on the joint surface, and the zone of cartilage analyzed. Furthermore, these changes in composition are a consequence of both extracellular, post-translational processing of the core protein of aggrecan and changes in the sulfotransferase activity of the chondrocyte.

Proteoglycans are major components of the extracellular matrix of articular cartilage and provide the tissue with many of its characteristic physicochemical properties, including its ability to generate an osmotic swelling pressure, which enables it to withstand a wide range of compressive loads (1). Proteoglycans are also known to directly influence chondrocyte activity, either through cell-matrix interactions or by binding specific growth factors in the extracellular matrix, thereby modifying their temporal and spatial effects. Many of these biological interactions are properties of the glycosaminoglycan chains, which are covalently attached to the protein cores of individual proteoglycan molecules. In articular cartilage, the glycosaminoglycans are mainly chondroitin and keratan sulfate chains, as well as a small proportion of dermatan sulfate chains, and they exert their action by virtue of the high electronegative charge and spatial arrangement of their constituent sulfate groups.

Aggrecan, the major type of proteoglycan found in articular cartilage, consists of a protein core to which are attached many chondroitin sulfate chains that are predominantly 4- or 6-sulfated (2). Aging of human articular cartilage is accompanied by many changes in the structure of aggrecan and the multimeric aggregate that it forms with hyaluronan and link protein. These molecular changes are a consequence of biosynthetic and catabolic events regulated by many cellular and extracellular events. The extent to which these mechanisms are expressed within articular cartilage is, therefore, not uniform, and factors such as species, site (which joint), zone (through the tissue depth), and region (topographical distribution) all dictate specific qualitative and quantitative changes in proteoglycan. However, it is the age of the individual that appears to have the most profound effect on the composition of cartilage (3–5). Joint diseases occur most frequently in the older age group, and it is important that the changes associated with joint pathology are identified and distinguished from normal, age-related events. Although age-related changes in the proportion of 4- and 6-sulfated disaccharides have been described in human articular cartilage, these have been carried out on a relatively small number of specimens from a mixture of joints and no attempt has been made to separate changes coincident with maturation from those associated with subsequent aging (4, 6–8). Moreover, the topographical and zonal variations in sulfation pattern have not been defined, and there are no data concerning the relative contribution of the biosynthetic and degradative pathways.

The need for a better understanding of the sulfation pattern of human aggrecan is prompted by two published observations. The first of these concerns the generally accepted view that osteoarthritic chondrocytes synthesize proteoglycans with an “immature” composition (9), in keeping with the hypermetabolic activity of the tissue and the hypertrophic response of the cells. This conclusion was based on analysis of the cartilage remaining on resected hip joints, which had an increased content of 4-sulfated disaccharide. However, the study took no account of the anatomical, morphological, or age patterns described above and made no attempt to differentiate between anabolic and catabolic events. The concept of a chondroblastic phenotype in osteoarthritis may be appealing in relation to tissue repair, but there is little direct evidence to support it. Clarification of these experimental findings is urgently required, not just to determine their place in the pathogenesis of osteoarthritis, but also because measurements of 4- and 6-sulfated disaccharides in joint fluids are now being published as “markers” of proteoglycan metabolism without reference to the
normal, macro- or micro-heterogeneity of these structures (10, 11).

The second of these observations is more recent and concerns the identification, using monoclonal antibodies, of atypical structures in the chondroitin sulfate chains of animal and human osteoarthritic cartilage (12–14). The monoclonal antibodies concerned do not recognize the regular repeating structure of chondroitin sulfate chains, but appear to be specific for selected (as yet uncharacterized) sequences of sulfation both within the chains and at the non-reducing terminal site of the chains, which are found infrequently. Although more recent studies have raised doubts about the validity of this interpretation (15), there is no doubt that in osteoarthritic abnormalities in the sulfation of chondroitin sulfate chains are related to attempts at matrix remodeling and repair, and it is an interesting possibility that these abnormal glycosaminoglycan chains may contain special properties, such as an increased affinity for growth factors or other cytokines that may facilitate matrix repair by the chondrocytes.

The studies described in this report address these questions and provide an analysis of normal human articular cartilage, which will enable investigations of diseased tissue to be interpreted more effectively.

**EXPERIMENTAL PROCEDURES**

Intact joints were obtained from amputations or massive replacements at operation for bone tumors not involving the joint space. Fresh specimens (within 1 h of operation) were dissected using sterile techniques, and full-thickness cartilage was removed from the femoral and tibial articular surfaces as described below.

**Total Tissue—**Full-thickness cartilage was removed from all of the femoral condyle and died into 1–2 mm² pieces. The tissue was thoroughly mixed to ensure that a representative sample could be taken, and then triplicate aliquots of approximately 50 mg wet weight were analyzed.

**Topographical and Zonal Studies—**A single full-depth plug of cartilage including the underlying subchondral bone was taken from the sites indicated in Fig. 2 with a sterile cork borer (3 mm diameter). After removing the bone, the cartilage plug was sectioned from the surface on a cryostat at 50 μm, and every four sections were pooled to give 200-μm zones of tissue.

**Radio labeling of Cartilage Explants—**The glycosaminoglycan chains of articular cartilage explants were radiolabeled with [35S]sulfate; the exact conditions of the culture depended on the subsequent analysis. When the rate of [35S]sulfate into cartilage explants was determined, tissue was cultured in the absence of serum, in Ham's F-12 medium containing 100 units/ml of penicillin G and 100 μg/ml of streptomycin. When analyzing the newly synthesized disaccharides of chondroitin sulfate chains or for investigating the structure of newly synthesized aggrecan, involved culturing tissue in Ham's F-12 medium containing 100 μg/ml of [35S]sulfate for 8 h.

**Extraction and Purification of Aggrecan—**Aggrecan (A1 fraction) was purified from 4 × guanidinium HCl extracts of cartilage of various ages (newborn, 9, 24, and 47 years) by associative CsCl equilibrium density gradient centrifugation (17).

**Agarose/Polyacrylamide Gel Electrophoresis, Autoradiography, and Western Blotting—**A 1 fraction of purified aggrecan (newborn, 9, 24, and 47 years) was dissociated in 8 M urea under reduced conditions and subjected to electrophoresis in low and high-gelling agarose/polyacrylamide gels and the outer lanes were stained with toluidine blue to identify the aggrecan subpopulations (18, 19). Individual subpopulations of aggrecan were cut from the gel with a scalpel, and the slices were extracted with 4 M guanidinium HCl prior to their analysis by capillary zone electrophoresis after chondroitin ABC lyase digestion of the samples with 25 milliunits of chondroitin ABC lyase overnight. An aliquot of supernatant was re-diluted to 100 μg/ml in 50 mM sodium acetate, pH 7.4 for 3 h at room temperature: HG1, monoclonal antibody recognizing the G1 domain of human aggrecan (20); MZ15, monoclonal antibody recognizing a pentasaccharide sequence present on keratan sulfate chains (21); 3B3, monoclonal antibody recognizing the terminal unsaturated 4-sulfated disaccharide remaining after digestion of chondroitin sulfate chains with chondroitin ABC lyase (22); 2B6, monoclonal antibody recognizing the terminal unsaturated 4-sulfated disaccharide remaining after digestion of chondroitin sulfate chains with chondroitin ABC lyase (22).

**Chondroitin ABC Lyase Digestion—**Chondroitin disaccharides were separated on a 270A-HT capillary electrophoresis system (Applied Biosystems, Warrington, Cheshire, United Kingdom (UK)) connected to a DS4000 chromatography work station (Drew Scientific, London, UK). The uncoated capillary was initially washed for 2 min with 100 mM NaOH and then conditioned for 5 min with 40 mM phosphate, 40 mM SDS, 10 mM borate adjusted to pH 9.0. The disaccharides released from chondroitin sulfate chains with chondroitin ABC lyase were introduced into the capillary using hydrodynamic loading for 2 s (approximately 8 nL) at 15 kV. The capillary was loaded for 15 min, the disaccharides were migrated past the detector window by the resultant electro-endosmosic flow. Detection was by UV absorbance at 232 nm. Peak identity was achieved by comparison of migration time with authentic reference standards (Δdi-0S, Δdi-6S, Δdi-4S) and by overspiking into chondroitin ABC lyase digests. Digest stability was determined by conducting repeat injections at hourly intervals over a 24-h period.

**Capillary Electrophoresis—**The disaccharides released from chondroitin sulfate chains with chondroitin ABC lyase were separated on a 270A-HT capillary electrophoresis system (Applied Biosystems) connected to a DS4000 chromatography workstation (Drew Scientific). The analysis was conducted at pH 9.0 to monitor nonsulfated and monosulfated disaccharides. The uncoated capillary (72 cm × 50 μm internal diameter, 0.62 mm inner diameter), was held at 25 °C, filled for 2 min with 100 mM NaOH and the sample introduced hydrodynamically for 2 s (8 μL), over which time the capillary eluent was monitored for UV absorbance at 232 nm. Disaccharides were eluted with 40 mM phosphate, 40 mM SDS, 10 mM borate adjusted to pH 9.0 for 5 min. The concentrations of Δdi-6S, Δdi-4S, and Δdi-0S were summed and expressed as a proportion of the total disaccharide composition. Recovery of exogenously added chondroitin chains was assessed by the use of papain. A batch of samples were initially subjected to a simple chondroitin ABC lyase digestion. After digestion, the tissue was removed, rinsed in water, dried on paper, and then subjected to papain digestion. Once the material had been digested, the papain was denatured by boiling for 5 min at 95 °C. The sample was then treated with chondroitin ABC lyase overnight. An aliquot of supernatant was removed and analyzed by capillary electrophoresis. No peaks were detected for Δdi-0S, Δdi-6S, or Δdi-4S, leading us to conclude that complete digestion of disaccharides is achieved with chondroitin ABC lyase treatment alone.

**HPLC Separation of Radiolabeled Disaccharides—**Radiolabeled cartilage was digested with papain in 0.1 M Tris acetate, 2.4 mM EDTA, 10 mM cysteine, pH 5.8, at 65 °C for 24 h. Samples were then dialyzed into 0.1 M Tris acetate, pH 8.0, and concentrated in a Centrucon ultrafiltration device containing a membrane with M, 10,000 exclusion. Concentration was achieved by centrifugation at 4600 × g for 40–45 min. Following digestion of the samples with 25 milliunits of chondroitin ABC lyase, they were mixed with four volumes of cold ethanol and stored overnight at 4 °C. After removal of a small pellet containing the enzyme and any undigested material, the supernatants were eluted from a Partisil 5-PAC column equilibrated in Tris borate buffer as described previously (23). The Δdi-6S and Δdi-4S disaccharides released from endogenous chondroitin sulfate chains were monitored by their UV absorbance at 232 nm, and the radiolabeled ([35S]sulfate) Δdi-6S and Δdi-4S disaccharides were analyzed using an in-line monitor (Berthold HPLC radioactivity monitor, LB 506 C-1) and their proportions were calculated relative to each other.

1 The abbreviations used are: Δdi-0S, Δdi-6S, and Δdi-4S, un saturated 0-sulfated, 6-sulfated, and 4-sulfated disaccharides, respectively, released from chondroitin sulfate by the action of chondroitin ABC lyase; HPLC, high performance liquid chromatography.
Immunohistochemistry—Full-depth blocks (1 mm × 2 mm) pieces of articular cartilage including the subchondral bone were taken from the femoral condyle with a scalpel and snap-frozen in n-hexane that was prechilled in a CO₂/ethanol slurry. Cryosections (7 μm) of the cartilage, mounted on glass microscope slides, were digested at room temperature for 30 min with 25 milliunits of chondroitin ABC lyase, and the terminal disaccharides remaining on the chondroitin sulfate chains were localized with the monoclonal antibodies 3B3 (6-sulfated) and 2B6 (4-sulfated) and reaction products were detected using a gold-enhancement-of-silver kit marketed by Amersham International Ltd. The specificity of the immunostaining was determined by digesting the tissue sections with chondroitin ACII lyase, which removes the final disaccharide remaining associated with the core protein of the proteoglycan after it is treated with chondroitin ABC lyase. This procedure completely abolished all of the 3B3(+) and 2B6(+) immunoreactivity.

RESULTS

Effect of Age on the Sulfation of Chondroitin Sulfate—The unsaturated disaccharides of chondroitin sulfate released from full-thickness slices of normal human cartilage by chondroitin ABC lyase were mainly the monosulfated and nonsulfated disaccharides Ddi-6S, Ddi-4S, and Ddi-0S. Disulfated and higher sulfated products were also identified, but they only accounted for a small proportion of the total disaccharide pool (<1%). The Ddi-6S content of cartilage increased with age up to 20 years of age, whereas the Ddi-4S decreased immediately after birth, up to 20 years of age (Fig. 1). During subsequent aging (20–85 years), there was little change in the overall 6- or 4-sulfation. In contrast, the content of Ddi-0S was relatively constant throughout life (2–5%), as was the proportion of higher sulfated products (results not shown).

Topographical and Zonal Variations in Sulfation—The schematic shown in Fig. 2 illustrates the different areas of the knee joint selected for analysis. Although all areas of cartilage expressed age-related changes, there was considerable variation in the Ddi-6S:Ddi-4S molar ratio of full-thickness cartilage and the value obtained depended on the region of the joint surface sampled (results not shown). These topographical differences in composition were more obvious in tissue obtained from immature joints, but they were also evident in mature specimens. The extent to which these variations reflected changes in the thickness of cartilage and, therefore, zonal changes in composition, was investigated by sectioning cartilage from the articular surface through the tissue depth and analyzing consecutive 200-μm layers. As an example of the site-dependent variability in composition, the results obtained for the posterior tip of the lateral femoral condyle from knee joints of four different ages, are shown in Fig. 3. It is apparent that Ddi-6S and Ddi-4S concentrations are not evenly distributed in different zones. In immature (9-year-old) cartilage, chondroitin sulfate chains in the upper half of the tissue were Ddi-6S-rich, whereas the lower half of the tissue contained chains that were predominantly Ddi-4-sulfated. Furthermore, the concentration of Ddi-6S increased from the mid-zones of the tissue toward the articular surface and the highest concentration of this disaccharide was located in the upper quartile of the tissue (800–1200 μm). The decreased content of 6-sulfation observed at the surface of cartilage was a consistent finding at all ages. By 14 years of age, the thickness of cartilage at this site had decreased by 50% to approximately 3 mm and there was a concomitant loss of the deeper, Ddi4-S-rich region observed in the
younger specimen, except for the zones directly adjacent to the subchondral bone. The remainder of the tissue retained a \( \Delta \text{di-6S} : \Delta \text{di-4S} \) ratio that was similar to the distribution measured in the upper half of the 9-year-old specimen. By 43 years of age, the cartilage at this site had reduced in thickness even further and the \( \Delta \text{di-6S} \) content of the middle and deep zones of the tissue had increased by 10–30%; the composition of the surface layers remained unchanged. These trends continued with advancing age until, at 60 years of age, the cartilage was only 1.25 mm thick and all zones had a \( \Delta \text{di-6S} \) content of approximately 85%, with the exception of the 200-\( \mu \text{m} \) surface layer of the tissue.

When this detailed analysis of disaccharide composition was extended to the other eleven areas of the joint, age-related variations were observed at all of these sites (Fig. 4). In addition, at both ages, there was a topographical variation in the zonal distribution of chondroitin sulfation. This was more pronounced in immature cartilage, where cartilage thickness in different areas of the joint was also most variable, but the same phenomenon was also evident at different sites on the adult knee joint.

The content of the 0-sulfated disaccharides generated by chondroitinase ABC digestion of chondroitin sulfate was also measured for the specimens described above. Although this disaccharide comprised only a small proportion of the total disaccharide pool, it also exhibited zonally related changes. These were mainly confined to the articular surface where they were present at a higher concentration compared with the remainder of the cartilage (results not shown).

**Synthesis of Chondroitin Sulfate Chains**—The rates of \([\text{35S}]\)sulfate incorporation into full-depth cartilage pieces (i.e., glycosaminoglycan synthesis) confirmed that the biosynthetic activity of the chondrocyte was higher during the years just prior to and during puberty (Fig. 5). This anabolic response decreased between 20 and 30 years of age and, although there was a transient increase in activity, at an age usually associated with the time of menopause in women (40–60 years), the rates of synthesis remained fairly constant during adult life. In order to determine the extent to which the aging and zonal changes described in the previous section were a consequence of anabolic or catabolic events, cartilage explants were radio-labeled with \([\text{35S}]\)sulfate and the disaccharide composition of the endogenous and newly synthesized chondroitin sulfate chains were compared. The results indicated that, at all ages, chondroitin sulfate chains from both pools have a similar disaccharide composition. Furthermore, when the same analysis was applied to different zones of cartilage, the \( \Delta \text{di-6S} \) and \( \Delta \text{di-4S} \) content of endogenous and newly synthesized chains had a similar composition (Fig. 6, a–c) at all ages.

**Analysis of Aggrecan Fractionated by Agarose/PAGE**—Purified aggrecan preparations were fractionated by electrophoresis in large-pore, agarose/polyacrylamide gels (Fig. 7). All of the toluidine blue-stained bands were immunoreactive with a polyclonal antiserum raised against the G1 domain of human aggrecan (Fig. 8). Whereas extracts of newborn cartilage (4 h old) contained only one major aggrecan species (band 1a), which migrated at a slower rate than band 1b in the preparations from the 9-, 24-, and 47-year-old specimens, with advancing age, bands 2 and 3 accounted for a higher proportion of the total aggrecan pool. The “free G1” domain, a normal turnover product of aggrecan that accumulates in cartilage during aging, was also identified as a diffuse, slower migrating, immunoreactive band in the gels. The presence of keratan sulfate in each aggrecan species was also confirmed by immunoreactivity with the monoclonal antibody MZ15.

The individual bands 1a, 1b, 2, and 3 from each specimen shown in Fig. 7 were extracted from the gels and re-electrophoresed (results not shown) to ensure that they were homogeneous, before releasing the sulfated disaccharides from the
constituent chondroitin sulfate chains with chondroitinase ABC. As expected, an age-related increase in the concentration of \(\Delta\text{di-6S}\) and in the ratio of \(\Delta\text{di-6S:}\Delta\text{di-4S}\) was measured, in keeping with the total tissue analysis, but this was generally higher than that obtained after digesting intact tissue samples (Table I). This finding may indicate that the proteoglycans

**FIG. 4.** The zonal distribution of \(\Delta\text{di-6S (closed symbols)}\) and \(\Delta\text{di-4S (open symbols)}\) through the depth of articular cartilage at 12 sites on the knee joint, in specimens of 9 years (●, ○) and 60 years (●, ○) of age. Full-depth samples of articular cartilage were taken from defined anatomical sites on the femoral condyles (as illustrated in Fig. 2), and each plug of tissue was sectioned at 50 μm. The disaccharides of chondroitin sulfate released by digestion of the tissues with chondroitin ABC lyase were analyzed by capillary electrophoresis.
remaining in the tissue residue after extraction (10–15% of the total glucuronate), presumably those that are intimately associated with the collagenous network, are Δdi-4S-rich. There was very little difference in the disaccharide composition of bands 1b, 2, and 3 at any one age. Furthermore, at each age there was also very little difference in the composition of each band indicating that, if they arise primarily by extracellular cleavage of the core protein, then the 6- and 4-sulfated disaccharides are very evenly distributed on the protein core of aggrecan at all ages.

Age-related changes in disaccharide composition were also observed when Western blots of the large pore gels were probed with the monoclonal antibodies 3B3 and 2B6 after chondroitinase ABC digestion of the membranes. These antibodies recognize the terminal unsaturated 6- or 4-sulfated disaccharide that remains associated with the protein core after digestion of aggrecan with chondroitinase ABC. Thus, there was an increase in the intensity of Δdi-6S staining of all bands with advancing age, indicating that the disaccharide in this position of the chondroitin sulfate chain is predominantly 6-sulfated (Fig. 8).

**Immunohistochemical Localization of the Reducing Terminal Disaccharide of Chondroitin Sulfate Chains**—The monoclonal antibodies 3B3 and 2B6 were also used to immunostain chondroitinase ABC-digested histological sections of cartilage in order to determine if there was any variation in the spatial distribution (pericellular versus intercellular) of chondroitin sulfate chains substituted with terminal unsaturated Δdi-6S or Δdi-4S disaccharides. The findings obtained for adult cartilage (23 years) demonstrated that the 4-sulfated disaccharide recognized by 2B6 was only localized intercellularly in the top and bottom 20 μm of the tissue, where the staining was very strong. The matrix in the remainder of the cartilage did not stain, and reaction product was confined to a pericellular location through most of the tissue depth. The 6-sulfated disaccharide recognized by 3B3 was also unevenly distributed. It showed both intercellular and pericellular staining at the surface and it was also concentrated in a region in the middle zones of the cartilage.

The Composition of Newly Synthesized and Endogenous Chondroitin Sulfate Chains Associated with Aggrecan—The similarity in the disaccharide composition of the endogenous and newly synthesized chondroitin sulfate chains, suggested that the same results should be expected for purified aggrecan. The metabolic relationship between newly synthesized aggrecan and the pool of proteoglycan pre-existing in the tissue, was investigated by pulse-labeling full-thickness samples of cartilage from donors of different ages with [35S]sulfate. The metabolic relationship between newly synthesized aggrecan and the pool of proteoglycan pre-existing in the tissue, was investigated by pulse-labeling full-thickness samples of cartilage from donors of different ages with [35S]sulfate. The labeling period was restricted to 4 h in order to minimize
turnover of aggrecan and avoid the production of degradation products. At all ages, autoradiography of the agarose/polyacrylamide gels that were used to fractionate aggrecan, showed that over 90% of the radioactivity was restricted to the slower migrating band 1 in each case (Fig. 9). However, extracts of the mature specimens did contain some labeled proteoglycan migrating at the position of bands 2 and 3. The latter finding was more pronounced when extracts were analyzed from different zones of cartilage. Whereas zones 2–8 (400 μm each) of the 9-year-old cartilage only contained one radiolabeled aggrecan band, zones 2–7 (200 μm each) from the 60-year-old specimen contained two bands, and in zone 8 of the 60-year-old specimen, three labeled bands were identified (Fig. 10). In marked contrast, three radiolabeled proteoglycan bands were observed in the surface zone of both the 9- and the 60-year-old specimens of cartilage. The specific banding pattern of the surface zone of the tissue was observed for all specimens regardless of their age (results not shown). In addition, the [35S]sulfate radiolabel in the surface slices was more evenly distributed between the three bands, compared with the bands from the other zones of the tissue.

**DISCUSSION**

Proteoglycans have a major role in maintaining the physicochemical, mechanical, and metabolic homeostasis of articular cartilage. In this report, we have concentrated on the age-related changes occurring in the major chondroitin sulfate containing proteoglycan, aggrecan. The constituent chondroitin sulfate chains of aggrecan impart the negative fixed charge density that is the basis of many of the molecule’s properties (1). By inference, it is generally accepted that normal age-related changes in the composition of chondroitin sulfate chains (e.g., sulfation pattern) reflect important, but as yet poorly understood, changes in the properties of the extracellular matrix. These changes are initiated by the resident chondrocytes, presumably to enable the tissue to respond more effectively to the altered biological environment that it encounters with advancing age. There are some published studies of the chondroitin sulfate-disaccharide composition of normal human articular cartilage (4, 8, 24). However, these either inves-
tigated a very limited age range, or they were primarily aimed at describing the changes in the osteoarthritic femoral head cartilage and suffered from the design flaws highlighted in the Introduction. Sophisticated methods have also been developed to enable the chondroitin sulfate chain size and the number of chains/protein core to be determined and major changes in these parameters in bovine cartilage were identified (25). In the study described here, no attempt was made to determine the size or number of chondroitin sulfate chains at different ages, but it is clear that changes in these parameters do occur in human articular cartilage (4). It is therefore likely that the physicochemical properties of aggrecan are influenced by these structural events.

We have shown that the major changes in sulfation pattern occur during maturation of the cartilage (up to approximately 20 years of age) and that this corresponds to the period when the chondrocyte is most actively synthesizing glycosaminoglycan chains. However, the more detailed analysis of regional and zonal changes emphasized how these parameters can influence the data. It was clearly shown that age-related changes in composition at different sites and in different compartments of the tissue continued to take place beyond the age indicated by the measurements of randomized, full-depth tissue samples. The data shown in Fig. 4 indicate that the thickness of the tissue may correlate with the mechanical load to which the different topographical regions of the joint are subjected and in this way the composition of the tissue may be affected. However, although there was very little difference in tissue thickness at sites 10 and 11 for the 9- and 60-year-old specimens, there was a dramatic change in the Δdi-6S:Δdi-4S ratio at these sites. Thus, metabolic differences cannot be attributed only to mechanical loading, and they often likely reflect metabolic changes that are a consequence of normal aging. It is of interest that Plaas et al. (26) also observed changes in the sulfation of the non-reducing termini of chains, later in life than the alterations in 4- and 6-monosulfation of the internal repeating disaccharides. Whether this biological feature of aging represents two different phases of tissue metabolism, which are influenced to different extents by anabolic and catabolic events, remains to be established.

Another anomalous result was obtained when the biosynthesis of chondroitin sulfate chains was studied. The similar disaccharide composition of newly synthesized and endogenous chains, analyzed in full-depth cartilage, suggested that most age-related and zonal changes were biosynthetic in origin, but the agarose/polyacrylamide gels (Figs. 7 and 9) indicated otherwise. The purified, newly synthesized aggrecan was largely confined to a single molecular species, whereas the pre-existing aggrecan was represented by up to three molecular species with different electrophoretic mobilities. Thus, it is coincidental that the composition of these different molecular pools were similar, and we conclude that the most likely mechanism giving rise to the heterogeneity observed in aggrecan molecular size is catabolic in origin. This would be in keeping with many other studies, which have provided structural and enzymatic evidence that is consistent with this hypothesis. Even so, it can be confidently stated that the data are consistent with the replacement of a considerable proportion of the chondroitin sulfate pool during the period from before to after puberty. This analysis also supports the hypothesis put forward by Lohmander et al. (27), which incorporated the concept of multiple turnover pools of proteoglycan in articular cartilage.

Furthermore, the appearance of multiple [35S]sulfate-labeled bands in the composite gels (Figs. 9 and 10) suggests that the structural changes can also arise via anabolic processes. It
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is also possible that the role of each of these metabolic processes will depend not only on the age of the individual, but also on the tissue compartment from which the molecule is derived (pericellular versus intercellular) and the zone of tissue analyzed. This hypothesis is certainly supported by the immunohistochemical studies and the Western blots of composite gels. It is worth noting that in both cases the distribution was very different to that obtained by chemical analysis of the tissue or analysis of purified aggrecan, suggesting that the terminal unsaturated disaccharide remaining after digestion of the chondroitin sulfate chains does not necessarily reflect the composition of the remainder of the chain (28). These studies also support the conclusions of Plaa et al. (15), who showed that the use of monoclonal antibodies alone can be very misleading and that, regardless of the important structural information they can impart, monoclonal antibody technology should only be used as an adjunct of detailed chemical analyses.

An additional interesting finding that has emerged from the current investigation is a zonal variation in disaccharide composition and the fact that this is not constant with age or topographical location on the joint surface. These analyses confirm that all three parameters can influence the sulfotransferase(s) activity of chondrocytes. In particular, the relatively high Δdi-4S content of the deep layers of articular cartilage before puberty suggests that this composition is a consequence of the hypertrophic properties of the chondrocyte and thus, the mineralization of the tissue, which is still active at this age. Analyses of the Δdi-6S:Δdi-4S ratio of articular cartilage and the growth (epiphyseal) cartilage from an 8-year-old specimen (1.90 and 0.77, respectively) also support this hypothesis, as do the measurements that Deutsch et al. (25) made of bovine tibial growth plates. However, the latter investigation again highlighted the problems that are encountered if the metabolic heterogeneity of chondrocytes is not taken into account. Deutsch et al. (25) found that, although the total tissue content of growth cartilage is rich in Δdi-4S, this composition is confined to the resting and upper proliferative zones of the tissue and the hypertrophic zones are in fact Δdi-6S-rich. It should be appreciated that these cartilages reside on opposite sides of the secondary center of ossification and that they are likely to exist under different mechanical and biological conditions that could influence the metabolic state of the resident chondrocytes. There are also changes in the chemistry of the growth plate depending on its stage of development. It is known that closure of the growth plate is not an even process, and the variation in Δdi-6S:Δdi-4S ratio measured in zones of cartilage derived from different topographical locations is consistent with this explanation. Furthermore, it is likely that there are also species and joint specificity associated with the chemistry. For example, Lemperg et al. (29) showed that articular cartilage from calves and heifers was almost exclusively chondroitin 4-sulfate and Platt et al. (30) demonstrated that the Δdi-6S:Δdi-4S ratio of normal articular cartilage from equine metacarpophalangeal joints decreased with advancing age, unlike that of the middle carpal joints, where there was an age-related increase in the ratio (31). Thus, our hypothesis concerning a simple association between mineralization of cartilage and the presence of Δdi-4S-rich chondroitin sulfate chains has not been adequately tested in the present study and will require further investigation.

Disaccharide measurement of tissue slices and the structural studies of aggrecan fractionated on composite gels also indicated that the synthesis products of chondrocytes in the surface layers of cartilage were different. First, it has been shown that decorin is enriched in these zones of cartilage and the chondroitin sulfate/dermatan sulfate chains associated with this core protein are known to be Δdi-4S-rich (32). Second, a recent publication from Schumacher et al. (33) described the purification of a high molecular weight proteoglycan from extracts of the surface layers of human articular cartilage, and it is likely that its presence in tissue digests contributed to the lower Δdi-6S:Δdi-4S at this site.

The main objectives of the present investigation were (i) to characterize the age-related, topographical, and zonal changes in the chondroitin sulfate disaccharide composition of human normal articular cartilage and to determine the extent to which this reflects the composition of the chains associated with aggrecan and (ii) to ascertain the extent to which these changes were anabolic or catabolic in origin. These objectives have been achieved, and the findings reported here provide a useful reference for characterizing the changes in composition and metabolism observed in osteoarthritic cartilage.

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