Characterization of a non-reducing terminal fragment from bovine articular cartilage keratan sulphates containing $\alpha(2-3)$ -linked sialic acid and $\alpha(1-3)$ -linked fucose

A sulphated variant of the VIM-2 epitope

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Alkaline-borohydride-reduced keratan sulphate chains were isolated from bovine articular cartilage (6–8-year-old animals) and digested with keratanase II, an endo- β -*N*-acetylglucosaminidase. The resulting oligosaccharides were borohydride-reduced and fractionated on a strong anion-exchange column. $^1\text{H-NMR}$ spectroscopic analysis of the products revealed one containing both $\alpha(2-3)$ -linked sialic acid and $\alpha(1-3)$ -linked fucose which was shown to have the structure (I) shown. This structure is a sulphated variant of the VIM-2 epitope (CD65), a putative

ligand of E-selectin. No oligosaccharide containing the sialyl-Le^x structure [NeuAc α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc β 1-] was identified in this study.

INTRODUCTION

Keratan sulphate (KS), a glycosaminoglycan, is based upon a repeating disaccharide motif of -3Gal β 1-4GlcNAc β 1- which is almost always sulphated on C(6) of *N*-acetylglucosamine, with further sulphate groups being present on C(6) of galactose [1]. KSs have been subclassified into KS-I from cornea and KS-II from skeletal tissues such as cartilage [2], and this latter type has been further subclassified into KS-IIA from articular cartilages and KS-IIB from non-articular types [3]. KS-IIA contains $\alpha(2-6)$ -linked sialic acid and $\alpha(1-3)$ -linked fucose which are not present in KS-IIB; however, both types contain $\alpha(2-3)$ -linked sialic acid. Chemical analyses have shown that there is more than one sialic acid residue per chain [4], and subsequent studies showed that these residues occupy non-reducing termini, both in the linkage region (to protein) and at the end of the poly(*N*-acetyl-lactosamine) repeat sequence [5–8].

Skeletal KSs have been widely studied because of their potential as early markers for osteoarthritis (see, e.g., [9]). During cartilage turnover, KS-bearing fragments are released into the synovial fluid and subsequently the blood, where they can be detected using anti-KS monoclonal antibodies [10]. In addition, corneal KSs and associated proteoglycans are now implicated in certain forms of macular corneal dystrophy [11]. KSs are present on the surfaces of many cell types, including tumour cells [12–15]; however, their functions in these tissues are not yet understood.

In the present study, KSs from bovine articular cartilage have been digested with the enzyme keratanase II and the resulting oligosaccharides reduced and separated using ion-exchange chromatography. Upon investigation by $^1\text{H-NMR}$ spectroscopy, one oligosaccharide was shown to contain both sialic acid and fucose and clearly derived from the non-reducing terminus of the KS chains. This oligosaccharide is a sulphated variant of the VIM-2 epitope [CD65; NeuAc α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)GlcNAc], a putative E-selectin ligand.

MATERIALS AND METHODS

Materials

Diphenylcarbamoyl chloride-treated trypsin (from bovine pancreas; EC 3.4.21.4) was purchased from Sigma. Chondroitin ABC lyase (*Proteus vulgaris*; EC 4.2.2.4) and keratanase II (*Bacillus* sp.) were obtained from ICN Biomedicals Ltd. (High Wycombe, Bucks., U.K.). All other chemicals and HPLC columns were as described in [7,8].

Isolation and purification of KS chains

KS chains were prepared from bovine femoral head cartilage (6–8-year-old animals) as described by Brown et al. [7,8] (based on Dickenson et al. [16]).

Preparative keratanase II digestion

KS chains (285 mg) were dissolved in 4.70 ml of 10 mM sodium acetate buffer, pH 6.5, and 0.6 unit of keratanase II added (dissolved in 300 μl of 10 mM sodium acetate buffer, pH 6.5). Digestion was performed at 37 °C for 30 h. The oligosaccharides were subsequently reduced and desalted on a Bio-Gel P-6 column as described by Brown et al. [7,8].

Strong anion-exchange chromatography

The oligosaccharides (140 mg) were dissolved in 800 μl of 2 mM LiClO₄, pH 5.0, and chromatographed in four separate runs on a Nucleosil 5SB strong anion-exchange column (250 mm $\times$ 10 mm) equilibrated in 2 mM LiClO₄, pH 5.0, and eluted with a linear gradient of 0–0.25 M LiClO₄, pH 5.0, from 15 to 255 min. The column eluate was monitored on-line at 206 nm.

Abbreviations used: KS, keratan sulphate; GlcNAc-ol, *N*-acetylglucosaminitol (2-acetamido-D-glucitol); Le^x, Gal β 1-4(Fuc α 1-3)GlcNAc; VIM-2 epitope, NeuAc α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4(Fuc α 1-3)GlcNAc.

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Figure 1 Nucleosil 5SB ion-exchange chromatogram of oligosaccharides produced by keratanase II digestion/borohydride reduction of bovine articular cartilage KSs

The column (250 mm $\times$ 10 mm) was eluted at a flow rate of 2 ml/min. The gradient program (shown by the broken line) was as follows: 15 min of 2 mM LiClO_4 , pH 5.0, and then 240 min of 0–100% 0.25 M LiClO_4 , pH 5.0. Oligosaccharide (I) was pooled as shown. Inset: re-chromatography of oligosaccharide (I) on a Spherisorb 5B SAX column. The column (250 mm $\times$ 4.6 mm) was eluted at a flow rate of 1 ml/min. The gradient program (shown by the broken line) was as follows: 5 min of 2 mM LiClO_4 , pH 5.0, followed by 25 min of 0–35% 0.25 M LiClO_4 and then 90 min of 35–50% 0.25 M LiClO_4 , pH 5.0. A purified oligosaccharide (I) was pooled as shown.

NMR spectroscopy

Samples were exchanged several times with 0.5 ml of 99.8% $^2\text{H}_2\text{O}$, buffered to pH 7 with phosphate (10 mM) and referenced with sodium 3-trimethyl[$^2\text{H}_4$]propionate as internal standard before final dissolution into 0.5 ml of 99.96% $^2\text{H}_2\text{O}$. High-field ^1H -NMR spectra at 600 MHz were acquired using a Bruker AMX600 spectrometer equipped with a 5 mm probe. Both one- and two-dimensional spectra were determined at 55–60 $^\circ\text{C}$. All chemical shifts are quoted relative to internal sodium 3-trimethyl[$^2\text{H}_4$]propionate at 0.0 p.p.m.

Spectra were reprocessed for presentation using the software packages NMR1 and NMR2, Versions 1-4-1, supplied by New Methods Research Inc., Syracuse, NY, U.S.A.

RESULTS

Keratanase II digestion of articular-cartilage KS yields a complex series of oligosaccharides [7,8], as revealed by strong anion-exchange chromatography (Figure 1). Fractions 104 and 105 were pooled as oligosaccharide (I). ^1H -NMR spectroscopic analysis revealed the presence of a second component, and therefore the mixture was rechromatographed on a Spherisorb S5 strong anion-exchange column (250 mm $\times$ 4.6 mm) with a shallow LiClO_4 gradient increasing from 35 to 50% 0.25 M LiClO_4 over 90 min. A purified oligosaccharide (I) was pooled as shown in the inset to Figure 1.

The 600 MHz ^1H -NMR spectrum of oligosaccharide (I) (Figure 2) shows the presence of four anomeric resonances and three separate *N*-acetyl methyl signals (not shown). Using both one-dimensional and two-dimensional COSY-45 data (Figures 2 and 3), almost complete chemical shift assignments can be made for this oligosaccharide (Table 1).

As this oligosaccharide is a product of keratanase II digestion, it would be expected that the reducing terminus would be occupied by an *N*-acetylglucosaminitol residue. Inspection of the one-dimensional spectrum shows the presence of a doublet of doublets at 4.040 p.p.m. characteristic of H(4) in an *N*-acetylglucosaminitol residue [8] which is strongly coupled to a neighbouring resonance slightly downfield. Using COSY-45 data, it is possible to identify an H(5) resonance at 4.087 p.p.m. The H(3) resonates at 4.243 p.p.m., showing a marked perturbation downfield (by approx. 0.26 p.p.m.) from that in an *N*-acetylglucosaminitol substituted only on C(4) by galactose [17]. The two-dimensional data provide connections from the H(5) resonance to a non-equivalent methylene pair of protons at 4.252 and 4.279 p.p.m. which can be assigned to H(6) and H(6') respectively, the downfield locations of which confirm that this residue is 6-*O*-sulphated.

Resonances at 1.805 and 2.760 p.p.m. (not shown) can be readily assigned to the H(3ax) and H(3eq) protons of an *N*-acetylneuraminic acid residue which is α (2-3)-linked to galactose [5,8]. This is confirmed by one-dimensional data which identify

Figure 2 Partial 600 MHz ^1H -NMR spectrum of oligosaccharide (I) obtained at 55 °C

The residual HO^2H signal has been artificially removed after processing for clarity.

a galactose H(3) resonance ($J_{2,3}$ 9.85 Hz, $J_{3,4}$ 3.25 Hz) at 4.124 p.p.m., shifted characteristically downfield by ~ 0.4 p.p.m. from that in either a non-reducing terminal or internal (i.e. 3-*O*-substituted) galactose. COSY-45 data for this galactose residue provide a connection via H(2) (at 3.569 p.p.m.) to the anomeric resonance at 4.600 p.p.m. In addition, although the $J_{4,5}$ coupling is small in this sugar (~ 1 Hz), it is possible to resolve a weak COSY-45 cross-peak from H(4) (at 3.989) to H(5) at ~ 3.73 p.p.m. Although further connections to H(6) and H(6') are lost within the complex envelope of signals at $\sim 3.70\text{--}3.85$ p.p.m., the location of this H(5) resonance confirms that this residue is unsulphated [7,8,17]. The H(5) in a sulphated sialylated galactose residue occurs approx. 0.23 p.p.m. further downfield at ~ 3.96 p.p.m. [8].

The anomeric resonance at 4.740 p.p.m. can be readily assigned to an internal (i.e. 4-*O*-substituted) *N*-acetylglucosamine [17,18]. COSY-45 data provide a connection to H(2) at 3.836 p.p.m., although further connections are not visible. However, close examination of the two-dimensional data indicate a non-equivalent methylene pair of protons at 4.313 and 4.415 p.p.m. which both connect to a resonance at 3.816 p.p.m. The shift positions are characteristic of the H(6), H(6') and H(5) protons respectively of a 6-*O*-sulphated internal *N*-acetylglucosamine residue.

The anomeric resonance at 4.572 p.p.m. can be readily assigned to a galactose residue occupying a position between the *N*-acetylglucosamine and *N*-acetylglucosaminitol residues. The chemical-shift location of H(4) at 4.181 p.p.m. confirms that this residue is linked at C(3) [17] [the H(4) resonance occurs at $\sim 3.95\text{--}4.00$ p.p.m. in a non-reducing terminal galactose]. Close examination of the COSY-45 data reveals an H(4) to H(5) cross-

peak partially obscured by the H(3) to H(4) connection, and locates H(5) at ~ 3.70 p.p.m. In addition, one of the H(6) methylene protons can be identified at ~ 3.805 p.p.m. These chemical shifts indicate that this galactose is also unsulphated [8].

The final anomeric resonance at 5.065 p.p.m. ($J_{1,2}$ 4.15 Hz) can be readily identified as that of an $\alpha(1\text{--}3)$ -linked fucose residue [7]. The H(6) methyl protons resonate at 1.228 p.p.m. ($J_{5,6}$ 6.60 Hz) and COSY-45 data locate H(5) at 4.288 p.p.m. These chemical shifts are all significantly different from those found for $\alpha(1\text{--}3)$ -linked fucose in intact KS chains (at 5.12, 1.17 and 4.76 p.p.m. respectively), indicating that the environment of this residue has changed significantly. The data demonstrate [8] that the fucose residue is linked to C(3) of the *N*-acetylglucosaminitol residue.

Oligosaccharide (I) is, therefore, a hexasaccharide with the structure:

DISCUSSION

Oligosaccharide (I) is unique among the 26 oligosaccharides isolated so far by keratanase II digestion of bovine articular cartilage KSs (see [7,8]) in containing both sialic acid and fucose

Figure 3 Partial 600 MHz COSY-45 spectrum of oligosaccharide (I) obtained at 55 °C

The spectral width was 2451 Hz, and 32 acquisitions for each of 815 increments were sampled into 1024 complex points. Data were reprocessed for presentation and detailed analysis using the software package NMR2. The array was zero-filled to 2048 × 2048 complex points and transformed in each dimension after application of a sinebell window function offset 2%. The spectrum has been symmetrized for presentation purposes after careful examination of unsymmetrized data.

residues. It is clearly derived from the non-reducing termini of the KS chains and provides the first evidence that fucose residues can occur within KS capping sequences. It is noteworthy that neither the sialyl-Le^x structure [NeuAc α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc β 1-] nor a similar structure with α (2-6)-linked sialic acid have been identified, although efforts were made to detect them. This possibly reflects the substrate specificities of the glycosyltransferases responsible for the biosynthesis of KS, i.e. these structures cannot be formed. If these latter structures were present in the original KS preparation then it is estimated that they occurred at concentrations below 0.2% (w/w). Based upon the total weight of all the oligosaccharides recovered, the oligosaccharide described here contributed approx. 1% of the weight of the original KS chains and represented approx. 4% of the capping sequences.

Many of the structures present within KS are similar to those recognized by the selectins, cell-surface lectins that mediate leucocyte adhesion to the vascular endothelium. For example, the immediate environment of the α (1-3)-linked fucose residue [i.e. Gal β 1-4(Fuc α 1-3)GlcNAc] is identical to that observed in the Le^x antigen (CD15) [19,20] except that the *N*-acetylglucosamine is sulphated. Similarly, the oligosaccharide described here is a sulphated variant of the VIM-2 epitope (CD65), a differentiation antigen on human myeloid cells [21] which is known to be a ligand for E-selectin, albeit with a 10–100-fold lower affinity than sialyl-Le^x [22]. Although it is known that sulphation of selectin ligands is important in recognition [23],

Table 1 Proton chemical shift assignments at 55 °C for oligosaccharide (I)

The structure of oligosaccharide (I) is:

Data for [C2] and [F3] are taken from ref. [8]:

[C2] = NeuAc α (2-3)Gal β (1-4)GlcNAc(6S) β (1-3)Gal β (1-4)GlcNAc(6S)-ol

[F3] = Gal β (1-4)GlcNAc(6S) β (1-3)Gal β (1-4)[Fuc α (1-3)]GlcNAc(6S)-ol

Oligosaccharide ...	Chemical shift (p.p.m.)		
	(I)	[C2]	[F3]
NeuAc (E)			
H(3ax)	1.805	1.805	
H(3eq)	2.760	2.760	
H(4)	3.709	~ 3.72	
H(5)	3.847	~ 3.82	
H(6)	3.665	3.663	
H(7)	3.600	3.598	
H(8)	~ 3.910	3.915	
H(9)	~ 3.665	~ 3.67	
H(9')	~ 3.89	3.876	
NAc	2.041	2.041	
Gal (D)			
H(1)	4.600	4.600	4.535
H(2)	3.569	3.570	3.552
H(3)	4.124	4.124	3.685
H(4)	3.989	3.989	3.948
H(5)	~ 3.73	—	—
H(6)	—	—	—
H(6')	—	—	—
GlcNAc (C)			
H(1)	4.740	4.741	4.754
H(2)	3.836	~ 3.835	~ 3.83
H(3)	—	—	—
H(4)	—	—	—
H(5)	3.816	~ 3.81	3.803
H(6)	4.313	~ 4.305	4.316
H(6')	4.415	~ 4.415	4.401
NAc	2.046	2.045	2.050
Gal (B)			
H(1)	4.572	4.560	4.572
H(2)	3.597	3.623	3.601
H(3)	3.713	3.721	3.718
H(4)	4.181	4.183	4.173
H(5)	~ 3.70	—	—
H(6)	~ 3.805	—	—
H(6')	—	—	—
GlcNAc(6S)-ol (A)			
H(1)	~ 3.765	~ 3.69	~ 3.77
H(1')	~ 3.780	~ 3.775	~ 3.77
H(2)	4.406	~ 4.275	4.408
H(3)	4.243	3.976	4.237
H(4)	4.040	3.934	4.039
H(5)	4.087	~ 4.145	4.081
H(6)	4.252	4.243	~ 4.265
H(6')	4.279	4.326	~ 4.265
NAc	2.053	2.059	2.053
Fuc (F)			
H(1)	5.065		5.065
H(2)	3.821		3.821
H(3)	3.895		3.893
H(4)	~ 3.83		—
H(5)	4.288		4.288
Me	1.228		1.228

further experiments will be required to determine whether oligosaccharide (I) is an effective ligand.

The VIM-2 structure may have an important role in the development of cartilage. Studies of human articular cartilage KS have indicated that the overall fucose content increases markedly at adolescence (G. M. Brown, unpublished work). Oligosaccharide profiling (see [23a]) of KSs from full-term human foetal cartilage has indicated that almost all of the fucose is present in the capping structure represented by oligosaccharide (I), even though the total fucose content of the KS is low. Therefore it is possible that VIM-2 type and Le^x antigens may be important at early stages of tissue development but are then subsequently masked by sulphation [24,25]. It is known that the sulphation level of KS increases with age but is low in foetal material [26,27].

It is clear that there is considerable structural diversity within the non-reducing termini of articular cartilage KS. To date, at least six different capping structures have been produced by keratanase II digestion which vary in their sulphate content and fucose and sialic acid substitution. As KSs are now known to be synthesized by many tissues and cell types, it is clear that the capping sequences have great potential for molecular interactions and recognition. Like other glycosaminoglycans, KS may prove to have interactions with growth factors, cytokines, extracellular matrix molecules, etc. However, elucidation of their structure–function relationships will require the detailed study of cap distribution within tissues and during development.

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