Sulphation heterogeneity in the trisaccharide (GalNAc β 1,4GlcA β 1,3GalNAcS) isolated from the non-reducing terminal of human aggrecan chondroitin sulphate

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We report here the isolation and sulphation isomer analyses of trisaccharides GalNAcS(β 1,4)GlcA(β 1,3)GalNAcS (in which S indicates sulphate) derived from the non-reducing termini of aggrecan chondroitin sulphate. Rat chondrosarcoma and human aggrecans were digested for 1 h at 37 °C with 30 μ -units of endo-chondroitinase ABC per μ g of chondroitin sulphate, and trisaccharides were isolated from the digests by ToyoPearl HW40S gel-filtration chromatography. Four trisaccharide species were identified; their sulphation isomer compositions, as determined by digestion with chondroitinase ACII and fluorescence-based ion-exchange HPLC, were GalNAc4S β 1,4-GlcA β 1,3GalNAc4S, GalNAc4S β 1,4GlcA β 1,3GalNAc6S, GalNAc4,6S β 1,4GlcA β 1,3GalNAc4S and GalNAc4,6S β 1,4-GlcA β 1,3GalNAc6S. The abundances of such sequences in chondroitin sulphate on aggrecan from normal (foetal to 72

years of age) and from osteoarthritic human knee cartilages were also established. The results showed that non-reducing terminal GalNAc4S or GalNAc4,6S can be linked to either a 4-sulphated or a 6-sulphated disaccharide, suggesting that the sulphation of the last disaccharide might not have a direct effect on the specificity of chondroitin sulphate terminal GalNAc sulphotransferases. Furthermore, for each aggrecan preparation examined, the 4S-to-6S ratio of all chain interior disaccharides was equivalent to that in the last repeating disaccharides at the non-reducing terminus, suggesting that neither chondroitin 4-sulphotransferase nor chondroitin 6-sulphotransferase shows preferential activity near the chain terminus.

Key words: chondroitin lyases, glycosaminoglycans, proteoglycans.

INTRODUCTION

Aggrecan is a major structural component of cartilage, providing compressive stiffness to the extracellular matrix through its substituent polyanionic glycosaminoglycan (GAG) chains. Chondroitin sulphate (CS) is the predominant GAG, and recently published cDNA sequence information of aggrecan core proteins from a range of species has shown that there are approx. 100 potential sites for CS substitution, all localized in the 'CS attachment region' [1–7]. Chemical analyses of extracted and purified aggrecan [8,9] furthermore suggest that *in vivo* most of these sites are used for substitution with a GAG chain. It has been observed that alterations in aggrecan CS chain size and sulphation pattern accompany cartilage remodelling at different stages of skeletal development and maturation [10–12], suggesting that functionally adapted 'glycoforms' of aggrecan are generated during post-translational modification, presumably by alterations in the activities of GAG polymerization and sulphation enzymes [13–16].

A number of pioneering studies on the regulation of CS biosynthesis were performed *in vitro* by assaying microsomal fractions enriched in GAG transferase activities on CS oligosaccharide substrates of defined structure [17–19]. A similar approach has been applied to the examination of substrate requirements of purified or recombinantly expressed glycosyltransferases [20] and sulphotransferases [21,22]. These studies suggested, for example, that reaction kinetics of GalNAc and GlcA transfer to the non-reducing ends of growing CS chains could be influenced by the size and sulphation of the polymer

acceptor [20,23,24] and that CS polymerization and sulphation occur in the same Golgi compartments [25,26]. However, the conclusions drawn from such assays *in vitro* by different laboratories have often been contradictory. This might have been due to variations in enzyme source, assay conditions, or purity of the oligosaccharide acceptors, as well as the general problem of reconstituting *in situ* conditions for GAG biosynthesis. For example, it is not clear whether CS elongation requires two transferases, or whether both steps can be performed by a single protein, as in the polymerization of both HS [27] and HA (reviewed in [28]). Moreover, the extent to which antiport transporter-mediated transfer of activated substrates into the Golgi lumen [29] might control chain polymerization events has not been elucidated.

The structural determination of specific sulphation sequences of GAGs extracted from tissues or produced in cell cultures has also given important clues to the mechanisms of the cellular regulation of biosynthesis, especially for GAGs such as heparan sulphate (HS) [30,31], keratan sulphate (KS) [32] and dermatan sulphate (DS) [33,34]. NMR structural analyses have identified sulphation sequences for CS oligosaccharides [35–37] as well as the sulphation of linkage-region oligosaccharides [38,39] and several adjacent repeat disaccharides [40,41]. Such fragments, mostly generated by chondroitin lyases, showed a notably high degree of diversity in the sulphation pattern of the linkage regions as well as the disaccharides near the core. However, no clear pattern has emerged that suggests a direct correlation between the sulphation isomers near the core linkage regions and those in the bulk of the polymer.

Abbreviations used: GAG, glycosaminoglycan; PG, proteoglycan; CS, chondroitin sulphate; endo-ChABC, protease-free chondroitinase ABC; ChACII, chondroitinase ACII; Δ , non-reducing terminal unsaturated glucuronic acid residue; TTS, terminal trisaccharide pool; HS, heparan sulphate.

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We report here the determination of the sulphation sequence for the non-reducing terminal trisaccharides GalNAcS β 1,4GlcA β 1,3GalNAcS (in which S stands for sulphate), prepared from human aggrecan CS, by a novel procedure using endo-chondroitinase ABC [42]. A comparison of the sulphation sequences of CS prepared from aggrecan from human knee cartilages of different ages [12] and from osteoarthritic joints [43] showed specific maturation-related and disease-related patterns. However, no one sulphation sequence in particular seemed to have a regulatory role for CS chain elongation or termination.

MATERIALS AND METHODS

Protease-free chondroitinase ABC (*Proteus vulgaris*) (endo-ChABC) and chondroitinase ACII (*Arthrobacter aurescens*) (ChACII) were purchased from Seikagaku Corporation (Rockville, MD, U.S.A.). All other chemicals, chromatography supplies and high-buoyant-density D1 preparations of human aggrecan were obtained as described in [12,43]. Superdex Peptide column (HR10/30) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.).

Digestion of purified aggrecan with chondroitin lyase

Aggrecan (containing 10 μ g of CS, as determined by the uronic acid method [12]) was dissolved in 50 μ l of 0.1 M ammonium acetate, pH 7.3. CS was digested by ChACII to internal Δ disaccharides (in which Δ indicates a non-reducing terminal unsaturated glucuronic acid residue) and the non-reducing terminal mono- and disaccharides as described [12]. Other portions were digested with endo-ChABC, using either a range of enzyme concentrations (20–5000 μ -units) for 1 h, or 300 μ -units of enzyme, for various incubation times (0.5–8 h). Reactions were terminated at 100 °C for 4 min, volatile buffer salts were removed by evaporation in a Speedvac, and products were derivatized with 2-aminopyridine/borane dimethylamine (15:1, w/w in glacial acetic acid) and analysed by ion-exchange HPLC [12].

Aggrecan preparations digested under the above conditions were filtered through MicroCon 3 units (Amicon, Beverly, MA, U.S.A.), and both filtrates (containing released CS digestion products) and retained materials (containing CS oligosaccharide stubs on the core protein) were assayed for uronic acid [43]. Under the conditions used here, more than 87% of the CS was removed from the aggrecan core protein after at least 1 h of digestion with more than 30 μ -units of endo-ChABC per μ g of CS.

Identification of oligosaccharide products from the non-reducing termini of CS

Adult human aggrecan (approx. 50 μ g of CS) was digested with 1.5 m-units of endo-ChABC for 1 h, and total digests were applied to a ToyoPearl HW40S gel-filtration column. Chromatography was performed in 0.1 M ammonium bicarbonate, pH 7.0 (flow rate 0.8 ml/min), and elution of Δ -products from the internal portions of the chains was detected by A_{232} . Eluate was collected (0.8 ml fractions), salts removed by evaporation in a Speedvac, and elution positions of non-reducing terminal products established by 2-aminopyridine/borane dimethylamine derivatization and ion-exchange HPLC before and after mercuric acetate treatment of the fluorescent products.

Preparation and compositional analyses of oligosaccharide products from the non-reducing termini

Various sulphation isomers of the CS trisaccharides GalNAcS β 1,4GlcA β 1,3GalNAcS- were identified and elution positions of

Table 1 Separation of 2-aminopyridine-derivatized CS non-reducing products by AS4A ion-exchange chromatography

Non-reducing terminal products	Elution time of fluorescent derivative (min)
Peak 1 GalNAc4S*	~ 6
Peak 2 GalNAc4,6S*	~ 24
Peak 3 GalNAc4S-Di4S†	~ 26
Peak 4 GalNAc4S-Di6S†	~ 27
Peak 5 GalNAc4,6S-Di4S†	~ 44
Peak 6 GalNAc4,6S-Di6S†	~ 45

* Generated in ChACII digests, see Figure 1(a).
† Generated in PFChABC digests, see Figures 3 and 4.

Figure 1 Cleavage of adult human aggrecan CS by ChACII and endo-ChABC

Adult human aggrecan (10 μ g portions) was digested for 2 h at 37 °C with either 100 m-unit of ChACII (a) or 300 μ -units of endo-ChABC (b); and products were analysed by fluorescence-based ion-exchange HPLC. Full-scale chromatography profiles are shown in the top traces to illustrate the elution of disaccharide Δ Di6S (from the chain interior) but are shown at a larger scale in the bottom traces to highlight the elution positions of the non-reducing terminal monosaccharides GalNAc4S and GalNAc4,6S. The putative oligosaccharide intermediates from the chain interior and the non-reducing termini are indicated by an asterisk in (b). (c) The abundances of all digestion products were quantified by fluorescence ion-exchange HPLC; the yields of Δ Di6S and sulphated monosaccharides (GalNAc4S and GalNAc4,6S) per μ g of uronic acid generated after 1–8 h by 100 m-unit of ChACII (○) or 300 μ -unit of endo-ChABC (●) are shown.

their fluorotagged derivatives on AS4A ion-exchange column established as follows: portions (100 μ g) of aggrecan (from adult human cartilage and rat chondrosarcoma tumour) were digested with 1.5 m-unit of endo-ChABC, and fractionated by ToyoPearl HW40S gel-filtration chromatography to prepare fractions enriched in trisaccharide size products (Figure 3A). Thus, trisaccharides designated terminal trisaccharide pool 2 (TTS2) (see Figures 3c and 3e) were prepared by combining eluates collected between 32 and 35 ml. For the isolation of TTS1 oligosaccharides (see Figures 3b and 3d), equivalent portions of aggrecan were digested with endo-ChABC but exposed to mercuric acetate before ToyoPearl chromatography. This treatment converted the relatively abundant chain internal Δ tetrasaccharides into tri-

Figure 2 Fractionation of endo-ChABC digests of adult human aggrecan by ToyoPearl HW40S gel-filtration chromatography

(a) Endo-ChABC digests of adult human aggrecan (see the Materials and methods section for details) were fractionated by gel-filtration chromatography on ToyoPearl HW40S; the eluate was monitored by measuring A_{232} . (b, c) Products in individual fractions collected between 30 and 39 ml were subsequently analysed by fluorescence-based ion-exchange HPLC. Typical chromatograms of fractions at 31.2, 34 and 38 ml before (b) and after (c) treatment with mercuric acetate are shown. Decomposition of Δ oligosaccharides to the corresponding monosaccharides or trisaccharides is also indicated in (c). Putative oligosaccharides containing non-reducing termini were eluted at 27.5 and 44.5 min and are marked by single and double asterisks respectively.

saccharides; these were then effectively separated from TTS1 oligosaccharides, which were collected in eluates between 30 and 32 ml.

For subsequent determinations of the sulphation isomers of isolated non-reducing terminal trisaccharides, pooled fractions from ToyoPearl chromatography were desalted by Superdex peptide chromatography in water; products were dried and redissolved in 100 μ l of 50 mM ammonium acetate, pH 7.3. Each sample was divided into two portions and incubated for 2 h at 37 °C in the absence or presence of 100 m-units of ChACII. Digestion products were analysed after fluorescent derivatization by AS4A ion-exchange chromatography.

The procedure for the preparation of non-reducing terminal oligosaccharides from the ChABC digests exposes digestion fragments for a prolonged period to ammonium-containing buffers. Kuster and Harvey [44] indicated that amination of the free reducing terminal sugars might occur under such conditions. To examine whether reducing terminal GalNAc residues in the ChACII and endo-ChABC products might be affected in this way before fluorescent derivatization, 10 μ g each of Δ Di4S and Δ Di6S (the structures of these disaccharides are given in [12]) were first subjected to digestion and chromatography under conditions identical with those used for oligosaccharide preparations; they were then derivatized with 2-aminopyridine/borane dimethylamine. The yield of fluorescent Δ disaccharides after pretreatment with ammonium buffers was identical with that of untreated Δ Di4S or Δ Di6S. Therefore the procedure used here provides a quantitative estimate of all chondroitinase products, regardless of the duration of incubation before fluorescent derivatization.

Identification and quantification of sulphation isomers of terminal trisaccharides from aggrecan in normal and osteoarthritic human cartilages

Portions (10 μ g) of aggrecan from normal human femoral condylar cartilages (foetal to 72 years of age [12]) and from degenerative femoral condylar cartilages [43] were digested with 0.15 m-unit of endo-ChABC for 1 h at 37 °C. Digests were derivatized with 2-aminopyridine and fractionated by AS4A ion-exchange HPLC. Non-reducing terminal monosaccharides (GalNAc4S and GalNAc4,6S) and trisaccharides were identified by their respective retention times (see Table 1) and quantified. For all endo-ChABC digests, 85–90% and more than 95% of GalNAc4S and GalNAc4,6S respectively were recovered in the trisaccharide products.

RESULTS

Cleavage of adult human aggrecan CS by ChACII and endo-ChABC

Hamai et al. [42] recently reported the purification of an endo-ChABC, which depolymerizes CS and DS to both Δ Di- and Δ oligosaccharides. Here we examined whether oligosaccharide intermediates could also be generated from the non-reducing termini of CS chains. Adult human aggrecan was incubated with either ChACII or endo-ChABC for various durations (1–8 h) and fluorescently derivatized products were analysed by ion-exchange HPLC (Figures 1a and 1b). As expected, ChACII depolymerized CS to Δ Di6S and the non-reducing terminal monosaccharides GalNAc4S and GalNAc4,6S (Figure 1a); each

product was generated quantitatively after 1 h (Figure 1c, ○). The Δ Di6S product was also detected in endo-ChABC digests (Figure 1b) but at a lower yield at all digestion times than under equivalent incubation conditions with ChACII (Figure 1c, left panel). Similarly, for 1–2 h digests with endo-ChABC, the non-reducing terminal monosaccharides GalNAc4S and GalNAc4,6S were barely detectable (Figure 1b) and were recovered only after 8 h (Figure 1c, middle and right panels). In contrast, in short incubations with endo-ChABC a number of unidentified fluorescent peaks were seen (Figure 1b) and their late elution times (26–28 min and 35–45 min) suggested their identification as oligosaccharide-sized products.

It was also noted that the saturated disaccharides Di4S and Di6S, previously determined as minor chain-terminating structures, were always recovered quantitatively after only 1 h of digestion (results not shown). This might have resulted from a rapid initial endolytic cleavage of such GlcA-terminating chains to liberate a hexasaccharide intermediate, which was further digested to a saturated disaccharide and a tetrasaccharide [41]. Alternatively, GlcA might be at the termini of chains that are more readily digested by endo-ChABC.

Identification of oligosaccharide products containing the non-reducing CS chain termini from adult human and rat chondrosarcoma aggrecan

A 1 h endo-ChABC digest of the adult human aggrecan was fractionated by ToyoPearl HW40S gel-filtration chromatography; the eluate was monitored by measuring A_{232} . The major detectable products were the Δ tetrasaccharides and Δ disaccharides (Figure 2a). Their fluorotagged derivatives were eluted at approx. 40 and 20 min respectively from ion-exchange HPLC, and were converted to the corresponding trisaccharides or monosaccharides after treatment with mercuric acetate (Figure 2c).

Oligosaccharides containing the non-reducing termini are not detectable by measuring A_{232} . However, when products in individually collected fractions were fluorotagged and subjected to ion-exchange HPLC, two additional minor components, with retention times of approx. 45.5 and approx. 27.5 min (Figure 2b, left and middle panels respectively) were seen. Their low abundance and unchanged elution times after treatment with mercuric acetate (Figure 2c, left and middle panels) suggested that these might be oligosaccharide products containing the non-reducing chain termini.

Such oligosaccharides were subsequently prepared from digests of adult human aggrecan in TTS1 and TTS2 oligosaccharide pools after ToyoPearl HW40S chromatography (see the Materials and methods section). Typical chromatography profiles of digests fractionated before and after treatment with mercuric acetate are illustrated in Figure 3(a). Ion-exchange HPLC of the fluorotagged products from the TTS1 pool were resolved into the putative terminal oligosaccharide with a 27.5 min retention time (see Figure 2b), as well as small amounts of co-eluting Δ DiS (18–20 min) and Δ tetraS (approx. 5–45 min) (Figure 3b, top trace). These products were also further digested by ChACII to give GalNAc4S and Δ Di6S as determined after fluorescent derivatization (Figure 3b, bottom trace). Because digestion of the co-purified Δ tetraS might have been responsible for the proportionally higher yield of Δ Di6S, the 27.5 min oligosaccharide peak was taken to represent the terminal trisaccharide GalNAc4S β 1,4GlcA β 1,3GalNAc6S. The TTS2 pool from adult human aggrecan was prepared after treatment of the endo-ChABC digests with mercuric acetate (see the Materials and methods section and Figure 3a). It contained the putative

Figure 3 Sulphation isomer sequence analyses of the trisaccharides from the non-reducing termini of rat and human aggrecan

(a) TTS1 and TTS2 oligosaccharide pools were prepared from adult human aggrecan by ToyoPearl HW40S gel-filtration chromatography of endo-ChABC digests subjected or not to pretreatment with mercuric acetate (see the Materials and methods section for details). Portions of oligosaccharide pools from human (b, c) or rat (d, e) chondrosarcoma aggrecan were analysed after fluorotagging by ion-exchange HPLC either before (upper traces) or after (lower traces) digestion with ChACII (ACII). (b, d) TTS1; (c, e) TTS2.

terminal oligosaccharide, which had an elution time from ion-exchange HPLC of 45.5 min (Figures 2b and 3c, top trace). Digestion of this oligosaccharide with ChACII generated GalNAc4,6S and Δ Di6S (Figure 3c, bottom trace), indicating the isolation of the terminal trisaccharide, GalNAc4,6S β 1,4GlcA β 1,3GalNAc6S, in this pool.

Because only trisaccharides with pre-terminal GlcA β 1,3GalNAc6S- were recovered from adult human CS digests, rat chondrosarcoma aggrecan CS, composed of approx. 95% 4-sulphated disaccharides, was used as a potential source of trisaccharides containing pre-terminal GlcA β 1,3GalNAc4S-. TTS1 and TTS2 oligosaccharide pools were prepared from these PGs in a manner identical with that shown in Figure 3(a) for human aggrecan. These TTS1 products were resolved into a major species that eluted at 26.5 min from the ion-exchange resin after fluorotagging. Digestion with ChACII generated GalNAc4S and Δ Di4S, consistent with identification of this product as GalNAc4S β 1,4GlcA β 1,3GalNAc4S (Figure 3d). From the rat chondrosarcoma TTS2 oligosaccharide pool, a major fluorescent product was generated that eluted at 44.5 min from ion-exchange HPLC. GalNAc4,6S and Δ Di4S (Figure 3e) were the predominant products after the digestion of TTS2 with ChACII, identifying the 44.5 min peak as the non-reducing terminal trisaccharide, GalNAc4,6S β 1,4GlcA β 1,3GalNAc4S. A summary

Figure 4 Ion-exchange HPLC of non-reducing terminal trisaccharides on CS from normal and osteoarthritic human aggrecan

Endo-ChABC digests of aggrecan from normal human cartilages (foetal, and 18 and 72 years of age) and from osteoarthritic cartilages (74 years of age) were fluorotagged and fractionated by ion-exchange HPLC. Examples of the chromatograms are shown to illustrate the separation and quantification of the non-reducing terminal monosaccharides (arrows 1 and 2) and trisaccharides (arrows T3 to T6; see Table 1).

of the compositional analyses of all detectable non-reducing terminal trisaccharides and elution times of their fluorescent derivatives is shown in Table 1.

Identification and quantification of CS non-reducing terminal trisaccharide sequences in human aggrecan from subjects of different ages

Human aggrecan CS (see the Materials and methods section for details), previously characterized for chain length, chain internal and non-reducing terminal sulphation [12,43], was treated with endo-ChABC and the non-reducing terminal trisaccharides were identified and quantified from the total digest by ion-exchange HPLC of fluorotagged products. Parts of typical chromatograms from foetal, adolescent, adult and OA aggrecan that showed the elution of non-reducing terminal monosaccharides and trisaccharides are shown in Figure 4. In all samples, a quantitative yield of all terminal trisaccharides was evident from the absence of peaks corresponding to the terminal monosaccharides GalNAc4S and GalNAc4,6S (Figure 4, arrows 1 and 2 respectively).

The two trisaccharides, GalNAc4S β 1,4GlcA β 1,3GalNAc4S- and GalNAc4S β 1,4GlcA β 1,3GalNAc6S- (Figure 4, arrows T3 and T4), were seen in digests of aggrecan prepared from foetal and postnatal growth cartilages (0–5 years), with both sulphation isomers in about equal abundance (Figure 5). Peaks corresponding to trisaccharides with terminal GalNAc4,6S were absent, as expected from our previous results [12] that

showed the lack of disulphated terminal GalNAc residues in foetal and juvenile CS.

In comparison with the juvenile aggrecan, the yield of trisaccharides per μ g of CS was higher in the adolescent (13–18 years) PGs, which is consistent with the decreased size in that age group [12]. Moreover, there was a change in the proportion of the two sulphation isomers, GalNAc4S β 1,4GlcA β 1,3GalNAc4S and GalNAc4S β 1,4GlcA β 1,3GalNAc6S; the latter constituted more than 80% of the total trisaccharides. It was noted that in one 15-year-old and one 18-year-old donor, a third trisaccharide, GalNAc4,6S β 1,4GlcA β 1,3GalNAc6S (Figure 4, arrow T6) accounted for approx. 5–10% of the total trisaccharides.

For normal adult (22–72 years) aggrecan, the yield of terminal trisaccharides per μ g of CS was also consistently higher than in foetal and juvenile aggrecan, but similar to that in adolescent aggrecan. Two types of terminal trisaccharide were recovered: GalNAc4S β 1,4GlcA β 1,3GalNAc6S and GalNAc4,6S β 1,4GlcA β 1,3GalNAc6S (Figure 4, arrows T4 and T6 respectively), which were present in about equimolar abundances. This would indicate the absence of CS chains with the terminal sulphation sequences GalNAc4S β 1,4GlcA β 1,3GalNAc4S- in normal adult cartilages (Figure 4, arrow T3). The sequence GalNAc4,6S β 1,4GlcA β 1,3GalNAc4S- (Figure 4, arrow T5), identified in digests of rat chondrosarcoma aggrecan, was not found in the human aggrecan preparations of the ages examined here.

Endo-ChABC digests of aggrecan from adult osteoarthritic cartilages also contained GalNAc4S β 1,4GlcA β 1,3GalNAc6S and GalNAc4,6S β 1,4GlcA β 1,3GalNAc4S (Figure 4, bottom traces), which constituted approx. 80% and approx. 20% of the

Figure 5 Proportional abundance of CS non-reducing terminal trisaccharide sequences in normal and osteoarthritic human aggrecan

The relative abundances of non-reducing terminal trisaccharides during cartilage growth, maturation and degeneration were determined by ion-exchange HPLC (see Figure 4): top panel, T3; middle panel, T4; bottom panel, T6. The results are expressed as percentages ($\pm$ S.D.) of CS on aggrecan terminating with the indicated sulphation sequences. Abbreviations: ND, not determined; OA, osteoarthritic.

total trisaccharides respectively. In the diseased cartilages, GalNAc4S β 1,4GlcA β 1,3GalNAc4S remained undetectable (Figure 4, bottom trace, arrow T3), lending further support to the suggestion [43] that the sulphation of aggrecan CS accumulated by chondrocytes during the anabolic phases in human osteoarthritis is not simply a reinitiation of the enzymic pathway active in foetal and juvenile growth cartilages, but rather reflects that displayed by adolescent cartilages.

DISCUSSION

In this study we report the preparation and structural analyses of trisaccharides from the non-reducing termini of aggrecan CS chains by using a combination of endo-ChABC and ChACII. Previously, other investigators have shown that a purified endo-ChABC produces Δ oligosaccharides from the CS chain interior [42] and that it can leave up to four repeating residues attached to the core-bound linkage region [41]. We demonstrate here, for the first time, that this enzyme also exhibits a unique digestion pattern at the non-reducing termini of CS. An initial endo-cleavage at the terminus occurs within the penultimate disaccharide to generate the trisaccharide GalNAcS β 1,4GlcA β 1,3GalNAcS- (Figures 1 and 2), which is subsequently digested to the monosaccharide and the Δ disaccharide (Figure 3). During this second step, trisaccharides containing terminal GalNAc4S were evidently cleaved faster than those ending in GalNAc4,6S (Figure 1c), which is entirely in keeping with the reported differences in digestion rates for chondroitin 4-sulphate and chondroitin sulphate E [42].

Conditions for digestion with endo-ChABC were established that allowed the recovery of more than 90% of all non-reducing terminal GalNAcS residues in trisaccharides (Figures 2 and 3). This required a low enzyme-to-substrate ratio, 1 h of incubation at 37 °C (Figures 1 and 2) and, most importantly, CS attached to the core protein. Thus the rapid and concerted release of trisaccharides (Figures 1 and 4) was not seen when isolated CS chains were digested under the same conditions. Instead, a significant proportion of the terminal GalNAc residues were liberated at the same time as the oligosaccharide products (results not shown); this might indicate that spatial orientation of CS can influence the initial stages of cleavage of the chains by endo-ChABC. Furthermore, under prolonged incubation conditions, endo-ChABC cleaved the trisaccharides into monosaccharides and disaccharides, which is in contrast with the capacity of the enzyme for completing the digestion of Δ oligosaccharides from the chain interior into Δ disaccharides (Figure 1c). Although the underlying mechanisms remain to be determined, it is possible that the enzyme has a higher accessibility to the GalNAc β 1,3GlcA linkage in a trisaccharide than in a tetrasaccharide.

Compositional analyses of terminal trisaccharides prepared from a range of aggrecan sources (Table 1) showed the presence of four major sulphation isomers. From this it was evident that both GalNAc4S and GalNAc4,6S can be linked to either a 4-sulphated or a 6-sulphated penultimate disaccharide; this in turn suggests that during CS synthesis, 4-sulphation or 4,6-sulphation of the terminal GalNAc residues is not critically regulated by sulphation of the GalNAc residue in the adjacent disaccharide. This finding is clearly distinct from reported data for several other sulphotransferases, principally those involved in HS synthesis, whose activities seem to be dependent on the prior modification of adjacent residues by epimerases or other sulphotransferases [31]. It should also be noted that the product GalNAc6S β 1,4GlcA β 1,3GalNAcS-, whose elution position was determined after treatment of the 6-sulphated Δ tetraS with mercuric acetate (results not shown), was not detected in endo-ChABC digests of aggrecan CS examined here, confirming our previous observation that GalNAc6S is rare as a terminal structure on human aggrecan CS.

The relative abundance of the terminal trisaccharide isomers was determined in digests of aggrecan from normal human knee cartilages (Figure 5) and found to be distinct between the three age groups foetal to 5 years (juvenile), 12–18 years (adolescent) and 22–72 years (adult). In keeping with previous analyses [43], trisaccharides from juvenile and adolescent growth cartilage CS all contained non-reducing terminal GalNAc4S, whereas about half of the trisaccharides from adult cartilages had the GalNAc4,6S terminal. In addition, these results provided for the first time definitive information on the 4-sulphation and 6-sulphation frequencies of the last repeating disaccharide of aggrecan CS. Thus, in growth cartilage CS, the 6S-to-4S ratio of the terminal disaccharides was approx. 0.85; this increased markedly to 15 in adolescent and 22 in adult CS populations. In all three age groups, these ratios compare well with those obtained for the 6S-to-4S ratios calculated for the chain-internal disaccharides, which were approx. 0.8 for growth cartilages, 12–15 for adolescent cartilages and approx. 26 for adult cartilages. This close agreement between the sulphation isomer ratios of the last repeating disaccharide and those of the chain-internal disaccharides suggests strongly that neither C4- nor C6-sulphotransferase enzymes [45,46] show a spatial preference for the sulphation of disaccharides towards the non-reducing terminus.

In summary, the identification and quantification of growth-specific and maturation-specific sulphation sequences in aggrecan CS have provided additional information to depict cartilage

matrix turnover events involving the GAG attachment domain of this PG, and differences in the glycosylation of aggrecan extracted from cartilages can be fully interpreted only if all preceding normal and pathological events are considered that might have altered both the anabolic processing and the catabolic processing of this matrix component. Indeed, taken together with the recently reported findings on the turnover time of protein-rich domains of aggrecan [47], the glycosylation changes for human aggrecan should provide a comprehensive picture of the regulatory mechanisms *in vivo* that modulate the charge density of the cartilage extracellular matrix.

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REFERENCES

- 1 Doege, K. J., Sasaki, M., Horigan, E., Hassell, J. R. and Yamada, Y. (1987) *J. Biol. Chem.* **262**, 17757–17767
- 2 Doege, K. J., Sasaki, M., Kimura, T. and Yamada, Y. (1991) *J. Biol. Chem.* **266**, 894–902
- 3 Chandrasekaran, L. and Tanzer, M. L. (1992) *Biochem. J.* **288**, 903–910
- 4 Walcz, E., Deak, F., Erhardt, P., Coulter, S. N., Fulop, C., Horvath, P., Doege, K. J. and Glant, T. T. (1994) *Genomics* **22**, 364–371
- 5 Valhmu, W. B., Palmer, G. D., Rivers, P. A., Ebara, S., Cheng, J. F., Fischer, S. and Ratcliffe, A. (1995) *Biochem. J.* **309**, 535–542
- 6 Watanabe, H., Gao, L., Sugiyama, S., Doege, K., Kimata, K. and Yamada, Y. (1995) *Biochem. J.* **308**, 433–440
- 7 Hering, T. M., Kollar, J. and Huynh, T. D. (1997) *Arch. Biochem. Biophys.* **345**, 259–270
- 8 Heinegard, D. and Axelsson, I. (1977) *J. Biol. Chem.* **252**, 1971–1979
- 9 Heinegard, D. (1977) *J. Biol. Chem.* **252**, 1980–1989
- 10 Roughley, P. J. and White, R. J. (1980) *J. Biol. Chem.* **255**, 217–224
- 11 Thonar, E. J., Buckwalter, J. A. and Kuettner, K. E. (1986) *J. Biol. Chem.* **261**, 2467–2474
- 12 Plaas, A. H. K., Wong-Palms, S., Roughley, P. J., Midura, R. J. and Hascall, V. C. (1997) *J. Biol. Chem.* **272**, 20603–20610
- 13 Roden, L. (1980) in *Biochemistry of Glycoproteins and Proteoglycans*, (Lennarz, W. J., ed.), pp. 267–371, Plenum Press, New York
- 14 Kitagawa, H., Ujikawa, M. and Sugahara, K. (1996) *J. Biol. Chem.* **271**, 6583–6585
- 15 Kitagawa, H., Tsutsumi, K., Tone, Y. and Sugahara, K. (1997) *J. Biol. Chem.* **272**, 31377–31381
- 16 Feyzi, E., Saldeen, T., Larsson, E., Lindhal, U. and Salmivirta, M. (1998) *J. Biol. Chem.* **273**, 13395–13398
- 17 Freilich, L. S., Lewis, R. G., Reppucci, A. C. and Silbert, J. E. (1975) *Biochem. Biophys. Res. Commun.* **63**, 663–668
- 18 Gundlach, M. W. and Conrad, E. (1985) *Biochem. J.* **226**, 705–714
- 19 Sugumaran, G., Humphries, D. E. and Silbert, J. E. (1989) *Methods Enzymol.* **179**, 422–427
- 20 Kitagawa, H., Tsutsumi, K., Ujikawa, M., Goto, F., Tamura, J., Neumann, K., Ogawa, T. and Sugahara, K. (1997) *Glycobiology* **7**, 531–537
- 21 Habuchi, O., Hirahara, Y., Uchimura, K. and Fukuta, M. (1996) *Glycobiology* **6**, 51–57
- 22 Habuchi, O., Suzuki, Y. and Fukuta, M. (1997) *Glycobiology* **7**, 405–412
- 23 Sugumaran, G. and Silbert, J. E. (1998) *Biochem. J.* **329**, 203–208
- 24 Silbert, J. E. (1978) *J. Biol. Chem.* **253**, 6888–6892
- 25 Sugumaran, G. and Silbert, J. E. (1991) *J. Biol. Chem.* **266**, 9565–9569
- 26 Cogburn, J. N. and Silbert, J. E. (1986) *Carbohydrate Res.* **151**, 207–212
- 27 Lind, T., Lindhal, U. and Lindholt, K. (1993) *J. Biol. Chem.* **268**, 20705–20708
- 28 Weigel, P. H., Hascall, V. C. and Tammi, M. (1997) *J. Biol. Chem.* **272**, 13997–14000
- 29 Toma, L., Pinhal, M. A., Dietrich, C. P., Nader, H. B. and Hirschberg, C. B. (1996) *J. Biol. Chem.* **271**, 3897–3901
- 30 Lindhal, U., Kusche-Gullberg, M. and Kjellen, L. (1998) *J. Biol. Chem.* **273**, 24979–24982
- 31 Rosenberg, R. D., Shwarak, N. W., Lui, J., Schwartz, J. J. and Zang, L. (1998) *J. Clin. Invest.* **99**, 2062–2070
- 32 Brown, G. M. and Nieduszynski, I. A. (1997) in *BioMethods*, (Jackson, P. and Gallagher, J. T., eds.), vol. 9, pp. 161–181, Birkhauser Verlag, Basel
- 33 Cheng, F., Heinegard, D., Malmstrom, A., Schmidtchen, A., Yoshida, K. and Fransson, L. A. (1994) *Glycobiology* **4**, 685–696
- 34 Kitagawa, H., Oyama, M., Masayama, K., Yamaguchi, Y. and Sugahara, K. (1997) *Glycobiology* **7**, 1175–1180
- 35 Cheng, F., Yoshida, K., Heinegard, D. and Fransson, L. A. (1992) *Glycobiology* **2**, 553–561
- 36 Chai, W., Kogelberg, H. and Lawson, A. M. (1996) *Anal. Biochem.* **237**, 88–102
- 37 Nakanaka, S. and Sugahara, K. (1997) *Glycobiology* **7**, 253–263
- 38 Shibata, S., Midura, R. J. and Hascall, V. C. (1992) *J. Biol. Chem.* **267**, 6548–6555
- 39 De Beer, T., Inui, A., Tsuda, H., Sugahara, K. and Vliegenhardt, J. F. (1996) *Eur. J. Biochem.* **240**, 789–797
- 40 Cheng, F., Heinegard, D., Fransson, L., Bayliss, M., Bielicki, J., Hopwood, J. and Yoshida, Y. (1996) *J. Biol. Chem.* **271**, 28572–28580
- 41 Huckerby, T. N., Lauder, R. M. and Nieduszynski, I. A. (1998) *Eur. J. Biochem.* **258**, 669–676
- 42 Hamai, A., Hashimoto, N., Mochizuki, H., Kato, F., Makiguchi, Y., Horie, K. and Suzuki, S. (1997) *J. Biol. Chem.* **272**, 9123–9130
- 43 Plaas, A. H. K., West, L. A., Wong-Palms, S. and Nelson, F. R. T. (1998) *J. Biol. Chem.* **273**, 12642–12649
- 44 Kuster, B. and Harvey, D. J. (1997) *Glycobiology* **7**, vii–ix
- 45 Fukuta, M., Uchimura, K., Nakashima, K., Kato, M., Kimata, K., Shinimura, T. and Habuchi, O. (1995) *J. Biol. Chem.* **270**, 18575–18580
- 46 Yamauchi, S., Hirahara, Y., Usui, H., Takeda, Y., Hoshino, M., Fukuta, M., Kimura, J. H. and Habuchi, O. (1999) *J. Biol. Chem.* **274**, 2456–2463
- 47 Maroudas, A., Bayliss, M. T., Uchitel-Kaushansky, N., Schneiderman, R. and Gilav, E. (1998) *Arch. Biochem. Biophys.* **350**, 61–71

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