Age-related changes in the structure of the keratan sulphate chains attached to fibromodulin isolated from articular cartilage

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Bovine articular cartilage fibromodulin has been isolated from animals aged 3 months to 8 years, and the attached keratan sulphate (KS) chains digested with keratanase II. The oligosaccharides generated have been reduced, examined by high-pH anion-exchange chromatography and their structures identified by comparison with standards. It has been shown that in fibromodulin from young articular cartilage, the KS chains do not possess either non-reducing terminal (α 2-6)-linked *N*-acetylneuraminic acid or fucose (α 1-3)-linked to sulphated

N-acetylglucosamine residues. However, an age-related increase has been observed in the abundance of both (α 2-6)-linked *N*-acetylneuraminic acid and (α 1-3)-linked fucose, neither of which is found in KS isolated from non-articular cartilage, irrespective of the age of the source. Interestingly, the KS chain length remains constant as a function of age, which possibly relates to a role in collagen fibril assembly. In addition, no significant age-related changes were identified in levels of galactose sulphation.

INTRODUCTION

Fibromodulin is a member of a group of small interstitial proteoglycans that share an amino acid homology. The group is extensive and includes decorin [1], biglycan [2], lumican [3–5], proline-arginine rich end leucine-rich proteoglycan [6], PG-Lb (epiphican) [7], chondroadherin [8,9] and keratocan [10], as well as fibromodulin [11]. The protein core of each contains leucine-rich tandem repeats [12], comprising up to 24 amino acids repeated up to 12 times. The role of this motif is not clear but it is possible that it may be involved in binding to other macromolecules [12].

Fibromodulin is exclusively glycosylated by short chains of keratan sulphate (KS) [13–16]. In addition, Plaas et al. [17] have demonstrated that, in 3-month-old bovine articular cartilage, only four out of the five potential glycosylation sites were substituted by either KS or an N-linked oligosaccharide.

Nieduszynski et al. [18] and Lauder et al. [15,16] respectively have demonstrated that, for O- and N-linked KS chains, there is a distinction between chains from articular cartilage, which can contain (α 2-6)-linked *N*-acetylneuraminic acid (NeuAc) chain caps and (α 1-3)-linked fucose, and those from non-articular cartilage which contain neither of these features. The presence of fucose as a component of KSs, representing approx. 1–2 % of the total weight, is well established [19,20]. Several workers [16,21,22] have found fucose residues, present as a branch, (α 1-3)-linked to sulphated *N*-acetylglucosamine residues in KSs from articular cartilage.

There is considerable evidence that fibromodulin, along with decorin, has a central role in the maintenance of the structure of the collagen fibrils, which make up the extracellular matrix of cartilage. Fibromodulin has been found to inhibit the formation of collagen fibrils *in vitro* [23,24], and binds to collagens I and II [24,25]. The core protein of fibromodulin binds to collagen monomers, but the attached KS chains apparently control fibril diameter and interfibrillar spacing [26]; thus changes in the

structure of the KS chains attached to fibromodulin may affect the structure of developing collagen fibrils.

Examination of the distribution of fibromodulin on collagen fibrils has found it to be localized mainly in the gap region, axially within the D-period [27]. These workers also found that, in articular cartilage, levels of fibromodulin were higher in the interterritorial matrix than adjacent to cells and at the cartilage surface than at deeper levels.

In this study fibromodulin has been isolated from bovine articular cartilage of different ages, and the structure of the KS chains has been determined by keratanase II digestion and profiling of the oligosaccharides produced by high-pH anion-exchange (HPAE) chromatography.

MATERIALS AND METHODS

Materials

The reagents used in this study are as reported previously [15,16].

Isolation of fibromodulin from bovine articular cartilage

Bovine articular cartilage fibromodulin was isolated and purified as described previously [15,16] from three age groups: 3 months ($n = 1$), 15–18 months ($n = 4$) and 6–8 years ($n = 5$). Briefly, diced bovine articular cartilage from animals of each age was extracted into 4 M guanidine hydrochloride with a protease-inhibitor cocktail [50 mM sodium acetate/100 mM 6-amino-caproic acid/10 mM EDTA/5 mM benzamidine hydrochloride (pH 6.8)], for 48 h at 4 °C. The extract was taken to associative conditions by dialysis against the protease-inhibitor cocktail, and solid CsCl was added to achieve a density of 1.48 g/ml. Following density-gradient centrifugation at 100 000 *g* in a Sorval T-865 Ti rotor for 48 h at 4 °C, the A3 and A4 fractions ($\rho < 1.53$ g/ml), which contain fibromodulin, were

Abbreviations used: KS, keratan sulphate; Gal A, galactose residue adjacent to the non-reducing terminal NeuAc of the KS chain; Gal B, galactose residue one removed from Gal A; Gal C, any galactose residue in the repeat region of the KS chain; HPAE, high-pH anion exchange.

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Table 1 HPAE chromatographic analysis of keratanase-II-derived oligosaccharides

Relative molar abundance of each oligosaccharide identified in the 3-month-old sample and in the representative examples of the 15–18-month-old and 6–8-year-old samples. The values are referenced to C1b as unity. R, repeat region oligosaccharide; C, capping oligosaccharide; F, fucose-containing oligosaccharide. GlcNAc, *N*-acetylglucosamine. GlcNAc-ol, *N*-acetylglucosaminol (2-acetamido-D-glucitol).

Code	Relative molar abundance			Oligosaccharide
	3 month	15 month	6 year	
RF1	—	—	5.23	Gal β ¹⁻⁴ (Fuc α ¹⁻³)GlcNAc(6S)-ol
R1	4.07	4.22	2.39	Gal β ¹⁻⁴ GlcNAc(6S)-ol
R3	4.86	7.15	7.62	Gal(6S) β ¹⁻⁴ GlcNAc(6S)-ol
RF2	—	—	0.39	Gal β ¹⁻⁴ (Fuc α ¹⁻³)GlcNAc(6S) β ¹⁻³ Gal β ¹⁻⁴ (Fuc α ¹⁻³)GlcNAc(6S)-ol
RF3	—	—	0.31	Gal β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal β ¹⁻⁴ (Fuc α ¹⁻³)GlcNAc(6S)-ol
RF4	—	—	0.52	Gal β ¹⁻⁴ (Fuc α ¹⁻³)GlcNAc(6S) β ¹⁻³ Gal β ¹⁻⁴ GlcNAc(6S)-ol
R2	0.49	0.52	1.38	Gal β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal β ¹⁻⁴ GlcNAc(6S)-ol
RF5	—	—	0.65	Gal(6S) β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal β ¹⁻⁴ (Fuc α ¹⁻³)GlcNAc(6S)-ol
RF6	—	—	0.25	Gal β ¹⁻⁴ (Fuc α ¹⁻³)GlcNAc(6S) β ¹⁻³ Gal(6S) β ¹⁻⁴ GlcNAc(6S)-ol
R4	0.28	0.29	0.80	Gal(6S) β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal β ¹⁻⁴ GlcNAc(6S)-ol
R5	0.27	0.44	0.43	Gal β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal(6S) β ¹⁻⁴ GlcNAc(6S)-ol
R6	0.47	0.65	0.31	Gal(6S) β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal(6S) β ¹⁻⁴ GlcNAc(6S)-ol
CF1	—	—	0.86	NeuAc α ²⁻³ Gal β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal β ¹⁻⁴ (Fuc α ¹⁻³)GlcNAc(6S)-ol
C1a	—	0.06	0.82	NeuAc α ²⁻³ Gal β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal β ¹⁻⁴ GlcNAc(6S)-ol
C1b	1	1	1	NeuAc α ²⁻³ Gal β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal β ¹⁻⁴ GlcNAc(6S)-ol
C2a	—	0.09	0.45	NeuAc α ²⁻³ Gal β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal(6S) β ¹⁻⁴ GlcNAc(6S)-ol
C2b	0.81	1.23	0.59	NeuAc α ²⁻³ Gal β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal(6S) β ¹⁻⁴ GlcNAc(6S)-ol
C3	0.09	0.04	0.11	NeuAc α ²⁻³ Gal(6S) β ¹⁻⁴ GlcNAc(6S) β ¹⁻³ Gal(6S) β ¹⁻⁴ GlcNAc(6S)-ol

pooled and exchanged into 6 M urea/0.15 M NaCl/50 mM Tris/HCl, pH 7.3.

This was subjected to ion-exchange chromatography on a Mono-Q HR 10/10 column, followed by size-exclusion chromatography on a Sephacryl S-300 column. Fibromodulin-containing fractions were identified by ELISA, using an antibody to fibromodulin's protein core, and purity was confirmed by reversed-phase chromatography on a Brownlee C4 column. A single peak of fibromodulin eluted at approx. 32 min, identical with that reported previously for fibromodulin [14].

Keratanase II digestion

Purified fibromodulin was subjected to exhaustive digestion with keratanase II (0.002 units/mg of fibromodulin) in 10 mM sodium acetate, pH 6.8, for 24 h at 37 °C.

Isolation of oligosaccharides

The oligosaccharides generated by keratanase II digestion were isolated as described previously [14]. Following digestion they were reduced by addition of NaBH₄ to 1 M. After 3 h at room temperature reduction was stopped by stepwise addition of acetic acid. The reduced oligosaccharides were isolated by size-exclusion chromatography on a Sephadex G-50 (medium) column and desalted on a Bio-Gel P-2 column, then freeze-dried.

HPAE chromatography

The composition of the reduced oligosaccharides was examined by HPAE chromatography on a Dionex IonPac AS4A-SC column (4 mm × 250 mm), with an AG4A-SC guard column (4 mm × 50 mm), maintained at 30 °C, and running at 1 ml/min.

Previous work [28] has determined response factors for each oligosaccharide generated by keratanase II digestion of KSs. These values were used in calculations of relative molar abundance and chain length reported in Tables 1 and 2, and galactose sulphation in Table 4. The average length of the antennae of KS

Table 2 Average antennar cap length at different ages

The average number of disaccharides isolated per NeuAc residue was determined by examination of the ratio of the abundance of repeat-region oligosaccharides to capping oligosaccharides. Each capping oligosaccharide contributes two disaccharides, as does each repeat-region tetrasaccharide and their fucosylated variants. Therefore the average number of disaccharides ($^{-3}\text{Gal}\beta^{1-4}\text{GlcNAc}\beta^{1-3}$)_n isolated per NeuAc residue is calculated by: $n = (\text{disaccharide type}) + (\text{tetrasaccharide type} \times 2) + (\text{pentasaccharide cap} \times 2)$: disaccharide type, disaccharides and fucosylated trisaccharide RF1, R1 and R3; tetrasaccharide type, tetrasaccharides and fucosylated penta- and hexasaccharides R2, RF2, RF3, RF4, RF5, R4, R5 and R6; pentasaccharide cap, pentasaccharide and fucosylated hexasaccharide caps CF1, C1a, C1b, C2a, C2b and C3. Values are means $\pm$ S.E.M. For codes, see Table 1.

Age	Average number of disaccharides per NeuAc
3 months	8.26
15 months	8.23 $\pm$ 0.61
6 years	8.19 $\pm$ 0.88

attached to each sample of fibromodulin has been estimated by calculating the ratio of capping oligosaccharides to repeat-region oligosaccharides.

NMR spectroscopy

Samples of fibromodulin, typically 2–5 mg, were buffered to pH 7 with phosphate, referenced with sodium 3-trimethylsilyl[²H₄]propionate as internal standard and dissolved in 0.5 ml of 99.96% (at. % ²H) ²H₂O after micro-filtration, several previous exchanges with 99.9% ²H₂O, and one using 99.96% ²H₂O. Proton NMR spectra were determined at 60 °C using a Bruker AM500 (500 MHz) or a Bruker AMX600 (600 MHz) spectrometer with a 5 mm probe. All chemical shifts are quoted relative to internal 3-trimethylsilyl[²H₄]propionate at 0.0 ppm.

RESULTS

Isolation of fibromodulin

Preliminary ^1H NMR spectroscopy (results not shown) revealed that the fibromodulin samples from each age group were broadly similar. However, the 3-month-old and 15–18-month-old samples lacked signals at approx. 1.7 and 2.7 ppm, characteristic of the H(3ax) and H(3eq) protons respectively of non-reducing terminal NeuAc (α 2-6)-linked to galactose [29], and at approx. 5.1 and 1.2 ppm, characteristic of H1 and H6 respectively of fucose (α 1-3)-linked to an *N*-acetylglucosamine residue [21,30]. Each of the samples displayed the distinctive signals at approx. 1.80 and 2.76 ppm of the H(3ax) and H(3eq) protons respectively of a non-reducing NeuAc linked to galactose in an (α 2-3)-linkage [31].

Digestion and analysis of KS

The reduced oligosaccharides isolated following keratanase II digestion and subsequent reduction of the KS attached to bovine articular cartilage fibromodulin of each age group have been examined by HPAE chromatography, and their structures assigned by analysis of the chromatograms and reference to standards. A portion of the chromatograms for the 3-month-old sample and representative examples of 15–18-month-old and 6–8-year-old samples are shown in Figure 1. The structures have been categorized as deriving from either the cap region [C], or from the repeat region [R] of the parent N-linked KS. The linkage region is not examined in this work. Some of the oligosaccharides have fucose residues [F], present as a branch, (α 1-3)-linked to sulphated *N*-acetylglucosamine or *N*-acetylglucosaminitol (2-acetamido-D-glucitol) [16].

The structures and codes for all of the individual oligosaccharides identified in this study are shown in Table 1.

Figure 1 Partial HPAE chromatograms of keratanase-II-derived oligosaccharides from 3-month-old, 15–18-month-old and 6–8-year-old bovine articular cartilage fibromodulin

A 20 μl aliquot of reduced oligosaccharides was suspended in water and applied to a Dionex AS4A-SC column. The eluent was monitored on-line by using a pulsed electrochemical detector, configured for integrated pulsed amperometry, using programme 1 (carbohydrates), and the following time/potential program: 0.00 s, +0.10 V; 0.50 s, +0.10 V; 0.51 s, +0.60 V; 0.59 s, +0.60 V; 0.60 s, –0.60 V; 0.65 s, –0.60 V, integration being 0.3–0.5 s. A 5 min isocratic period of 5% 1 M NaOH/95% water was followed by a linear gradient of 0–95% 1.5 M sodium acetate and constant 5% NaOH over 50 min. A short portion of the chromatogram is shown, within which examples of both fucose-containing repeat-region fragments and NeuAc elute are given. For codes, see Table 1.

Table 3 Abundance of articular-cartilage-specific moieties

The percentage of antennae capped by (α 2-6)-linked NeuAc and the percentage of fucosylated *N*-acetylglucosamine residues is shown. Values are means $\pm$ S.E.M.

Age	α (1-3)-Linked fucose (%)	α (2-6)-Linked NeuAc (%)
3 months	0	0
15–18 months	2 ± 0.5	5 ± 2
6–8 years	17 ± 11	26 ± 8

Table 4 Percentage galactose sulphation

Values are means $\pm$ S.E.M.

Age	Galactose sulphation (%)		
	Gal A and Gal B	Gal B only	Repeat region (Gal C)
3 months	5	43	54
15–18 months	2 ± 0.4	54 ± 8	62 ± 15
6–8 years	2.5 ± 0.5	40 ± 13	56 ± 13

Examination of HPAE chromatograms

Preliminary examination of the HPAE chromatograms (Figure 1) shows that those from younger samples show fewer peaks. In particular, there are notable differences in the abundance of both (α 1-3)-linked fucose and (α 2-6)-linked NeuAc-containing oligosaccharides (see Table 1). It is clear that the samples from the 6–8-year-old group have a greater abundance of oligosaccharides containing (α 1-3)-linked fucose and (α 2-6)-linked NeuAc.

The KS from 3-month-old animals shows no evidence for either (α 1-3)-linked fucose or (α 2-6)-linked NeuAc chain caps. In the samples from 15–18-month-old animals there is some evidence for (α 1-3)-linked fucose (Table 3), with approx. 2% of the *N*-acetylglucosamine residues fucosylated; on average 5% of the chains are capped by (α 2-6)-linked NeuAc. In the mature bovine sample from animals of 6–8 years olds there is evidence of much increased fucosylation, although the level is highly variable, $17 \pm 11\%$ (Table 3). In this older group there is a much higher incidence ($26 \pm 8\%$) of chains that are capped by (α 2-6)-linked NeuAc.

Chain length

The average length of the antennar cap and repeat region from the KS chains attached to fibromodulin from bovine articular cartilage is approx. 8 disaccharides (Table 2), irrespective of the age of the source.

Galactose sulphation

Analysis of the HPAE chromatography data (Table 4) demonstrates that Gal A, the residue adjacent to the capping NeuAc, is sulphated in only a small fraction (2–5%) of the chains. The level of sulphation of the galactose residue one removed from Gal A (Gal B) is much higher in each sample (approx. 43–54%) and in

each case the average level of sulphation of the galactose residues within the repeat region (Gal C) is even higher (approx. 54–62%). There is no evidence for any changes in the sulphation level or pattern with age.

DISCUSSION

Isolation of fibromodulin

Fibromodulin has been isolated from bovine articular cartilage, of different ages, by dissociative extraction and CsCl density-gradient centrifugation. The fibromodulin has been purified by ion-exchange and size-exclusion chromatography, a protocol previously shown to allow the isolation of intact fibromodulin from tracheal and articular cartilage [14,15].

Digestion and examination of KS

The oligosaccharide composition of the KS attached to bovine articular cartilage fibromodulin of each age group has been examined by HPAE chromatography, and the results were confirmed by the ^1H NMR spectroscopic data from intact fibromodulin samples. KSs from young (3-month-old and to a lesser extent 15–18-month-old) articular cartilage fibromodulin lack features normally associated with articular tissue, i.e. (α 1-3)-linked fucose and (α 2-6)-linked NeuAc chain caps. KSs, both O-linked and N-linked, from non-articular cartilage lack (α 1-3)-linked fucose and (α 2-6)-linked NeuAc, irrespective of the age of the source [14,15,18]. In the 6–8-year-old group there is evidence of considerable amounts of (α 1-3)-linked fucose and (α 2-6)-linked NeuAc chain caps. The appearance of these features in the older articular cartilage suggests that they may be part of a developmentally regulated response.

The biological significance of (α 2-6)-linked NeuAc is seen in recent work, which has demonstrated it to be important in renal function [32] and as a marker for aggressive malignancy in colorectal carcinoma [33,34].

Chain length

The average length of the antennar cap and repeat region from the KS chains attached to fibromodulin from bovine articular cartilage is approx. 8 disaccharides (Table 2), irrespective of the age of the source. These data confirm our previous work, examining the structure of the KS attached to fibromodulin from mature cartilage, in which it was found that the length of the repeat region and antennar cap were approx. 5–7 disaccharides from bovine tracheal cartilage [14,15] and approx. 8–9 disaccharides in bovine, equine [16] and human articular cartilage [35]. Thus it appears that the length of the KS chains attached to fibromodulin is tightly controlled, there being little variation between species (bovine, equine and human), tissues within a single species (tracheal and articular), or ages.

These data, showing the KS antennae attached to fibromodulin to be short, differ from early work by Plaas et al. [36], which found the chains to be 14–27 disaccharides in length. In that work, the total galactose content was estimated, giving a value for the overall chain length. In the present work, the length of the keratanase-II-susceptible portion of each chain has been determined by comparison with the abundance of NeuAc, giving a minimum value for the length of each antenna. Taken together, these data point to the presence of a biantennary chain in which each antenna comprises approx. 8–10 disaccharides.

Other workers, who examined human articular cartilage fibromodulin by SDS/PAGE using an antibody to its protein core, found that whereas fibromodulin from young individuals mi-

Figure 2 General structure of the N-linked KS attached to articular cartilage fibromodulin

The general structure of articular cartilage fibromodulin is shown. The galactose residues referred to as Gal A, Gal B and Gal C are indicated.

grated with an apparent size of 70–110 kDa [37] or 60–90 kDa [38], mature human fibromodulin migrated as a band of either 67 kDa or 58 kDa respectively.

Roughley et al. [37] were unable to detect any keratanase II or endo- β -galactosidase-susceptible KS attached to the fibromodulin that they isolated from mature human cartilage. However, in this study it has been shown that in adult bovine cartilage fibromodulin does have KS attached, in agreement with our previous work with fibromodulin from mature bovine tracheal cartilage [15] and human articular cartilage [35] isolated from elderly individuals. Plaas et al. [39] have also demonstrated by direct chemical means that adult bovine articular cartilage fibromodulin contains both galactose and glucosamine, suggesting the presence of the KS identified in this study.

The differences in these data may be due to species differences or the methods employed to examine the fibromodulin. In this study, the fibromodulin was isolated as a proteoglycan, whereas Roughley et al. [37] examined a whole cartilage extract using an anti-fibromodulin antibody.

Galactose sulphation

The HPAE chromatography data demonstrate that the galactose residue adjacent to the terminal NeuAc (Gal A) is sulphated on approx. 2–5% of the chains (Figure 2). This is in agreement with the levels of sulphation of this important sub-terminal residue in mature bovine tracheal [15] and articular [16] cartilage fibromodulin. However, very much higher levels of sulphation (approx. 30%) of this residue have been observed in fibromodulin from mature equine [16] and human [35] articular cartilage. The level of sulphation of the galactose residue one removed from Gal A (Gal B) is much higher (approx. 40–55%) than that of Gal A, and that of galactose residues within the repeat region (Gal C) is even higher in each case (approx. 40–60%). There is no evidence for any changes in galactose sulphation with age.

Age-related changes: relationship with other macromolecules

Several studies have identified age-related changes in proteoglycan structure [40], synthesis [41,42] and abundance [43]. In aggrecan there are age-related changes in the pattern of glycosylation (decrease in chondroitin sulphate and increase in KS). Other workers have reported age-related changes in the sulphation pattern of articular cartilage dermatan sulphate proteoglycans [44], or the ratio of galactose C6/C4 sulphation in articular cartilage chondroitin sulphate chains. The iduronate/glucuronate ratio of chondroitin/dermatan sulphate chains has also been reported to increase with age [43,44].

The functional relevance of the appearance of (α 2-6)-linked NeuAc and (α 1-3)-linked fucose is not yet clear. It is significant that the KS chains are modified in a very specific manner, there

being no significant change in either the antennar length or the sulphation pattern. It seems possible that these structural features can confer properties relating either to the chain's resistance to degradation or to specific recognition phenomena. In the case of the (α 2-6)-linked NeuAc it may be that the cap affects the clearance rate of the KS or fibromodulin. In the case of the (α 1-3)-linked fucose, its presence may increase resistance to glycolytic enzymes. However, alternatively, the appearance of these features may be related to loading or some other developmentally regulated phenomena.

Fibromodulin is believed to have a role in collagen formation [24]. Further, it is known that the (α 2-6)-linked NeuAc cap has a significantly different conformation to that adopted by (α 2-3)-linked NeuAc [45]. Therefore it is possible that chains capped by (α 2-6)-linked NeuAc may have an altered ability to influence fibril diameter and interfibrillar spacing, which are both known to increase during aging [46].

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