

Age-related changes in the sulphation of the chondroitin sulphate linkage region from human articular cartilage aggrecan

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The chondroitin sulphate (CS) linkage regions have been isolated from human articular cartilage aggrecan (from 10- to 72-year-olds) by chondroitin ABC endolyase digestion and size-exclusion chromatography. Linkage region hexasaccharides have been characterized and their abundance estimated by high-pH anion-exchange chromatography. The basic structure for the CS linkage region oligosaccharides identified from human aggrecan is as follows: $\Delta\text{UA}(\beta 1-3)\text{GalNAc}[0\text{S}/4\text{S}/6\text{S}](\beta 1-4)\text{GlcA}(\beta 1-3)\text{Gal}[0\text{S}/6\text{S}](\beta 1-3)\text{Gal}(\beta 1-4)\text{Xyl}$, where ΔUA represents 4,5-unsaturated hexuronic acid, 4S and 6S represent an O-ester sulphate group on C-4 and C-6 respectively, and 0S represents zero sulphation. There are significant age-related changes in the abundance of the various *N*-acetylgalactosamine (GalNAc) sulphation forms identified, occurring up to approx. 20 years old. During the period from 10 to 20 years old the level of GalNAc 6-sulphation at the linkage region increases from approx. 43% to approx. 75%, while there is a corresponding reduction in

unsulphated (approx. 30% to approx. 20%) and 4-sulphated (approx. 25% to approx. 6%) GalNAc residues. There is also an increase in the incidence of linkage region galactose 6-sulphation (approx. 2% to approx. 10%) which was only observed in linkage regions with GalNAc 6-sulphation. Beyond 20 years old there are few changes in the relative abundance of these GalNAc sulphation variants; however, there is a slight increase in the abundance of 6-sulphation between approx. 20 years old and approx. 40 years old and a slight decrease in its abundance beyond approx. 40 years old. Our data show that in the majority of chains from tissues of all ages the GalNAc residue closest to the linkage region is 6-sulphated, but the level of GalNAc 6-sulphation within the linkage region is lower than the average level observed within the repeat region.

Key words: ABC lyase, chondroitin sulphate, endolyase, glycosaminoglycan, osteoarthritis.

INTRODUCTION

The sulphated glycosaminoglycan (GAG), chondroitin sulphate (CS), is an abundant component of the large aggregating proteoglycan aggrecan, and it is known to contribute to the load-bearing properties of articular cartilage. The specific sulphation patterns in CS are important in macromolecular interactions and often the presence of 4-sulphated *N*-acetylgalactosamine (GalNAc) and the absence of 6-sulphation governs the interaction [1]. Such interactions include cytoadherence of malaria-infected red blood cells [2], the adherence of the malaria parasite to human placenta [3,4], and the regulation of neurite outgrowth [5]. However, it is a chondroitin 6-sulphate-containing epitope of the proteoglycan DSD-1, which is reported to influence neurite outgrowth [6]. In addition, the expression of some CS epitopes in the rodent are known to be developmentally regulated [7,8].

CS chains from articular cartilage comprise a linkage region, a repeat region and a chain cap. The repeat region consists of a repeating disaccharide $[-4)\text{GlcA}(\beta 1-3)\text{GalNAc}(\beta 1-)]_n$, which may be sulphated at C-4 and/or C-6 of GalNAc and at C-2 of glucuronic acid (GlcA). In adult articular cartilage CS 6-sulphation dominates (approx. 95%) over both unsulphated (approx. < 1%) and 4-sulphated (approx. 5%) GalNAc residues, and the level of GlcA sulphation is also low [9]. The linkage region has the general structure $\text{Gal}(\beta 1-3)\text{Gal}(\beta 1-4)\text{Xyl}(\beta 1-O)\text{Ser}$ [10]; a variety of CS linkage region structures with various sulphation patterns have been identified in articular cartilage [11], bovine nasal septum cartilage [12,13], rat chondrosarcoma

[14] and whale cartilage [15]. In addition, there are reports of galactose (Gal) 4- and 6-sulphation in CS isolated from aggrecan of shark cartilage [16,17], bovine nasal septum [12,13] and bovine articular cartilage [11]. For a review of CS linkage region structures see <http://www.glycoforum.gr.jp/science/word/teoglycan/PGA06E.html>. Clearly, further studies are required in order to elucidate the reasons for these, possibly tissue-related, variations in linkage region sulphation.

The linkage region described above is common to CS, dermatan sulphate, heparan sulphate (HS) and heparin. Several studies have shown that the amino acid sequence of the protein core may have an effect upon the GAG synthesized [19,20]; however, the detailed structure of the priming oligosaccharide is also important [21,22]. The structure of the repeat region close to the linkage region of CS from adult bovine articular cartilage has been shown to differ from the bulk of the repeat region; it contains a higher than average level of unsulphated and 4-sulphated GalNAc residues and a concomitantly lower level of 6-sulphation [11,23,24]. In contrast, the CS chain cap has been found to have a high sulphation density with a 4,6-disulphated GalNAc being abundant [25,26].

There is mounting evidence for age-related changes in the structure of GAGs. Bayliss et al. [27] reported an age-related increase in the ratio of 6- to 4-sulphated GalNAc residues in human articular cartilage CS, while Plaas et al. [25,26] reported an age-related increase in the abundance of 4,6-disulphated GalNAc at the caps of such chains. The capping structure of articular cartilage keratan sulphates (KSs) also shows an age-

Abbreviations used: CS, chondroitin sulphate; GAG, glycosaminoglycan; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; HPAEC, high-pH anion-exchange chromatography; HS, heparan sulphate; KS, keratan sulphate; ΔUA , 4,5-unsaturated hexuronic acid (4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid).

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related change [28,29], with an increasing level of $\alpha(2-6)$ -linked *N*-acetylneuraminic acid [28,29], while the repeat region shows an increasing chain length, level of Gal sulphation and fucosylation [29]. Each of these studies found that in the human the greatest changes in the abundance of these structures occurred in the period up to 20 years old.

The sulphation profile of CS, both at different positions along a chain and at different ages, is of considerable interest, as it has been proposed that determination of average 4- or 6-sulphation levels may be an appropriate marker of articular cartilage turnover and of osteoarthritis [30]. In the present study the structure and age-related changes in the CS linkage regions from human aggrecan are examined.

MATERIALS AND METHODS

Sepharose CL-6B was purchased from Pharmacia (Uppsala, Sweden), Toyaparl HW-40s was obtained from Tosohaas via Anachem (Luton, Herts., U.K.), and Bio-Gel P2 was purchased from Bio-Rad (Watford, Herts., U.K.). Diphenylcarbamyl chloride-treated trypsin (bovine pancreas; EC 3.4.21.4), guanidinium chloride (practical grade) and mixed whale and shark CS were purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.). Chondroitin ABC endolyase (protease-free) (*Proteus vulgaris*; EC 4.2.2.4), keratanase II and chondroitin ACII lyase (*Arthrobacter Aurescens*; EC 4.2.2.5) were obtained from Seikagaku Corp. (Chuo-Ku, Tokyo, Japan). CsCl was purchased from Fluka chemicals (Gillingham, Dorset, U.K.), and NaOH (A. R. 46/48%) was obtained from Fisons Scientific Equipment (Loughborough, Leics., U.K.). All other chemicals were of analytical grade.

Cartilage source

Each linkage region sample, with one exception, was derived from a single human femoral head. The exception was pooled material (mean age = 72 ± 12 years old, $n = 7$) for which fibromodulin structure has already been reported [31].

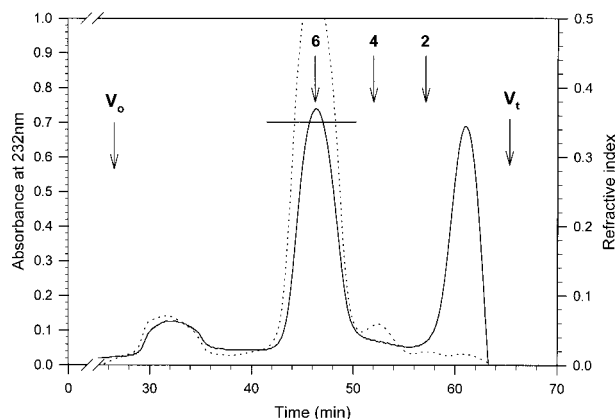


Figure 1 HW-40s size-exclusion chromatography of linkage region oligosaccharides

The linkage region oligosaccharides were isolated for subsequent HPAEC analysis by size-exclusion chromatography on a column (50 cm \times 1 cm) of Toyaparl HW-40s eluted with 0.5 M ammonium acetate at 0.4 ml/min. The eluate was monitored on-line by measuring the refractive index (continuous line) and absorbance at 232 nm (broken line), and the hexasaccharide linkage regions were pooled as shown. The elution positions of known hexa-, tetra- and di-saccharides (6, 4 and 2 respectively) are shown. The hexasaccharides represent > 98% of the CS oligosaccharides present by estimation of 232 nm absorbance. V_0 , void volume; V_t , total volume.

Isolation of linkage region fragments

The large aggregating proteoglycan, aggrecan, was isolated from human femoral head cartilage by standard methods, and the CS linkage regions were isolated as previously described for bovine and equine materials [11]. Briefly, following extraction of the comminuted cartilage with 4 M guanidinium chloride in the presence of a cocktail of protease inhibitors, the extract was subjected to associative CsCl density gradient centrifugation. CS-rich material (6B2 fraction) was obtained from the A1 fraction by chondroitin ABC endolyase and trypsin digestions followed by size-exclusion chromatography on a column of Sepharose CL-6B as previously described [11,32,33].

The CS linkage regions in the 6B2 fraction were released from the peptide by β -elimination with 1 M sodium borohydride in 0.05 M NaOH at 45 °C for 48 h [34], the reaction being terminated by the careful addition of 2 M acetic acid. The released CS oligosaccharides were separated from other materials by size-exclusion chromatography on a column (50 cm \times 1 cm) of Toyaparl HW-40s eluted with 0.5 M ammonium acetate at 0.4 ml/min [9,11,23]. The eluate was monitored by measuring the absorbance at 232 nm and the refractive index. The hexasaccharide-sized CS oligosaccharides (see Figure 1) were pooled and the volatile ammonium acetate was removed by repeated freeze-drying and resuspension in distilled water prior to examination by high-pH anion-exchange chromatography (HPAEC) fingerprinting [9,11,23].

HPAEC

The reduced linkage region oligosaccharides were examined by HPAEC on a calibrated Dionex chromatography system using a method previously described for CS linkage regions [9,11] and for repeat region di-, tetra- and hexa-saccharides [23,35]. The Carbopac PA1 column (4.6 mm \times 250 mm) was calibrated with reduced repeat region di- and tetra-saccharides along with linkage region standards [9,11]. The relative abundance of each oligosaccharide identified was determined by estimation of the peak area and the application of a response factor [23,36].

NMR Spectroscopy

Heterogeneous linkage region samples (75–145 μ g) isolated following HW-40s size-exclusion chromatography were resuspended in 0.5 ml of 99.8% $^2\text{H}_2\text{O}$, buffered to pH 7 with 10 mM sodium phosphate and referenced with sodium 3-trimethylsilyl [$^2\text{H}_4$]propionate as an internal standard. After micro-filtration through 0.45 μ m nylon filters, samples were freeze-dried and exchanged several times with 0.5 ml of 99.8% $^2\text{H}_2\text{O}$ and once with 0.5 ml of 99.96% $^2\text{H}_2\text{O}$ before final dissolution into 0.7 ml of 99.96% $^2\text{H}_2\text{O}$.

High-field ^1H -NMR spectra at 600 MHz were performed at 43 °C using a Varian Unity INOVA spectrometer equipped with a 5 mm probe capable of field-gradient experiments. All chemical shifts are quoted relative to internal sodium 3-trimethylsilyl [$^2\text{H}_4$]propionate at 0.0 p.p.m. The spectra were acquired using a 7000 Hz spectral width and approx. 28672 complex points, and were processed by application of a 0.2 Hz exponential window function and Fourier transformation into 65536 complex points.

The abundance of each form of the repeat region GalNAc present, i.e. 4-, 6- or un-sulphated, was estimated by integration of the responses for H-4 within the terminal 4,5-unsaturated hexuronic acid (Δ UA), which is known to be sensitive to the sulphation of the adjacent GalNAc [24,37].

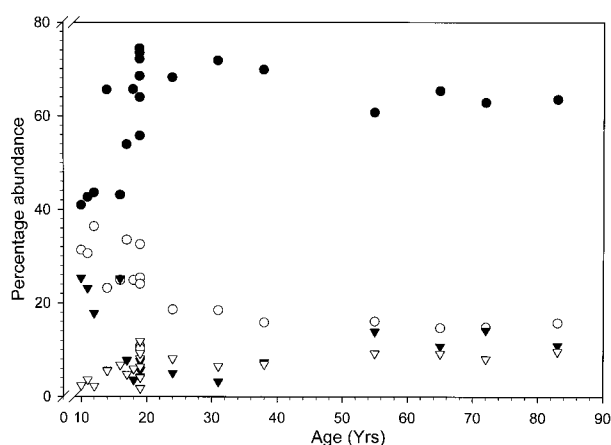


Figure 5 Age-related changes in the sulphation of CS linkage regions from human articular cartilage aggrecan

○, Δ UA(β 1-3)GalNAc(β 1-4)GlcA(β 1-3)Gal(β 1-3)Gal(β 1-4)Xyl-ol; ●, Δ UA(β 1-3)GalNAc6S(β 1-4)GlcA(β 1-3)Gal(β 1-3)Gal(β 1-4)Xyl-ol; ▼, Δ UA(β 1-3)GalNAc4S(β 1-4)GlcA(β 1-3)Gal(β 1-3)Gal(β 1-4)Xyl-ol; ○, Δ UA(β 1-3)GalNAc6S(β 1-4)GlcA(β 1-3)Gal6S(β 1-3)Gal(β 1-4)Xyl-ol.

Table 1 Percentage abundance of linkage regions estimated by NMR and HPAEC

	Percentage abundance			
	10-Year-old		72-Year-old	
GalNAc sulphation	NMR	HPAEC	NMR	HPAEC
Unsulphated	26.0	31.4	13.5	14.9
4-Sulphated	30.0	25.3	14.0	14.2
6-Sulphated	44.0	43.3	72.5	70.9

the linkage region sulphation profile did not differ from that seen following analysis of linkage regions from the CS-rich region (6B2 fraction) of the same aggrecan sample (results not shown).

We have examined the one-dimensional $^1\text{H-NMR}$ spectra of the heterogeneous mixtures of linkage regions, and partial spectra of samples from a 10-year-old and a 72-year-old may be seen in Figure 3. We have previously characterized the $^1\text{H-NMR}$ spectra for the octa-, hexa- and tetra-saccharide linkage regions of CS from articular cartilage aggrecan [11,24], and reported that the signals from H-1 and H-4 of the non-reducing terminal Δ UA were differently perturbed dependent upon the sulphation status of the adjacent GalNAc residue. Comparison of the one-dimensional spectra obtained from human samples with our previous data [24] shows that the abundance of linkage regions in which the GalNAc is 6-, 4- or un-sulphated may be estimated by examination of H-4 signals from the adjacent non-reducing terminal Δ UA. The abundance of each structure was estimated by integration of H-4 signals from the terminal Δ UA and the data were found to be in close agreement (Table 1) with the data derived from HPAEC analysis (Figure 2) of the same samples.

DISCUSSION

We have used an HPAEC fingerprinting method [9,11,23] for the analysis of CS linkage region hexasaccharides from human articular cartilage aggrecan of various ages. Linkage region

structures have been identified (Figure 4) in which the GalNAc residue may be either 4-, 6- or un-sulphated; in addition, Gal B 6-sulphation, always found in conjunction with GalNAc 6-sulphation, has been identified.

It has been shown that there are significant age-related changes in the abundance of each linkage region identified (Figure 5). These dramatic changes occur primarily during the period up to 20 years old, and both the level and profile of sulphation of the CS linkage regions change over this period. There is a significant increase in the abundance of GalNAc 6-sulphation, with a concomitant decrease in the levels of both 4- and un-sulphated residues.

The total sulphation level (abundance of 4- plus 6-sulphated GalNAc residues) increases from approx. 70% at 10 years old to approx. 80–90% at 20 years old, although through this period the level of 4-sulphation falls. These data are in agreement with those of Cheng et al. [13] who found that this residue was sulphated in almost 100% of chains from old human articular cartilage. The level of GalNAc 6-sulphation for older humans compares well with data from both bovine and equine samples [11].

The sharp rise in GalNAc 6-sulphation at the linkage region slows at approx. 20 years old (Figure 5). Structural changes in other cartilage GAGs have also been found to occur primarily during the period from birth to 20 years old, with a dramatic slowing in the rate of change after this age. Examples of structures which undergo such changes include CS cap structure [25,26] and repeat region sulphation [27], and KS cap, linkage [28,29] and repeat region sulphation, fucosylation and chain length [29]. The pattern of age-related changes seen at the linkage region in the present study, i.e. an increase in GalNAc 6-sulphation, is also observed within the repeat region [27] and at the chain caps [25,26].

No data are available for linkage region sulphation at ages below 10 years old; however, the sharp rise in GalNAc 6-sulphation seen between 10 and 20 years old suggests that at younger ages there may be an even lower level of GalNAc 6-sulphation. This would be in accord with age-related changes in both the repeat region [27] and chain caps [25,26], which both show a low level of GalNAc 6-sulphation that increases from birth to approx. 20 years old.

The data suggest that between 20 years old and approx. 40 years old there are few significant changes in either the total level of sulphation or distribution between 4-sulphation and 6-sulphation; however, during the later years, approx. 40–80 years old, there is a slight decrease in the level of GalNAc 6-sulphation with a concomitant increase in the levels of 4-sulphated residues; there is little change in the abundance of unsulphated GalNAc residues. Further data are required to determine if this change is widely observed and reflects normal aging, or is an early response to a pathological condition such as osteoarthritis. Plaas et al. [25,26] have reported that in osteoarthritis the abundance of 4,6-disulphated CS chain caps falls with a concomitant increase in 4-sulphated residues. It is noteworthy that in the linkage region there is no significant change in unsulphated residues, which are abundant in young tissues.

Comparison of the level of linkage region GalNAc 6-sulphation observed at each age with the data of Bayliss et al. [27] for the levels of GalNAc 6-sulphation within the repeat region shows that the level of linkage region GalNAc 6-sulphation is always lower than the average level of GalNAc 6-sulphation along the chain. This preferential localization of 4- and un-sulphated GalNAc residues close to the linkage region, at all ages [11], is in sharp contrast with the highly sulphated chain caps [25,26]. It is clear that the sulphation profile of the linkage region

of CS chains from articular cartilage aggrecan of human, as well as bovine and equine, origin does not reflect the composition of the remainder of the chains.

In human articular cartilage CS linkage region Gal **B** may be 6-sulphated, but only in conjunction with 6-sulphation of the GalNAc residue. Furthermore, we have shown that the abundance of this novel structure [11] increases with age.

There was no evidence of any uronic acid sulphation close to the linkage region at any age, in agreement with data for adult bovine and equine articular cartilage aggrecan [11], along with other studies of CS linkage region structure ([16,17] and see <http://www.glycoforum.gr.jp/science/word/proteoglycan/PGA06E.html>), none of which report the observation of a sulphated uronic acid close to the linkage region of CS. However, in heparin and HS a sulphated uronic acid has been observed as the non-reducing terminal residue of linkage region oligosaccharides, which are of octasaccharide size or larger [38].

The sulphation pattern of the CS linkage regions from the CS-rich region of aggrecan (6B2 fraction) and the KS-rich region (6B1 fraction) were examined in selected samples. There were no differences in the CS linkage regions of these two regions of aggrecan. These data suggest that there is no variation in the sulphation of CS linkage regions at different locations along the aggrecan monomer.

Data from the present study, and elsewhere [11], show that the linkage region of articular cartilage CS has a lower than average level of sulphation, whereas the chain cap has been shown to have a high level of sulphation [25,26]. KS from cornea [39] along with that from articular and tracheal cartilage fibromodulin [31,40–42] has also been shown to have levels of sulphation which are lower at the linkage region than towards the chain cap.

Many of the reported properties of CS, such as the cytoadherence of malaria-infected red blood cells [2], the adherence of the malaria parasite to human placenta [1,3,4] and the regulation of neurite outgrowth [5], involve GalNAc residues that are 4-sulphated. Indeed 6-sulphated GalNAc residues have been shown to abolish the ability of CS to inhibit malaria parasite binding to human placenta [3,4]. Thus the region of the CS chain which is closest to the linkage region, and which has a lower level of GalNAc 6-sulphation, may be able to participate in interactions and have properties and functions which differ from the rest of the chain. In addition, with aging, as the level of GalNAc 6-sulphation increases the extent of such interactions would change. The observation of altered sulphation at the linkage region has also been reported for HS [43], heparin [44] and KS [40].

In addition to the estimation of the abundance of each linkage region by HPAEC (Figure 2), selected linkage region mixtures have been examined using high-field NMR spectroscopy (Figure 3) and may be compared with the data from our previous NMR study of pure isolated linkage regions [24]. The data derived from NMR spectroscopy were found to be in close agreement (Table 1) with data from HPAEC analysis (Figure 2). This agreement confirms the validity of each method and demonstrates that NMR spectroscopy, although requiring greater amounts of material (> approx. 75 µg), is useful in the non-destructive estimation of the sulphation pattern for the GalNAc residues close to the CS linkage regions. NMR spectroscopic approaches may allow the characterization of the abundance of discrete structures within a mixed population [45].

In conclusion, it is shown that the pattern of sulphation close to the linkage region of human articular cartilage aggrecan CS chains differs markedly with age. With increasing age, up to approx. 20 years old, there is an increase in the relative abundance of 6-sulphated GalNAc residues and a concomitant reduction

in both 4- and un-sulphated residues. There is also an increase in the abundance of Gal 6-sulphation. Beyond 20 years old there is a slight reduction in the abundance of 6-sulphated GalNAc. This analysis of the normal age-related changes in sulphation of CS linkage regions will allow the interpretation of data from pathological samples.

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