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The structure of the keratan sulphate chains attached to fibromodulin from human articular cartilage

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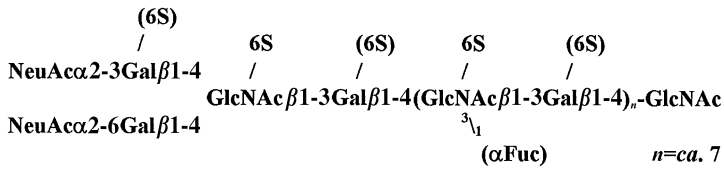
The small keratan sulphate proteoglycan, fibromodulin, has been isolated from pooled human articular cartilage. The main chain repeat region and the chain caps from the attached N-linked keratan sulphate chains have been fragmented by keratanase II digestion, and the oligosaccharides generated have been reduced and isolated. Their structures and abundance have been determined by high pH anion-exchange chromatography.

These regions of the keratan sulphate from human articular cartilage fibromodulin have been found to have the following general structure:



Significantly, both *α*(2-6)- and *α*(2-3)-linked *N*-acetyl-neuraminic acid have been found in the capping oligosaccharides. Fucose, which is *α*(1-3)-linked as a branch to *N*-acetylglucosamine, has also been found along the length of the repeat region and in the capping region. The chains, which have been found to be very highly sulphated, are short; the length of the repeat region and chain caps is *ca.* nine disaccharides.

These data demonstrate that the structure of the N-linked keratan sulphate chains of human articular cartilage fibromodulin is similar, in general, to articular cartilage derived O-linked keratan sulphate chains. Further, the general structure of the keratan sulphate chains attached to human articular cartilage fibromodulin has been found to be generally similar to that of both bovine and equine articular cartilage fibromodulin.

***Keywords*: fibromodulin, small proteoglycan, keratan sulphate, glycosaminoglycan, sulphation, keratanase II, human**

***Abbreviations*: KS, keratan sulphate; IEC, ion-exchange chromatography; ELISA, enzyme linked immunosorbent assay; Gal, *β*-D-galactose; Fuc, *α*-L-Fucose; GlcNAc, *N*-acetylglucosamine (2-acetamido-*β*-D-glucose); GlcNAc-ol, *N*-acetylglucosaminitol (2-acetamido-D-glucitol); NeuAc, *N*-acetyl-neuraminic acid; 6S/(6S), O-ester sulphate group on C6 present/sometimes present; NMR – nuclear magnetic resonance; HPAE, high pH anion-exchange; PED, pulsed electrochemical detection; HPLC, high performance liquid chromatography**

Introduction

Fibromodulin, the product of the *FMOD* gene which has recently been localized to human chromosome Iq32 [1], was first isolated [2] from bovine articular cartilage, and subsequently cloned and sequenced [3] from bovine tracheal cartilage, a tissue known to be rich in fibromodulin [2]. When first isolated fibromodulin was termed 59 kDa matrix protein [2] because of its mobility on SDS PAGE. The name fibromodulin relates to its ability to modulate (inhibit) the formation of collagen fibrils *in vitro* [3].

Fibromodulin has a 42.2 kDa core protein, composed 375 amino acids, the central portion of which is made up of 10 leucine-rich tandem repeats of 23 amino acids. The repeats were found to have a conserved leucine (or leucine-like amino acid), and a highly conserved asparagine, in positions

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previously reported for a diverse collection of leucine-rich repeat proteins [4]. The amino acid sequence homology between human [5] and bovine [3] fibromodulin is *ca.* 95%, with the homology in the central leucine repeats being almost 100%. Fibromodulin also has significant amino acid sequence homology with lumican, decorin and biglycan. In each case the homology is highest in the central repeat region, and very low outside it. Decorin, biglycan, lumican and fibromodulin are similar in size, 357–375 amino acids, and have 6 cysteine residues in conserved locations [4].

Fibromodulin has a tyrosine-rich domain at its N-terminus, in which some of the tyrosine residues are sulphated [6]. There are 20 tyrosine residues in total, 13 in the tyrosine rich region, of which seven to nine may be sulphated. Fibromodulin is glycosylated by N-linked keratan sulphate [7]. In fibromodulin from 3-month old bovine articular cartilage only four out of five potential sites for *N*-glycosylation were found to be substituted [7]. Significantly, it was found that each of these sites may be substituted either by an N-linked oligosaccharide or a keratan sulphate chain. Thus each fibromodulin molecule may have between zero and four keratan sulphate chains at any combination of four glycosylation sites, the others being glycosylated by an N-linked oligosaccharide. The chains which are elongated to keratan sulphate are short, comprising between six and nine disaccharide units per *N*-acetyl-neuraminic acid cap [8–10]. The residues which may be substituted by an N-linked oligosaccharide, or keratan sulphate chain (Asn 126, 165, 200 and 290) are in, or close to, the leucine-rich repeat region, in contrast to both decorin and biglycan, in both of which the chondroitin/dermatan sulphate chain(s) are close to the N-terminus.

There is considerable evidence that fibromodulin is involved in regulating the formation of the network of collagen fibrils which makes up the extracellular matrix of cartilage. Fibromodulin, along with the related small proteoglycan decorin, inhibits the formation of collagen fibrils *in vitro* [11, 12], and has been found to bind to collagen types I and II [12, 13]. The binding site for fibromodulin on collagen has not yet been identified, however it is probably distinct from that of decorin as the two molecules have a synergistic effect in the collagen fibrillogenesis assay [12].

Immuno-cytochemical localization studies have found that fibromodulin is localized mainly in the gap region, axially within the D-period of collagen fibrils [14]. These workers also found that, in articular cartilage, levels of fibromodulin were higher in the interterritorial matrix than adjacent to cells, and were higher at the cartilage surface that at deeper levels.

Keratan sulphates have been classified according to their mode of linkage to protein as KS-I for N-linked chains, and KS-II for the O-linked chains from skeletal tissues such as cartilage [15]. A third type, O-linked mannose to serine or threonine, has been isolated from brain tissue [16]. Keratan sulphate is known to be based upon a repeating *N*-acetyllactosamine sequence of $(\text{-3Gal}\beta\text{1-4GlcNAc}\beta\text{1-})_n$ which is usually sulphated on C-6 of *N*-acetylglucosamine, and further sulphate groups may be present on C-6 of galactose [17].

Recent studies [18, 10] have suggested that there is a distinction between chains from articular cartilage, which contain both α(1-3)-linked fucose and α(2-6)-linked *N*-acetylneuraminic acid, and those from non-articular cartilage which contain neither of these features. Keratan sulphate chains, both N- and O-linked, can be conveniently considered to have three domains: the chain cap, a linkage region which connects the chain to the protein core and an intervening repeat region.

Keratanase II, an endo-β-*N*-acetylglucosaminidase, has been found to cleave the β(1-3)-glycosidic bond of a 6-O-sulphated *N*-acetylglucosamine in keratan sulphates [19]. Tetra- and disaccharides are generated from the sulphated poly-*N*-acetyllactosamine repeat region, and in the case of the sialic acid-containing capping oligosaccharides pentasaccharides are recovered [20].

In this study fibromodulin has been isolated from pooled human articular cartilage, and the attached keratan sulphate chains have been degraded by keratanase II. The oligosaccharides generated have been isolated and their structures and abundances determined by HPAE chromatography.

Experimental

Materials

Sephacryl S-300 and a Mono-Q column were purchased from Pharmacia (Uppsala, Sweden). Keratanse II was from ICN Biomedicals (High Wycombe, Bucks, UK), and as a gift from Dr K. Yoshida of the Seikagaku Corporation (Tokyo, Japan). An IonPac AS4A-SC column with an AG4A-SC guard column was purchased from Dionex (Camberley, Surrey, UK). Guanidine hydrochloride (practical grade) was from Sigma Chemical Co. (Poole, Dorset, UK). Sodium hydroxide (A.R. 46/48%) was from Fisons Scientific Equipment (Loughborough, Leics, UK). Sodium acetate was $> 99.5\%$ Microselect grade from Fluka (Gillingham, Dorset, UK). The antibody to the protein core of fibromodulin used in this study was a kind gift of Dr A.H.K. Plaas (Shriners Hospital, Tampa FL, USA). All other chemicals and reagents were of analytical grade.

Isolation of fibromodulin from human articular cartilage

Human femoral heads ($n = 7$ mean age 72 ± 12) were obtained, following amputation or hip replacement following fracture, from the Royal Preston Hospital. All were frozen following excision, and were considered normal by visual inspection. The cartilage was scraped off and the diced cartilage was extracted into 4 M guanidine hydrochloride plus a protease inhibitor cocktail (50 mM sodium acetate,

100 mM 6-aminocaproic 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 added to achieve a density of 1.48 g ml^{-1}. Following density gradient centrifugation at 100 000 × **g** (37 000 rpm) in a Sorval T-865 Ti rotor for 48 h at 4 °C, the A3 and A4 fractions ($\rho < 1.53$) were pooled and exchanged into 6 M urea/0.15 M NaCl/50 mM Tris/HCl, pH 7.3.

The A3 and A4 fractions were subjected to ion-exchange chromatography on a Mono-Q HR 10/10 column which had previously been equilibrated with 6 M urea/0.15 M NaCl/50 mM Tris/HCl, pH 7.3. Following loading, the column was washed with 30 ml of running buffer, and bound material eluted with a linear gradient of 0.15 M–1 M NaCl in 6 M urea/50 mM Tris/HCl, pH 7.3. The absorbance of the eluate was monitored on line at 280 nm, and for fibromodulin by an ELISA

Fibromodulin-containing fractions were pooled, exchanged into 4 M guanidine hydrochloride/50 mM Tris/HCl, pH 7.3, and subjected to size exclusion chromatography on a Sephracyl S-300 column run in 4 M guanidine hydrochloride/50 mM Tris/HCl, pH 7.3, at 9 ml h^{-1}, fractions being collected every 20 min. The absorbance of the eluate was monitored at 280 nm and for fibromodulin by an ELISA Fibromodulin-containing fractions were pooled and dialysed overnight against 0.2 M NaCl and then extensively against water.

Analytical methods

Glycosaminoglycan concentrations were monitored on microtitre plates using a 1,9-dimethymethylene blue assay [21]. Fibromodulin levels were determined with a microtitre plate ELISA using an antibody specific to the protein core of fibromodulin as previously described [8, 7].

Keratanase II digestion

Purified human articular cartilage fibromodulin (78 mg) was subjected to exhaustive digestion by keratanase II (0.002 U mg^{-1} 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 previously described [8]. Briefly, following digestion they were reduced by addition of $NaBH_4$ 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 (84 cm × 1.5 cm) run in 0.15 M NaCl at 9 ml h^{-1}, fractions being collected over 20 min intervals. The oligosaccharides were pooled and desalted by chromatography on a Bio-Gel P-2 column (11 cm × 1 cm) run in water at 12 ml h^{-1}, then lyophilized.

High pH anion-exchange chromatography

The composition of the reduced oligosaccharides was examined by HPAE chromatography on a Dionex chromatography system comprising of Rheodyne 7125 injection valve, Advanced Gradient Pump, Eluant degas module, Pulsed Electrochemical Detector module using a PAD II flow cell with a gold working electrode and a combination (pH/Ag-AgCl) reference electrode, along with an advanced computer interface with AI-450 software.

The oligosaccharides were separated on an 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^{-1}. 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% 1 M NaOH over 50 min. The eluted oligosaccharides were monitored on-line by the PED, 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.

Previous work in this laboratory [20] has determined PED response factors for each oligosaccharide generated by keratanase II digestion of keratan sulphates. These values were used for the calculations of relative oligosaccharide abundance and chain length reported in Tables 1 and 2, and for the calculations of average galactose sulphation in Figure 3.

Results

Preliminary examination

Preliminary ^{1}H-NMR spectroscopy of human articular cartilage fibromodulin revealed signals at *ca.* 1.7 ppm and 2.7 ppm, characteristic of the H(3ax) and H(3eq) protons, respectively, of *N*-acetyl-neuraminic acid α(2-6)-linked to galactose [22], and at *ca.* 5.1 ppm and 1.2 ppm, characteristic of the H1 and H6 respectively, of fucose α(1-3)-linked to a reduced *N*-acetylglucosaminitol or *N*-acetylglucosamine residue [23, 24]. Also observed were signals at *ca.* 1.80 ppm and 2.76 ppm from the H(3ax) and H(3eq) protons, respectively, of a non-reducing *N*-acetyl-neuraminic acid linked to galactose in an α(2-3)-linkage [25].

HPAE chromatography

The oligosaccharides have been categorized as deriving from the cap region [C], or from the repeat region [R] of the parent N-linked keratan sulphate, and in some fucose has been identified, CF or RF (Table 1). The linkage region is not examined in this work. The oligosaccharides are sequentially numbered according to their origin and elution order. This numbering is used solely for reference within this work, although it is compatible with our previous work [8–10].

Table 1. HPAE chromatography PAD analysis of oligosaccharides derived by keratanase II digestion of human articular cartilage fibromodulin.

CODE[a]	*Abundance*	*Oligosaccharide*
RF1	0.15	Galβ1-4(Fucα1-3)GlcNAc(6S)-ol
R1	8.88	Galβ1-4GlcNAc(6S)-ol
R3	7.03	Gal(6S)β1-4GlcNAc(6S)-ol
RF2	0.09	Galβ1-4(Fucα1-3)GlcNAc(6S)β1-3Galβ1-4(Fucα1-3)GlcNAc(6S)-ol
RF3	0.08	Galβ1-4GlcNAc(6S)β1-3Gaβ1-4(Fucα1-3)GlcNAc(6S)-ol
RF4	0.43	Galβ1-4(Fucα1-3)GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol
R2	2.30	Galβ1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol
RF5	0.12	Gal(6S)β1-4GlcNAc(6S)β1-3Galβ1-4(Fucα1-3)GlcNAc(6S)-ol
RF6	0.17	Galβ1-4(Fucα1-3)GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol
R4	1.08	Gal(6S)β1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol
R5	0.89	Galβ1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol
R6	2.43	Gal(6S)β1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol
CF1	0.35	NeuAcα2-3Galβ1-4GlcNAc(6S)β1-3Galβ1-4(Fucα1-3)GlcNAc(6S)-ol
C1a	0.32	NeuAcα2-6Galβ1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol
C1b	1	NeuAcα2-3Galβ1-4GlcNAc(6S)β1-3Galβ1-4GlcNAc(6S)-ol
C2a	0.96	NeuAcα2-6Galβ1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol
C2b	0.54	NeuAcα2-3Galβ1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol
C3	1.29	NeuAcα2-3Gal(6S)β1-4GlcNAc(6S)β1-3Gal(6S)β1-4GlcNAc(6S)-ol

Analysis of the HPAE chromatograms of keratanase II derived oligosaccharides. The relative molar abundance of each oligosaccharide identified has been calculated by measurement of peak area, and application of a correction factor as described in methods. The values are referenced to C1b as unity.
[a]R, repeat region oligosaccharide; C, capping oligosaccharide; F, fucose containing oligosaccharide.

Table 2. Average length of keratan sulphate chains attached to human articular cartilage fibromodulin.

Repeat region disaccharides and their fucosylated variant[a]	3.87
Repeat region tetrasaccharides and their fucosylated variants[b]	1.82
Capping oligosaccharides[c]	1.00
Average number of disaccharides per *N*-acetyl-neuraminic acid	9.51

Relative abundance of each oligosaccharide type identified *ie* repeat region disaccharides and tetrasaccharides along with their fucosylated variants, and capping oligosaccharides. The average number of disaccharides isolated per *N*-acetyl-neuraminic acid 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 $(\text{-3Gal}\beta\text{1-4GlcNAc}\beta\text{1-})_n$ isolated per *N*-acetyl-neuraminic acid residue is calculated by:
n = (disaccharide type[a]) + (tetrasaccharide type[b] $\times$ 2) + (pentasaccharide cap[c] $\times$ 2)
[a]RF1, R1 and R3.
[b]R2, RF2, RF3, RF4, RF5, R4, R5 and R6.
[c]CF1, C1a, C1b, C2a, C2b and C3.

The reduced oligosaccharides derived following keratanase II digestion of the N-linked keratan sulphate attached to human articular cartilage fibromodulin have been isolated and examined by HPAE chromatography (Figure 1), and the oligosaccharides present identified by comparison with standards. The chromatographic profile is very complex, showing the presence of many oligosaccharides (Figure 1).

Two features of the chromatogram are obvious; firstly, the pentasaccharide capping fragments (C1a, C1b, C2a, C2b and C3) together comprise a significant portion of the total oligosaccharides. Secondly, the highly sulphated oligosaccharides C3 and R6 are also highly abundant. This chromatogram may be compared with that for bovine articular cartilage O-linked keratan sulphate derived from aggrecan (Figure 2). It is important to note that the detector response, although linear for each oligosaccharide, differs between oligosaccharides. However, it is clear that in the sample of O-linked keratan sulphate the capping oligosaccharides are significantly less abundant.

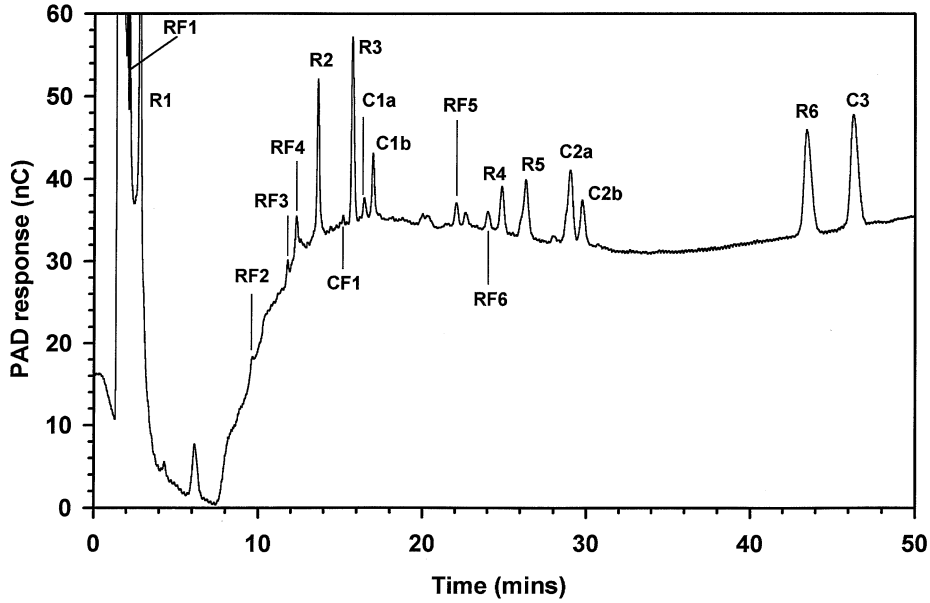


Figure 1. HPAE chromatography of the reduced oligosaccharides derived from fibromodulin isolated from pooled human articular cartilage. A 20 µl aliquot of reduced oligosaccharides, resuspended in water, was applied to an Dionex AS4A-SC column (4 mm × 250 mm) and the oligosaccharides separated by HPAE chromatography on a Dionex chromatography system. The eluent was monitored on-line by a pulsed electrochemical detector using integrated amperometry. An 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.

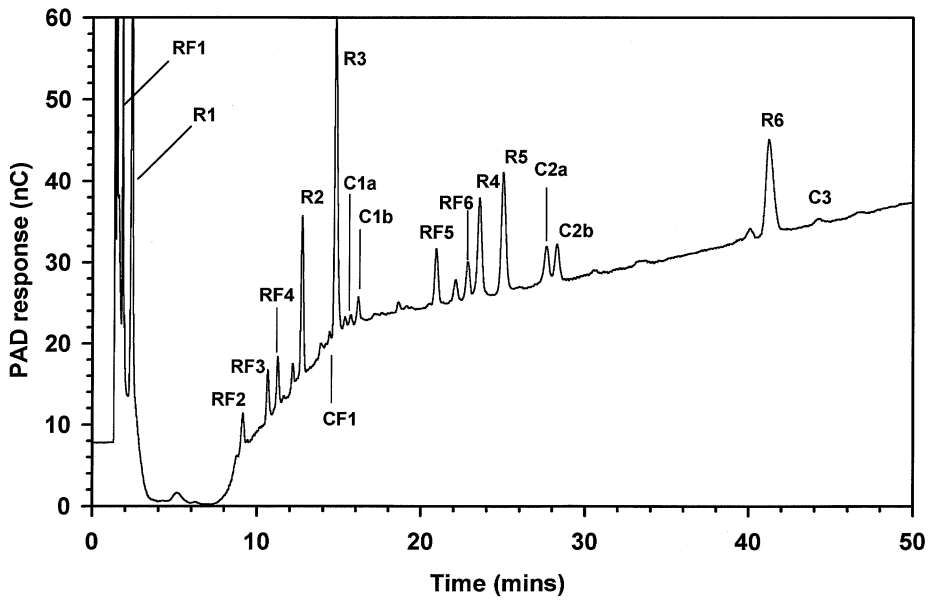
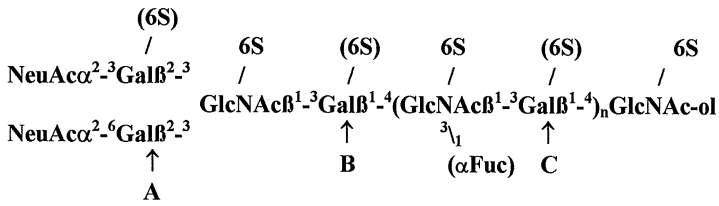


Figure 2. HPAE chromatography of the reduced oligosaccharides derived from O-linked keratan sulphate isolated from bovine articular cartilage. For caption see Figure 1.

Integration of dionex peaks

The abundance of each oligosaccharide derived by keratanase II digestion of the fibromodulin from human articular cartilage has been estimated by measurement of the peak areas (Figure 1), and the application of a response factor (as described in Methods). The results of these analyses are displayed in Tables 1 and 2.

		%Gal sulphation - all chains considered		
	% α(2-6)-linked NeuAc Caps	Gal A and B	Gal B only	Gal C
Human	31%	31%	37%	45%
Bovine (from 10)	33%	3%	58%	40%
Equine (from 10)	27%	30%	49%	42%



	%Gal sulphation - only α(2-3)-linked NeuAc		
	Gal A and Gal B	Gal B only	Gal C
Human	45%	19%	-
Bovine Tracheal cartilage (from 9)	1 - 2%	*ca.* 50%	*ca.* 60%
Bovine (from 10)	4%	44%	-
Equine (from 10)	40%	29%	-

Figure 3. General structure, and galactose sulphation level of the N-linked keratan sulphate attached to articular cartilage fibromodulin. The general structure of articular cartilage fibromodulin is shown, along with the frequency of sulphation of galactose residues at the chain cap and along the repeat region.

Chain length

Quantitative analysis confirms that the majority of the length of the chain is contained within the pentasaccharide caps (C1a, C1b, C2a, C2b and C3), *ie* the chains are short. The average length of the combined chain cap and repeat region from the N-linked keratan sulphate chains attached to human articular cartilage fibromodulin is *ca.* 9 disaccharides (Table 2). Evidence from high field ^{1}H-NMR spectra of human articular cartilage fibromodulin also indicates that the attached keratan sulphate chains are short (data not shown).

Galactose sulphation

The levels of galactose sulphation in the repeat region and at the chain cap of fibromodulin is shown in Figure 3. Analysis of the HPAE chromatography data (Figures 1 and 3) demonstrates that Gal A is sulphated in *ca.* 31% of the chains. If those terminating in α(2-6)-linked *N*-acetyl-neuraminic acid, in which Gal A cannot be sulphated (on chains capped with α(2-6)-linked *N*-acetyl-neuraminic acid the 6 position of the adjacent galactose is involved in the linkage to the *N*-acetyl-neuraminic acid and so cannot be sulphated), are removed from this calculation then this residue is sulphated in *ca.* 45% of those chains for which sulphation at this site is possible.

Levels of galactose sulphation increase at B and then increase further at C, in the repeat region. If we once again consider only those chains capped by α(2-3)-linked *N*-acetyl-neuraminic acid then only 19% of these chains are sulphated at Gal B and not Gal A.

Discussion

Fibromodulin has been isolated from pooled human articular cartilage by dissociative extraction and CsCl density gradient centrifugation. The fibromodulin has been purified by a combination of ion-exchange, and size exclusion chromatography.

During ion-exchange chromatography on a Mono-Q 10/10 column, human fibromodulin elutes in at least five partially resolved fractions. This is in agreement with previous observations on bovine tracheal and bovine and equine articular cartilage fibromodulin [9, 10]. It is likely that this heterogeneity is principally due to differing numbers of keratan sulphate chains attached to the protein core of fibromodulin [7], rather than heterogeneity in the protein core, as recent work has shown that the amino acid composition of the fibromodulin core from each population is substantially the same [10].

The keratan sulphate chains attached to human articular cartilage fibromodulin have been digested by keratanase II, and the resulting oligosaccharides reduced and then isolated. These oligosaccharides have been examined by HPAE chromatography, and their abundance determined.

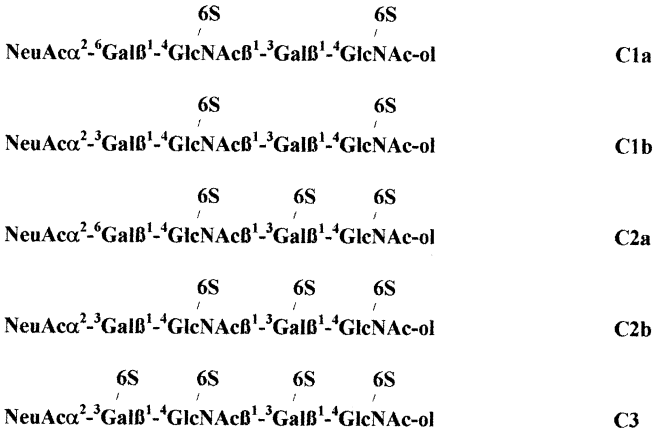


Figure 4. Capping oligosaccharides.

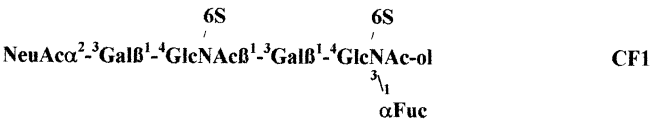


Figure 5. Fucosylated capping oligosaccharide.

Six capping oligosaccharides have been identified and characterized in human articular cartilage fibromodulin, each is based upon a core pentasaccharide structure, which may be extended by the addition of a branching fucose residue α(1-3)-linked to the *N*-acetylglucosaminitol residue. Three of these capping oligosaccharides, C1b, C2b and C3, are pentasaccharides in which the non-reducing terminal *N*-acetyl-neuraminic acid is α(2-3)-linked to the adjacent galactose, which in the case of C3 is sulphated. Two further oligosaccharides, C1a and C2a, have been identified in which the non-reducing terminal *N*-acetyl-neuraminic acid is α(2-6)-linked to the adjacent galactose (Figures 4 and 5).

In previous studies of the keratan sulphate chains attached to fibromodulin isolated from bovine tracheal cartilage [8, 9] *N*-acetyl-neuraminic acid α(2-3)-linked to the adjacent galactose was found to be the sole chain terminating structure. The keratan sulphate of aggrecan from non-articular cartilage has also been found to be capped exclusively by α(2-3)-linked *N*-acetyl-neuraminic acid [18].

Our previous work on the fibromodulin from bovine and equine articular cartilage demonstrated the presence of α(2-6)-linked *N*-acetyl-neuraminic acid in the N-linked keratan sulphate chains of fibromodulin. It is also noteworthy that in O-linked keratan sulphate chains, from bovine articular cartilage aggrecan, the *N*-acetyl-neuraminic acid chain terminator may be also be either α(2-3)- or α(2-6)-linked to the adjacent galactose.

Of the five potential sites for keratan sulphate attachment along the fibromodulin core protein [3], four are known to be glycosylated [7]. As yet it is not known if all of the keratan sulphate chains attached to a single fibromodulin protein core are capped by the same chain terminator, *ie* either α(2-6)- or α(2-3)-linked *N*-acetyl-neuraminic acid. Tai *et al.* [26] demonstrated that the O-linked keratan sulphate chains of articular cartilage aggrecan may have one antenna capped by α(2-3)-linked *N*-acetyl-neuraminic acid, and the other by α(2-6)-linked *N*-acetyl-neuraminic acid.

A further capping oligosaccharide, CF1, has been identified in this study. This oligosaccharide, which is related to C1b, has a fucose residue α(1-3)-linked to the sulphated *N*-acetylglucosaminitol residue and is a sulphated variant of VIM-2 antigen [27]. This novel oligosaccharide is fully described in a separate publication [28].

Twelve repeat region oligosaccharides have been identified in this work. Of these, six (R1–R6) (Figure 6), have been previously isolated and characterized from tracheal and articular cartilage fibromodulin [9, 10]. Six further repeat region oligosaccharides RF1–RF6 (Figure 7), have been identified; each has at least one α(1-3)-linked fucose residue as a branch on an *N*-acetylglucosamine or *N*-acetylglucosaminitol. In each of these oligosaccharides the galactose on the non-reducing side of the fucosylated *N*-acetylglucosamine or *N*-acetylglucosaminitol is unsulphated.

It has been previously noted that the activity of keratanase II is dependent upon the size of the oligosaccharide which is the substrate. Thus, it is unable to further cleave a repeat region tetrasaccharide into smaller oligosaccharides. In the case of the capping oligosaccharides the internal sulphated *N*-acetylglucosamine is also resistant to keratanase II cleavage, presumably due to the proximity of the *N*-acetyl-neuraminic acid residue [29]. However, it is clear that fucose residues do not hinder the activity of keratanase II upon the parent keratan sulphate, as they can be found in several repeat region oligosaccharides (RF1, 2, 3, and 5), and in the capping oligosaccharide CF1, α(1-3)-linked to the reducing terminal *N*-acetylglucosaminitol residue, at which the enzyme has cleaved the parent keratan sulphate. Such fucose residues have been shown to prevent the action of keratanase [30], and protects the parent keratan sulphate from hydrazinolysis/nitrous acid cleavage [31].

It is as yet unknown if the fucose residues are distributed randomly within the keratan sulphate, or are concentrated within a specific sub-population. Because keratanase II cleaves the parent keratan sulphate into tetra- and di-saccharides, it is not possible to obtain any information concerning the position within the parent keratan sulphate formerly occupied by a particular oligosaccharide.

The α(1-3)-linked fucose in the N-linked keratan sulphate from articular cartilage fibromodulin is in an environment similar to that observed in the Le^x antigen/CD15 [32]. It is, however, a sulphated variant which is attached to fibromodulin. In the mouse, expression of this structure was observed transiently during embryogenesis and it has been

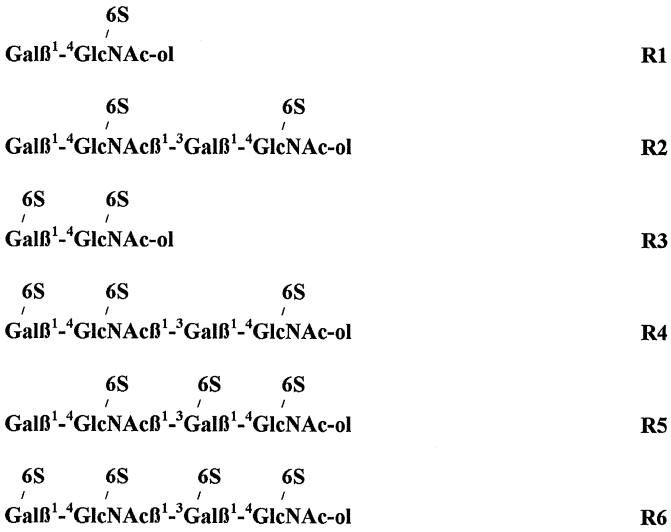


Figure 6. Repeat region oligosaccharides.

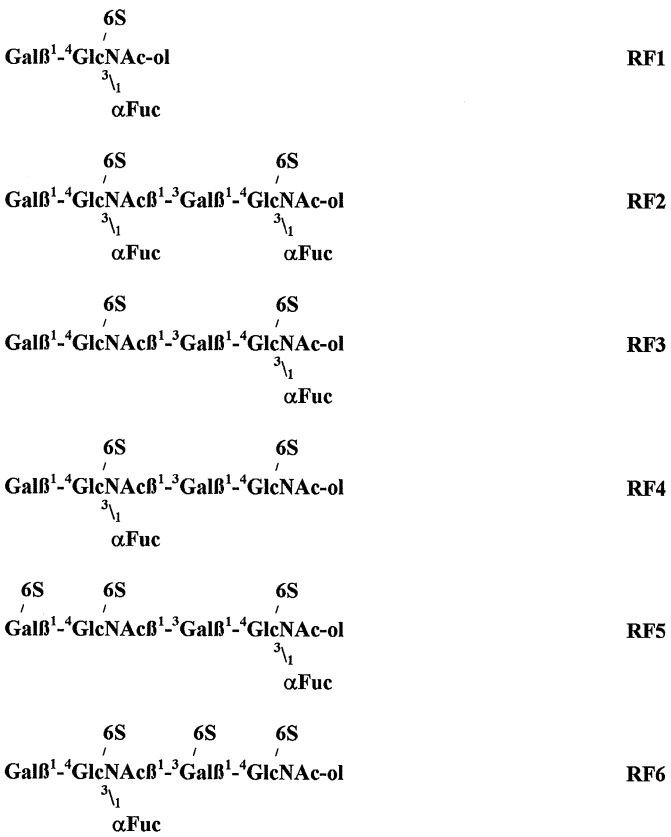


Figure 7. Fucosylated repeat region oligosaccharides.

termed SSEA-1 (stage-specific embryonic antigen) [33]. This raises the possibility of a developmental role for this sulphated variant on fibromodulin.

It is striking that the galactose residue on the non-reducing side of the fucosylated *N*-acetylglucosamine is always unsulphated. Whether fucosylation prevents sulphation, or *vice versa*, remains unclear.

In this study, as with previous work in this laboratory on the N-linked keratan sulphate from fibromodulin and the O-linked keratan sulphate from aggrecan, there was no evidence of any unsulphated *N*-acetylglucosamine residues. There is however less than 100% sulphation of galactose residues, and evidence for differential sulphation along the keratan sulphate chain.

Oligosaccharide C3, in which the terminal *N*-acetyl-neuraminic acid is α(2-3)-linked to a sulphated galactose, has been previously observed in fibromodulin from bovine tracheal cartilage [9] and articular cartilage [10].

Analysis of the HPAE chromatography data (Figures 1 and 3) demonstrates that the galactose residue adjacent to the non-reducing terminus (Gal A) is sulphated in *ca.* 31% of the chains. If those terminating in α(2-6)-linked *N*-acetyl-neuraminic acid, in which this galactose cannot be sulphated (in chains capped with α(2-6)-linked *N*-acetyl-neuraminic acid the 6 position of the adjacent galactose is involved in the linkage to the *N*-acetyl-neuraminic acid and so cannot be sulphated), are not considered, then this residue is sulphated in *ca.* 45% of those chains for which sulphation is possible; a very high level of sulphation. Our previous work examined the keratan sulphate chains attached to fibromodulin from bovine tracheal cartilage and bovine articular cartilage [9, 10], found this galactose residue to be sulphated in only *ca.* 1–2% and 2–3% of chains respectively. A sample of equine articular cartilage has recently been examined [10] which was also found to have a high level of sulphation, Gal A was sulphated in *ca.* 30% of the chains. That this very high level of sulphation is observed in a sample of fibromodulin pooled from seven individuals, makes it unlikely that any single anomalous sample has significantly altered the composition.

It is of considerable interest to note that even in this sample of articular cartilage, which is obviously highly sulphated at the chain cap, there was no evidence of sulphation of Gal A in the absence of sulphation of Gal B, in agreement with previous work on bovine and equine cartilage fibromodulin [8–10].

The level of Gal sulphation in O-linked keratan sulphate from a 38-year-old human articular cartilage is very similar to that reported here. In that study Gal A was found to be sulphated in 33% of the chains overall and in 49% of the chains with an α(2-3)-linked *N*-acetyl-neuraminic acid cap [31].

In keratan sulphate from human fibromodulin the average level of sulphation of Gal B is 36%, higher overall than that of Gal A. This is in agreement with previous data on the keratan sulphate attached to fibromodulin with bovine and equine articular and tracheal cartilage [9, 10], in which the sulphation of Gal B was always higher than that of Gal A. However, if we consider only the caps with α(2-3)-linked *N*-acetyl-neuraminic acid, then the level of Gal B sulphation

is low, at only 19%. Clearly as the level of Gal A sulphation increases the number of chains remaining with Gal B sulphated but not Gal A, will decrease.

The level of sulphation of galactose residues within the repeat region, was found to be *ca.* 45%. This is similar to keratan sulphate from bovine and equine articular cartilage fibromodulin, in which, on average, *ca.* 40–45% of the galactose residues were sulphated [10], but lower than that of keratan sulphate from bovine tracheal cartilage fibromodulin, in which, on average, *ca.* 59–63% of the repeat region galactose residues were sulphated [9].

Because keratanase II digestion generates tetra and disaccharides, along with their fucosylated variants, from the repeat region of the parent keratan sulphate, it is not possible to determine from these data the level of sulphation of the galactose residues along the repeat region. However, previous work has reported increasing levels of galactose sulphation along the repeat region towards the non-reducing terminus (the chain cap), with a very sharp decline at the galactose adjacent to the non-reducing terminus, *ie* Gal A [8, 9]. These data are generally in agreement with those of Oeben *et al.* [34], who also reported increasing levels of galactose sulphation towards the chain cap of pig corneal N-linked keratan sulphate compared to the repeat region.

The data presented here support this general model of galactose sulphation, *ie*, increasing levels of galactose sulphation towards the chain cap within the keratan sulphate chains of human articular cartilage fibromodulin. However, as with equine articular cartilage the situation is more complex; the drop at Gal A is only slight, and, if only chains capped by α(2-3)-linked *N*-acetyl-neuraminic acid are considered then the level of Gal sulphation appears to drop at Gal B and then rise again at Gal A. The functional significance of the high level of Gal A sulphation is not clear. It will be of considerable interest to know if this high level of sulphation is present in young human articular cartilage.

The average length of the chain cap and repeat region from the N-linked keratan sulphate chains attached to human articular cartilage fibromodulin has been determined to be *ca.* eight disaccharides (Table 2). Evidence from high field ^{1}H-NMR spectroscopy of human articular cartilage fibromodulin also indicates that the keratan sulphate chains are short (data not shown). That the chains are short, is in agreement with previous work examining the structure of the keratan sulphate attached to fibromodulin from various sources [8–10]. It has been consistently found that the repeat region and chain cap of fibromodulin from tracheal cartilage is ca. two to seven disaccharides in length. From articular cartilage the chains are, on average, slightly longer at seven to nine disaccharides.

The structure of O-linked keratan sulphate chains from bovine nasal septum have been reported by Stuhlstatz *et al.* [35] who found that the chains derived from this tissue were also short, having a repeat region seven to nine disaccharides in length. However, Oeben *et al.* [34], in a study of N-linked keratan sulphate chains from pig cornea, found chains with repeat regions which were 10–52 disaccharides in length. These workers found no *N*-acetyl-neuraminic acid chain caps, and so the chains may be even longer *in vivo*.

The repeat region and chain caps of the N-linked keratan sulphate attached to fibromodulin from pooled human articular cartilage have been found to have the general structure shown in Figure 3. The keratan sulphate attached to fibromodulin from articular cartilage has been found to contain α(2-6)-linked *N*-acetyl-neuraminic acid caps and has branching fucose residues α(1-3)-linked to *N*-acetyl-glucosamine residues both along the repeat region and within chain caps. The presence of these structural features, which are also found in O-linked keratan sulphate from articular cartilage aggrecan, confirms the broad structural similarity of the repeat region and chain caps of N-linked KS-I and O-linked KS-II from articular cartilage. These data also highlight the importance of tissue specific fucosyl- and sialyl-transferase enzymes in the elaboration of both N-linked and O-linked keratan sulphates.

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