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Detection of oligosaccharide ligands for Hepatocyte growth factor/Scatter factor (HGF/SF), Keratinocyte growth factor (KGF/FGF-7), RANTES and Heparin cofactor II by neoglycolipid microarrays of glycosaminoglycan-derived oligosaccharide fragments

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Abstract Neoglycolipid technology is eminently adaptable for microarray design for high-throughput detection and specificity assignments of carbohydrate-protein interactions. Dermatan sulfate (DS) is known to play an important role because of its ability to bind growth factors as well as chemokines and to modulate their biological activities during inflammation and response to injury. We prepared various iduronic acid-rich fragments from DS by complete digestion with chondroitinase ACI, and investigated whether the DS-binding proteins, such as HGF/SF, RANTES, KGF/FGF-7 and HCII, can detect their oligosaccharide ligands in a neoglycolipid microarray. First, a comparison of the intensity of binding signals obtained from chondroitin oligosaccharides with those of heparin oligosaccharides showed that our microarray system is feasible not only to single-out the oligosaccharide ligands, but also to detect the difference between an intrinsic interaction unrelated only to electrostatic interaction and non-specific electrostatic interaction. Second, HGF/SF, KGF/FGF-7 and HCII showed preferential binding to iduronic acid-rich fragments of DS oligosaccharides that are greater than 8-mers in lengths. In contrast, RANTES binding seemed to depend only on the negative charges; their binding intensity towards the DS oligosaccharides was somewhat stronger than the binding of HGF/SF, KGF/FGF-7 and HCII. Third, the use of polyvinylpyrrolidone-40 (PVP-40), ovalbumin (OV) and Tween 20 in place of BSA as a blotting agent was useful in these glycosaminoglycan dependent reactions to minimize background due to non-specific interactions.

Keywords Oligosaccharide-microarray · Hepatocyte growth factor/scatter factor · Keratinocyte growth factor · RANTES · Heparin cofactor II · Dermatan sulfate · Neoglycolipid

Abbreviations

ADHP	N-aminoacetyl-*N*-(9-anthracenylmethyl)-1,2-dihexadecyl-sn-glycerol-3-phosphoethanolamine
CSA	chondroitin sulfate A
CSC	chondroitin sulfate C
DHPE	1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine
DS	Dermatan sulfate
ΔUA	unsaturated uronic acid
GAG	glycosaminoglycan
HCII	Heparin cofactor II
HGF/SF	Hepatocyte growth factor/Scatter factor
KGF/FGF-7	Keratinocyte growth factor or fibroblast growth factor-7
HS	heparan sulfate
IdoA	iduronic acid
mer	number of monosaccharides (i.e. degree of polymerization)

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NC	nitrocellulose
NGL	neoglycolipid
OV	ovalbumin
PBS	phosphate buffered saline
PVP-40	polyvinylpyrrolidone-40
RANTES	Regulation upon Activation, Normal T cell Expressed and presumably Secreted
PG	proteoglycan
ΔUA	unsaturated hexauronic acid
α-ΔCS	a mixture of anti-proteoglycan ΔDi4S and ΔDi6S with a ratio of 1:1
HPTLC	high performance thin-layer chromatography

Introduction

Proteoglycans (PGs) are ubiquitous and important components of the extracellular matrix as well as cell membranes. Each consists of a core protein to which one or more glycosaminoglycan (GAG) chains are covalently attached. The common GAG types in PGs are chondroitin sulfates A and C (CSA and CSC, respectively), dermatan sulfate (DS, also known as chondroitin sulfate B), heparan sulfate (HS) and keratan sulfate.

Because of the high content of sulfate and carboxyl groups in their GAG moieties, PGs are the most negatively charged glycoproteins in living tissues. This property enables them to interact with proteins that have clusters of positively charged amino acids. Among these are certain growth factors, cytokines, and chemokines. DS is mainly composed of repeating disaccharide units of iduronic acid (IdoA) and *N*-acetylgalactosamine sulfated at C4 position. The presence of IdoA in DS likens it to heparin and HS. It is also well known that IdoA residue appears to play an important role in formation of the binding site specificity for GAG-binding proteins such as heparin cofactor II (HCII), a series of fibroblast growth factors, cytokines and chemokines (for a review, see the reference 1). Recently WISP-1, a member of the CCN family of growth factor that are thought to mediate, has been shown to interact with decorin and biglycan through DS chains, although a heparin binding consensus sequence is absent in WISP-1 [2]. DS differs in sugar composition from other GAGs, such as chondroitin sulfates A and C, heparin and heparan sulfate. DS contains iduronic acid rather than the glucuronic acid contained in CSA and CSC. It contains *N*-acetylgalactosamine rather than *N*-acetylglucosamine, which is different from HS and heparin. DS is considered to play an important role in many cellular processes.

The neoglycolipid (NGL) technology for generating lipid-linked oligosaccharide probes has many features that render it adaptable for carbohydrate ligand detection [3,4]. Recently we have demonstrated that the NGL approach for generating an oligosaccharide microarray system is applicable both to structurally defined oligosaccharides and to oligosaccharide mixtures derived from biological sources, glycoproteins, GAG, and even a whole organ for the high-throughput detection of ligands for carbohydrate-binding proteins, even when only one pmol of oligosaccharides per spot was spotted (5, for a review, see reference 6). Using the oligosaccharide microarray system it was also shown that the binding profiles of L- and E-selectins and the signal intensities visualized were corresponded perfectly with earlier assignments [7]; initial exploratory experiments were also carried out with the chemokines-RANTES (acronym for Regulation upon Activation, Normal T cell Expressed and presumably Secreted), and the cytokine interferon gamma [5]. Therefore, this microarray seemed to be applicable to compare the binding intensity among ligand oligosaccharides as well as to search for specific oligosaccharide ligands.

To further examine the applicability of the NGL based microarray approach as well as to elucidate the protein interactions with CSA, CSC and DS, we focused on the interactions of four well-known DS-binding proteins, such as RANTES, Hepatocyte growth factor/Scatter factor (HGF/SF), Keratinocyte growth factor or fibroblast growth factor-7 (KGF/FGF-7), and Heparin cofactor II (HCII). Until now, the preference of iduronic acid residues and the smallest length of oligosaccharides needed for this binding were poorly understood, except for HGF/SF and KGF/FGF-7.

Because of the high sulfate and carboxyl group content in GAG molecules, we must distinguish the protein-carbohydrate interaction between the intrinsic interaction unrelated only to electrostatic interaction and non-specific electrostatic interaction. It is well known that chondroitinase ACI, which only catalyzes the cleavage of the N-acetylgalactosaminide linkage to glucuronic acid found in common chondroitin sulfate chains, creates a series of oligosaccharides rich in internal iduronic acid from DS [8,9]. Therefore, for this purpose we prepared a series of oligosaccharides from CSA, CSC and DS with chondroitinase ACI digestion, which enabled us to obtain a series of iduronic acid-rich fragments from DS. This study has shown that carbohydrate-protein interactions can be clearly detected with one pmol oligosaccharide spot on nitrocellulose (NC) membranes, and suggested that the minimum size of DS required for binding to HGF/SF, KGF/FGF-7 and HCII was at least 8- or 10-mers with almost all of the internal uronic acid residues being iduronic acid. As a particularly important finding, we also suggest that our microarray is quite feasible for the detection of a specific oligosaccharide when the oligosaccharides under investigation were far less than one pmol, because Maimone and Tollefson [10] have shown that the portion of specific oligosaccharide chains to which HCII binds has the characteristic feature of a sulfate group at the

C2 position of iduronic acid, and the percentage of the chains was merely 5% in DS.

In the course of the preparation of heparin oligosaccharide-microarray, we produced a polyclonal antibody that recognizes the non-reducing termini of heparin oligosaccharides with an unsaturated uronic acid residue at the non-reducing terminal. Our microarray suggested that the major epitopic structure for this antibody might be larger than trisaccharide in length as well as the structural feature of either ΔUA(2S)-GlcNS(6S)-GlcA-GlcNS(6S) or ΔUA(2S)-GlcNS(6S)-IdoA-GlcNS(6S).

Materials and methods

Materials

Murine monoclonal antibodies anti-proteoglycan ΔDi4S and ΔDi6S, BSA(fraction V), OV(crystallized), DS(pig skin), chondroitinase ACI, chondroitinase B and heparinase (Flavobacterium heparinum) were purchased from Seikagaku Kogyo, Japan. Chondroitin sulfate A (Bovine trachea) and C (Shark cartilage), DS (porcine intestinal mucosa), heparin (porcine intestinal mucosa), polyvinylpyrrolidone-40 (PVP-40), and FAST 3,3′-diaminobenzidine (DAB-FAST) reagent were from Sigma, USA. Recombinant human HGF/SF, monoclonal antibodies anti-human HGF/SF, recombinant human KGF/FGF-7, anti-human KGF/FGF-7, recombinant human RANTES, anti-human RANTES were obtained from R & D Systems, USA. Mouse anti-immunoglobulins/HRP, polyclonal rabbit anti-goat immunoglobulins/HRP, and polyclonal goat anti-rabbit immunogloblins/HRP were from DakoCytomation, Japan. HCII was from CALBIOCHEM, Germany. Goat anti-human heparin cofactor II was purchased from Affinity Biochemicals, Canada. NC membranes (Trans-Blot transfer medium, 0.45 μm) were from Bio-Rad, USA. 1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (DHPE) was from Fluka, Japan. *N*-aminoacetyl-*N*-(9-anthracenylmethyl)-1,2-dihexadecyl-snglycerol–3-phosphoethanolamine (ADHP) was prepared from DHPE according to the methods of Stoll *et al*. [11].

Partial depolymerization of chondroitin sulfates A and C, DS and heparin by chondroitinase ACI and heparinase, respectively, and fractionation of the oligosaccharide fragments by the gel filtration on Bio-gel P-10 column

Chondroitin sulfates A and C and heparin (50 mg) dissolved in 2 ml of 40 mM Tris-HCl buffer, pH 7.3 containing 0.04 M sodium acetate and 0.1 mg/ml BSA were partially digested by limited digestion at 37°C with 250 mU of chondroitinase ACI or 0.05 units of heparinase, respectively, and the reaction stopped at around 40% completion (usually 1–2 h incubation). DSs (derived from pig skin and porcine intestinal mucosa) dissolved in the above buffer were exhaustively digested with 250 mU of chondroitinase ACI at 37°C for 30 h to obtain the iduronic acid-rich fragments by cleavage at isolated internal glucuronic acid residues. The digests were fractionated on a Bio-Gel P-10 column (1.5 × 95 cm) eluted with aqueous 10% ethanol containing 1 M NaCl. The oligosaccharide fractions obtained were desalted on a Sephadex G-10 column (1.5 × 35 cm) eluted with water. The eluate was monitored by absorption at 232 nm.

Preparation and fractionation of fluorescent NGLs

Fluorescent NGLs were prepared as described previously [11] and purified by HPTLC. In brief, typically, to the 50 nmol of lyophilized oligosaccharides 75 μl of 2 mM *N*-aminoacetyl-*N*-(9-anthracenylmethyl)-1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (ADHP) in chloroform/methanol/water, 10:10:1, v/v and 15 μl of freshly prepared tetrabutylammmonium cyanoborohydride solution (28.2 mg/ml of methanol) was added to convert into NGLs by conjugating to the fluorescent lipid, ADHP. The mixture was incubated at 60°C for 72–96 h. Reaction mixtures were resolved by HPTLC using chloroform/methanol/water, 105:100:28, v/v as a solvent for 2-mer to 6-mer, or 50:60:20, v/v for 8-mer to 12-mer, and then the fluorescent NGLs were visualized under UV light and they were extracted from the bands with chloroform/methanol/water, 25:25:8, v/v. The fluorescent NGLs were quantified with fluorescent densitometry at 254 nm. In this paper, we used a mixture of anti-proteoglycan ΔDi4S and ΔDi6S with a ratio of 1:1, which was designated α-ΔCS in this paper, in order to confirm the existence of chondroitin sulfate oligosaccharides in ADHP-conjugated NGLs.

Preparation of oligosaccharide microarray

The 0.5 pmol/μl of NGLs in chloroform/methanol/water, 25:25:8, v/v were applied by jet spray as a 2 mm band for 30 s at a rate of 70 nl/s with a sample applicator (Linomat V, Camag, Switzerland) as described previously (7). For the detection of binding signals, 1 pmol of NGLs was applied per spot, while 10 pmol of fluorescent NGLs were spotted only when the test of fluorescent NGLs stability was performed, due to the highly fluorescent background of the NC membrane.

Carbohydrate-binding assays

Oligosaccharide microarray binding assays were performed essentially as described previously [5], except that PVP-40

and OV were used in place of BSA due to having the relatively higher hydrophobic nature of HGF/SF. Fluorescent NGLs spotted NC membranes were first immersed in 10 mM Tris-HCl buffer, pH 7.4, containing 1% OV and 0.05% Tween 20 in 150 mM NaCl, 2 mM $CaCl_2$, 0.8 mM $MgCl_2$ (TNCM-ov) supplemented with 1% PVP-40 for 1 h, and then with TNCM-ov for 2 h to blot the NC membranes. The NC membranes were overlaid either with diluted antibodies in TNCM-ov at room temperature with a suitable concentration of chemokines and growth factors (ie, 25 ng/ml of HGF/SF, 0.5 μg/ml of RANTES, 25 ng/ml of KGF/FGF-7, and 0.5 μg/ml of HCII) in TNCM-ov for 16 h at 4°C. In order to evaluate the effect of ionic strength on the carbohydrate-protein interaction, the NaCl concentration in TNCM-ov to be overlaid was increased stepwise up to 0.35 M. After the incubation, the NC membranes were washed three times with 10 mM Tris-HCl buffer, pH 7.4, containing 20 mM NaCl, 5 mM $CaCl_2$ and 2 mM $MgCl_2$ (TNCM-L) in order to minimize the elution of the bound protein from the ligand oligosaccharides as described previously [5]. Binding signals were detected by overlaying with a monoclonal antibody recognized HGF/SF, RANTES and KGF/FGF-7, at 1 μg/ml in TNCM-L containing 1% OV and 0.05% Tween 20 for 2 h, followed by overlay with HRP-conjugated anti-mouse immunoglobulins. In the case of monoclonal antibodies and antiserum that recognized the non-reducing terminal of chondroitin sulfate and heparin oligosaccharides, we used HRP-conjugated anti-mouse immunoglobulins and HRP-conjugated anti-rabbit immunoglobulins, respectively. The binding signals of antibodies, growth factors, cytokines and anticoagulant factor were visualized by using FAST 3,3′-diaminobenzidine reagent (DAB-FAST, Sigma, Japan) according to the manufacture's instructions.

Antiserum (polyclonal antibody) that recognizes heparin-derived oligosaccharides with unsaturated uronic acid at its non-reducing termini

Polyclonal antibody that recognizes the heparin oligosaccharides having unsaturated uronic acid residues at its nonreducing terminal was produced in a rabbit. In brief, to prepare a synthetic antigen, 1 μmol of the heparin 4-mer fraction obtained from heparinase digests was incubated with 1 mg of Keyhole limpet hemocyanine and 100 μmol of sodium cyanoborohydride at 4°C for 3 weeks. After the reaction mixtures were dialyzed against PBS, one third of it was emulsified with adjuvant and was given to one rabbit with multiple sc injections in the back. This procedure was repeated three times every three weeks before bleeding.

Although the epitopic structure recognized by this antiserum remains to be determined, a preliminary inhibition experiment showed that the epitope structure for this antiserum seemed to be more than a 3-mer in length and was suspected to have the structure of ΔUA(2S)-GlcNS(6S) at the non-reducing terminal, because the antibody binding to the heparin 4-mer NGLs was completely inhibited by the heparin 4-mer, and weakly by a disaccharide of ΔUA(2S)-GlcNS(6S), but not ΔUA(2S)-GlcNS and ΔUA-GlcNS(6S) (data not shown). Since there was some reliable evidence that the heparinase only works to eliminative cleavage both at the α-GlcNS(6S) linkage to IdoA(2S) and α-GlcNS linkage to IdoA(2S) [12], the third sugar from the non-reducing terminal should be either IdoA or the GlcA residue as far as the heparin 4-mer is concerned. Therefore, the epitopic structure is tentatively assigned as ΔUA(2S)-GlcNS(6S)-IdoA or ΔUA(2S)-GlcNS(6S)-GlcA.

Results

Depolymerization of chondroitin sulfates A and C, and DSs by chondroitinase ACI and fractionation of the oligosaccharide fragments

Oligosaccharide fractions were prepared from partially depolymerized chondroitin sulfates A (bovine trachea) and C (shark cartilage), and from completely depolymerized DSs (pig skin and porcine intestinal mucosa) by digestion with chondroitinase ACI followed by gel filtration on Bio-Gel P-10 column. Based on the unsaturated uronic acid content measured by the absorption of 232 nm, the oligosaccharides from skin DS and mucosa DS were found to be iduronic-acid-rich fragments; the treatment with chondroitinase ACI gave about 15%, and about 2% unsaturated uronic acid (ΔUA) of the uronic acid content of untreated pig skin DS and porcine intestinal mucosa DS, respectively. Those values were in good agreement with the uronic acid content reported previously; that is, 7–15% for pig skin DS and 2.3–2.8% for porcine intestinal mucosa DS [13,14]. Therefore, it was deduced that almost all of the oligosaccharides obtained from the exhaustive digestion with chondroitinase ACI were composed of iduronic-acid-rich fragments, and also suggested that all of the internal glucuronic acid residues in DS were cleaved quantitatively in this chondroitinase ACI digestion. In fact, all oligosaccharide fractions derived from DS were completely converted into disaccharides by the treatment with chondroitinase B, which selectively cleaves the GalNAc-IdoA linkage (data not shown). Gel filtration chromatography of the digests on Bio-Gel P-10 revealed predominant disaccharide (2-mer to 6-mer) peaks with a series of well-resolved peaks corresponding to 8-mer to 12-mer (Figure 1), although 8-mer to 12-mer fractions contained not only the same size of GAG oligosaccharides but also some larger and smaller oligosaccharides

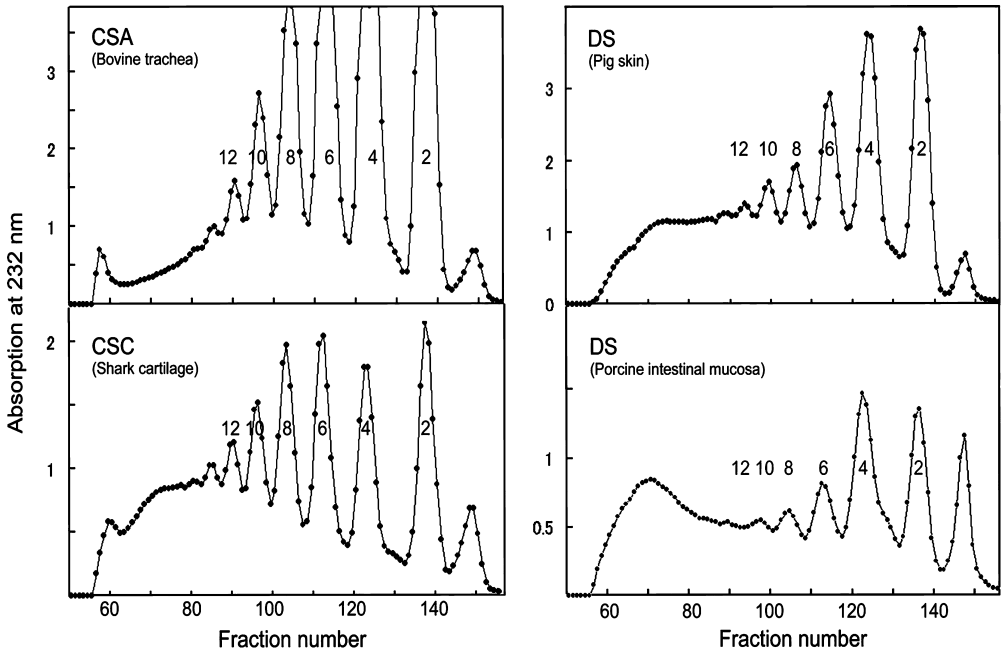

Fig. 1 Gel filtration profiles on Bio-Gel P-10 column of chondroitin sulfates A and C, and two kinds of DSs after treatment with chondroitinase ACI. Chondroitin sulfates A and C were partially digested with chondroitinase ACI and the reaction was stopped at around 40% completion, whereas DSs derived from either pig skin and porcine intestinal mucosa were exhaustively digested, leaving behind the iduronic acid-rich DS oligosaccharides by cleavage at the isolated internal glucuronic acid residues. The resulting oligosaccharides were fractionated on a column of Bio-Gel P-10 in aqueous 10% ethanol containing 1 M NaCl. The oligosaccharide concentration of each fraction was determined by UV absorption at 232 nm. The number of disaccharide units is indicated at each peak. The oligosaccharide populations corresponding to 2mer-12mer were individually collected, freeze-dried and desalted on Sephadex G-10 column.

other than the oligosaccharides indicated as the number of monosaccharides.

Preparation of fluorescent neoglycolipids (NGLs) and oligosaccharide microarray

The individual oligosaccharides corresponding from 2-mer to 12-mer were converted into fluorescent NGLs by conjugating with ADHP according to the method of Stoll *et al.*, [11]. The resulting NGLs were separated from excess ADHP and minor byproducts by HPTLC. To confirm that the major fluorescent bands on TLC plates are the ADHP-conjugated oligosaccharides, immunostainings were performed on HPTLC plates with a mixture of anti-proteoglycan ΔDi4S and ΔDi6S with a ratio of 1:1, which is designated α-ΔCS in this paper. As an example, Figure 2 shows the chromatograms of NGLs synthesized from CSA oligosaccharides, and the immunostaining profiles with the mixed antibodies. The major fluorescent bands that migrated slower than the free ADHP are ADHP-conjugated oligosaccharides, because these bands were in good correspondence to those from the immunostaining experiment. After the ADHP-conjugated oligosaccharides were developed on HPTLC plates, they were extracted from the corresponding bands using a solvent of chloroform/methanol/water (25:25:8, v/v). Then the oligosaccharide microarray was prepared as described previously [5]. First, we evaluated the retention of NGLs printed on the NC membranes during the incubation. For this, the NGLs (2- to 12-mer fractions) were spotted on duplicate sheets. One sheet, on which 10 pmol of fluorescent NGLs were spotted, were subjected to assay conditions similar to those in carbohydrate-binding assays. After that, the fluorescence intensity of each spot was examined under UV lamp at 254 nm. As shown in Figure 3a, every NGL was found to be retained equally on the NC membrane. By scanning these spots in fluorescent mode with a Shimazu CS 9000 scanning densitometer, the amount of retained NGLs on NC membrane was calculated to be about 60% against those of NGLs before the treatment of washing procedure. The values (about 60%) were quite similar to those shown in the case of the non-fluorescent-NGLs (DHPE-conjugated oligosaccharides) printing experiment [5]. Next, we examined another sheet, on which 1 pmol of NGLs per spot was printed, to confirm immunochemically that the NGLs were retained equally on the NC membrane. By overlaying with α-ΔCS, which recognizes the disaccharide structure of unsaturated uronic acid residues linked to either 4-sulfated or 6-sulfated GalNAc at its reducing terminal, every spot gave similar antibody binding signals among the spots apart from the 2-mer fractions in spite of being of different origins (Figure 3b).

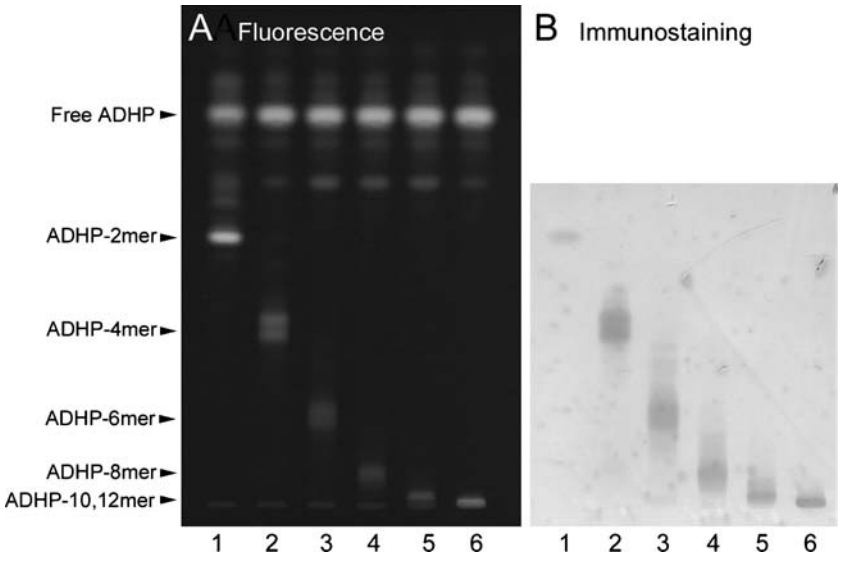

Fig. 2 Thin-layer chromatograms of the ADHP-conjugated chondroitin sulfate A oligosaccharides and immunostaining with antibody mixture,α-ΔCS. (A) Reaction mixtures were subjected to HPTLC in a solvent of chloroform/methanol/0.2% $CaCl_2$ aqueous, 105:100:28 v/v, and visualized under a UV lamp. (B) Immunostaining with α-ΔCS. The position of free ADHP and NGLs (2-mer to 12-mer) are indicated.

Binding of HGF/SF, RANTES, KGF/FGF-7 and heparin cofactor II to oligosaccharides arrayed on the NC membrane

Binding by HGF/SF

At first, (25 ng/ml) of HGF/SF was overlaid onto the arrays with 3% BSA as a blocking agent. Although we observed somewhat stronger binding signals to the DS oligosaccharides of 8-mer to 12-mer than those from CSA and CSC, we could not clearly show the selectivity of the interaction among them (as shown in Figure 3c) in spite of there being definite differences in the carbohydrate structure, such as the position of the sulfate moieties and the presence of iduronic acid residues, as well as in chain length. As the N-domain (hairpin loop) of HGF/SF, which has a helix-extended-helix motif with a cluster of hydrophobic residues and a cluster of positively charged residues considered to be the binding domain of HGF/SF receptor and heparin, the non-specific binding observed in Figure 3c might be attributed to the nature of HGF/SF molecules. Especially, the presence of the Anthracene group on the aglycon portion of ADHP-conjugated oligosaccharides is expected to confer more hydrophobicity on NGLs than those of DHPE-conjugated oligosaccharides. Therefore, we examined several blocking agents (such as casein, skim milk, OV and PVP-40) with nonionic detergents (such as Tween 20), to minimize the non-specific hydrophobic interaction. Finally, the use of PVP-40 and OV plus Tween 20 for the blocking agent was found to be suitable for the assay of the NGLs-HGF/SF interaction. PVP-40 was also known to be a good blocking agent for glycolipids in solid-phase immunostaining studies [15].

After blotting the NGL spotted NC membranes with 10 mM Tris buffer containing 1% PVP-40 and 1% OV plus 0.05% Tween 20, the NC membranes were overlaid with HGF/SF in the above blocking solution except for PVP-40. As shown in Figure 3d, the use of PVP-40, OV and Tween 20 was found to reduce the nonspecific hydrophobic interaction markedly when we compared the signal intensities of 2- and 4-mers between Figure 3c and Figure 3d. The use of PVP-40, OV and Tween 20 enabled us to show that HGF/SF bound preferentially to DS oligosaccharides, especially to the spots that were more than 8-mer, even under the conditions of physiological pH and ionic strength. Therefore, in the following experiments we used 1% PVP-40 and 1% OV plus 0.05% Tween 20 solution as a blocking agent to compare the binding intensities among chondroitin sulfates without regard to whether the proteins to be overlaid had clusters of hydrophobic residues or not.

In order to better distinguish the more strongly positive signals from DS compared with those from CSA and CSC, asterisks are written down at the side of the band that give significantly higher binding signals than those of CSA (lane A) and CSC (lane C) corresponding to DS (lane Bs and Bm).

Next, to discriminate whether the binding signals obtained were ascribed to the intrinsic interaction or merely electrostatic interaction, the ionic strength in the overlay solution was increased stepwise. As shown in Figure 3d to Figure 3f, the increase in ionic strength from 0.15 M up to 0.35 M NaCl revealed that HGF/SF was bound preferentially to DS oligosaccharides with higher affinity than those of corresponding to CSA and CSC, and also shown that the smallest oligosaccharides bound to HGF/SF under 0.35 M NaCl were the 8-mer fractions from both of DS oligosaccharides.

Binding by RANTES

When 0.5 μg/ml of RANTES was overlaid onto the array under physiological ionic strength (Figure 3g), the binding signals were only observed in the spots larger than 8-mer,

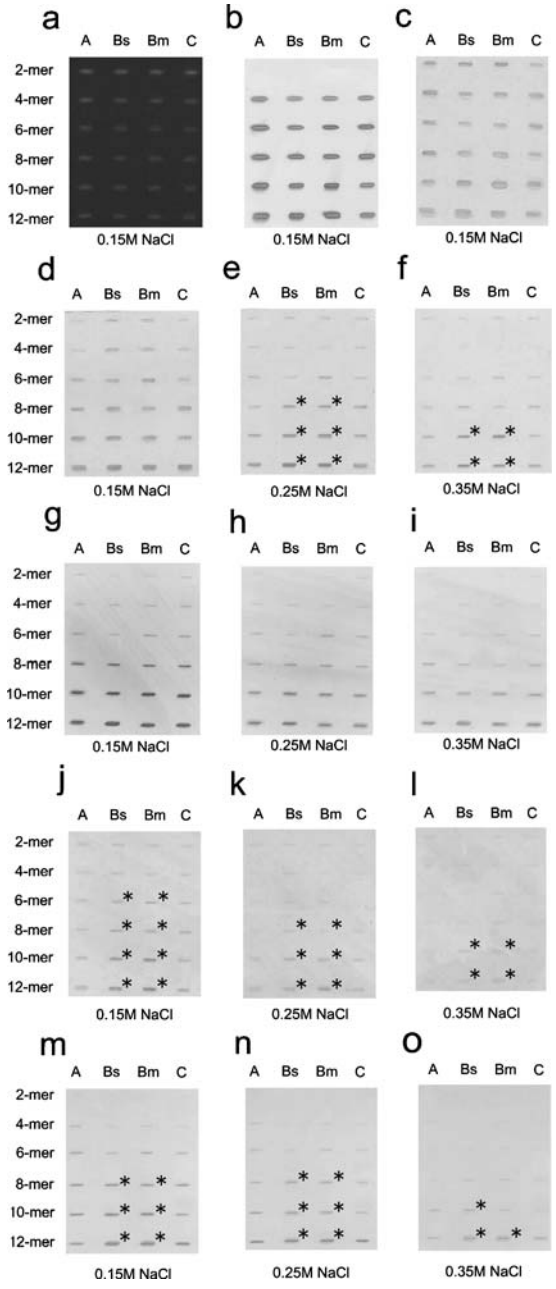

Fig. 3 Detection of epitope or ligand-bearing oligosaccharides on neoglycolipid microarray. ADHP-NGLs of 2-mer to 12-mer oligosaccharides derived from chondroitin sulfate A (lane A), pig skin and intestinal mucosa DSs (lanes Bs, and Bm, respectively) and chondroitin sulfate C (lane C) were printed onto nitrocellulose membrane. (a) The retention of NGLs after the treatment of the washing procedure similar to those in carbohydrate-binding assays was determined by fluorescence. As a high background of NC membranes prevent us from the detecting the fluorescence, 10 pmol of ADHP-NGLs were printed on. (b) Immunostaining with α-ΔCS, (c-f) probing with HGF/SF, (g-I) RANTES, (j-l) KGF/FGF-7, and (m-o) HCII. Printing of ADHP-NGLs was at 10 pmol per spot (a), 1 pmol per spot (b-o). The concentration of NaCl in the overlaying solution is indicated at the bottom of each figure. The spots of DS oligosaccharides to which these ligands bound specifically or intrinsically higher than those of CSA and CSC are marked with an asterisk.

and the binding intensities given by DS oligosaccharides were the same as those of chondroitin sulfates A and C. As shown in Figures 3h and 3i, these binding signals gradually reduced depending on the NaCl concentration. These results indicate that the interaction between these oligosaccharides and RANTES may be due to electrostatic, but not intrinsic, because the binding signals seem to depend merely on the number of sulfate and carboxyl residues, or chain length.

Binding by KGF/FGF-7

It has been shown that DS in human wound fluid can promote FGF-2- and KGF/FGF-7-dependent proliferation of keratinocytes as a physiological relevant cofactor [16,17]. So, we examined the interaction between DS oligosaccharides and KGF/FGF-7. When 25 ng/ml of KGF/FGF-7 was overlaid onto the array under physiological ionic strength, the binding signals observed were slightly weaker than those of HGF/SF, but the spots of DS oligosaccharides with more than 6-mer gave distinct signals towards DS oligosaccharides (Figure 3j). Although the binding intensities of those spots were reduced gradually when the NaCl concentration was raised to 0.25 M and 0.35 M (Figure 3k and 3l, respectively), KGF/FGF-7 was bound preferentially to DS with higher affinity than chondroitin sulfates A and C, and it was also shown that the smallest oligosaccharides bound in the presence of 0.35 M NaCl were the 10-mer (marked with asterisks in Figure 3l). These results indicate that KGF/FGF-7 seems to bind preferentially to DS oligosaccharides, but the interaction between DS oligosaccharide and KGF/FGF-7 is not so strong as that in the case of HGF/SF.

Binding by Heparin cofactor II (HCII)

It has been well known that DS increases the rate of inhibition of thrombin by heparin cofactor II (HCII) approximately 1000-fold by providing a catalytic template to which both the inhibitor and the protease bind [18]. However, the percentage of specific structure for the HCII-binding in pig skin DS, which has been shown to be comprised of hexasaccharides and octasaccharides with clusters of a sulfate in the C2 of iduronic acid and C4 of GalNAc, represents only 5% of the intact DS [10]. Moreover, the purified oligosaccharide fragments were estimated to be 20 times less active than native DS. This evidence have suggested that our microarray assay may be prevented from detection of binding signals due to having a lower amount of HCII-binding sites. At first, 50μg/ml HCII was overlaid onto microarrays, because the concentration of HCII in human serum is known to be around 50 μg/ml. However, 50 μg/ml of HCII overlaid onto the arrays gave nonspecific binding signals that were too high as well as a high background to compare the difference between GAG (data not shown). Then, 0.5 μg/ml of HCII was overlaid

onto the microarray. As shown in Figure 3m, the HCII binding signals were observed in every spot larger than a 6-mer, and the intensity was gradually increased in proportion to the increase in oligosaccharide chain length. However, we could not detect any significant difference in the binding signals among CSA, DSs and CSC, although the oligosaccharides with more than 10-mer both from skin and intestinal DSs gave somewhat stronger binding signals than those of CSA and CSC. When the NaCl concentration was raised to 0.25 M, DS oligosaccharides with more than 8-mer still retained a strong signal, while the binding signals found in those of CSA and CSC decreased under 0.25 M NaCl.

These results not only confirmed previous data that the carbohydrate structure that HCII recognizes are clusters of disulfated disaccharides (especially those having C2-sulfated iduronic acid), but also have shown that this oligosaccharide microarray technique was possible to detect the binding signals, even when less than 1 pmol, because the clusters of disulfated disaccharide content were known to be about one-twentieth of the intact skin and intestinal DS molecules.

Depolymerization of heparin by heparinase and fractionation of the oligosaccharide fragments

In order to compare the binding intensity between DS and heparin, heparin was depolymerized by partial digestion with heparinase and then the resulting oligosaccharides were fractionated by gel filtration on the column of Bio-Gel P-10 (Figure 4). Prior to the preparation of the microarray, the purity of NGLs was confirmed by using anti-heparin antiserum that had been prepared by immunizing rabbits with heparin 4-mer fractions conjugated with Keyhole limpets as an antigen. When oligosaccharide microarrays on which 1 pmol of fluorescent NGLs were spotted were overlaid with anti-heparin antiserum, every spot of heparin oligosaccharides, except for 2-mer gave equal binding signals, but not the spots derived from DSs and chondroitin sulfates (Figure 5c). In contrast to this, α-ΔCS gave no binding signals towards any spot of heparin-NGLs (Figure 5b).

Comparison of HGF/SF, RANTES, KGF/FGF-7 and Heparin cofactor II (HCII) bindings between DS and heparin oligosaccharides

We next examined the binding intensities to heparin-derived NGLs to determine whether the affinities of HGF/SF, RANTES, KGF/FGF-7 and HCII to heparin oligosaccharides differ from those of DS. When we carried out comparative experiments, the NaCl concentration to be overlaid was decided to be the condition that was as high as possible, to eliminate non-specific electrostatic interactions. When HGF/SF was overlaid under 0.35 M NaCl (Figure 5d), the binding to 10-mer and 12-mer of heparin oligosaccharides was somewhat stronger than those of DS with same chain length. The 6-mer to 8-mer of heparin oligosaccharides gave a definite binding signal, although the binding signals towards skin DS oligosaccharides were relatively weak and only found for the bands with more than 8-mer (as similar distinct signals as those seen in Figure 3f). Based on the indistinguishable binding nature among the oligosaccharides of CSA, DS and CSC, the binding them to RANTES appeared to merely be an electrostatic interaction. The comparison with DS and heparin also shows that it might be true. In fact, the oligosaccharides having the same number of negative charges gave similar binding intensities, regardless of the difference in the carbohydrate structure between DS and heparin. As shown in Figure 5e, in the presence of 0.25 M NaCl, the heparin oligosaccharides with more than 4mer gave a more distinctive binding signal than those of the corresponding DS spots, while the intensity of binding signals seemed to correspond to the amount of negative charge. For example, the binding intensities for 6-mer and 8-mer heparin oligosaccharides, which have 12–15 and 16–20 negative charged moieties were similar to those of 8-mer and 12-mer DS oligosaccharides,

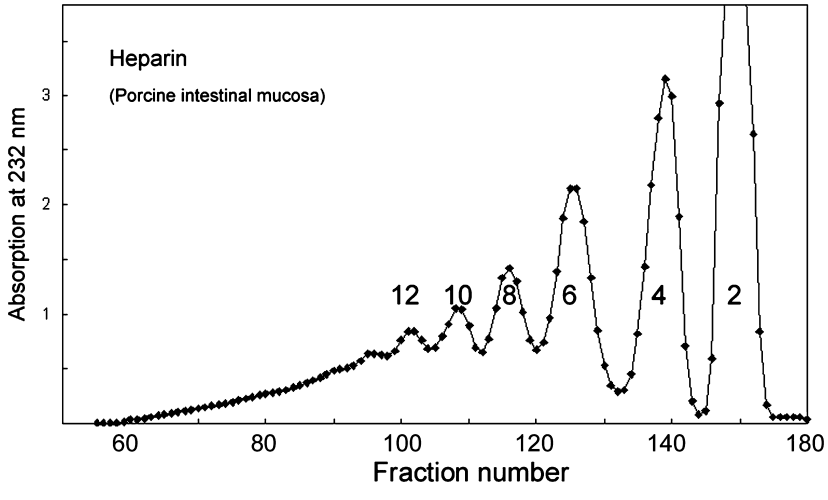

Fig. 4 Size fractionation of heparin oligosaccharides on the column of Bio-Gel P-10. Heparin was partially digested with heparinase at around 40% completion, and the resulting oligosaccharides were fractionated on the Bio-Gel P-10 column. The number of disaccharide units is indicated at each peak. The oligosaccharide populations corresponding to 2mer-12mer were individually collected, freeze-dried and desalted on a Sephadex G-10 column, as described in Figure 1.

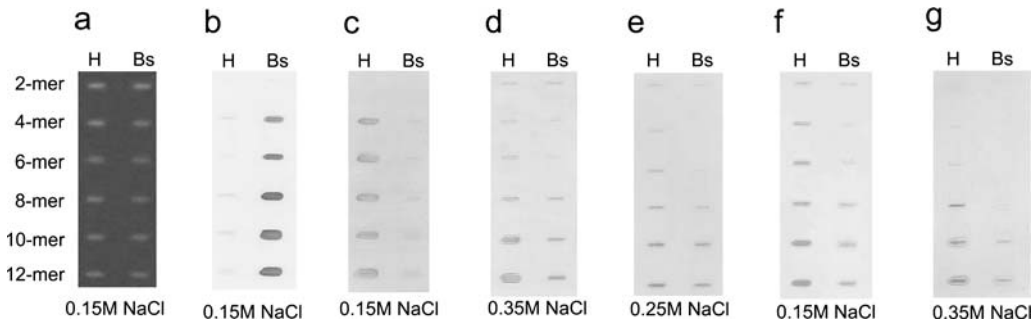

Fig. 5 Comparison of HGF/SF, RANTES, KGF/FGF-7, and heparin cofactor II bindings between pig skin DS and heparin oligosaccharides. ADHP-NGLs of 2-mer to 12-mer oligosaccharides from pig skin DS (lane Bs) and heparin (lane H) printed on to NC membranes for fluorescence detection (a), and for probing with α-ΔCS (b), with anti-heparin antiserum (c), with HGF/SF (d), with RANTES (e), with KGF/FGF-7 (f), and with HCII (g). NGLs were printed at 10 pmol in (a) at 1 pmol in (b-g). The concentration of NaCl in the overlaying solution is indicated at the bottom of each figure.

which have 16 and 24 negative charged moieties, respectively. From the binding experiments of KGF/FGF-7 under 0.15 M NaCl (Figure 5f) and HCII under 0.35 M NaCl (Figure 5g), heparin was also found to have a higher affinity for them.

Discussion

The neoglycolipid technology has been shown to be an extremely useful tool for the detection and characterization of bioactive oligosaccharides that are extremely minor components [4]. The oligosaccharide microarray on which NGLs were immobilized has become a more sensitive assay system than those from affinity chromatography and ordinary ELISA methods, and has also enabled us to achieve the high-throughput detection of ligand carbohydrate, even in heterogeneous glycan populations [5]. Therefore, in this paper, we first tried to show the powerful advantage of oligosaccharide microarray for using several carbohydrate-binding proteins such as growth factors, chemokines, which have been shown to have relatively less affinity than those of antibodies, and secondly to elucidate the specificity of the ligand carbohydrate structure for GAG binding proteins, especially for the proteins that target the continuous iduronic acid containing regions in DS.

The oligosaccharides that play the role of biological function are often believed to be monosaccharides to decasaccharides in size. Therefore, we tried to compare the binding strength with a series of oligosaccharides with unsaturated uronic acid at the nonreducing terminal from chondroitin sulfates A and C, and DS. DS, also known as chondroitin sulfate B, is defined as a chondroitin sulfate due to the presence of *N*-acetylgalactosamine. As mentioned earlier, chondroitinase ACI, which only catalyzes the cleavage of the *N*-acetylgalactosaminide linkage to glucuronic acid found in common chondroitin sulfate chains, gives a series of oligosaccharides rich in internal iduronic acid from DS [8,9]. Moreover, the presence of iduronic acid in DS likens it heparin and heparan sulfates, which also contain this residue. Lyon *et al*. [19] and Bećhard *et al*. [20] have pointed out that charge groups of iduronate carboxyls and hexosamine sulfates in DS might have a similar spatial disposition within the solution conformation of a (-4IdoAα1-3GlcNS(6S)α1-)*n* sequence in HS as they do in a (-4IdoAα1-3GalNAc(4S)α1-)*n* sequence in DS. Recently, there has been the accumulation of evidence that DS chains and/or DS proteoglycans are implicated in diverse biological processes through binding to heparin/heparan sulfate-binding proteins such as growth factors, cytokines, chemokines and blood anticoagulant factors (1).

HGF/SF, one of the plasminogen related growth factor family, is composed of six domains (N-domain or hairpin loop, four Kringle domains and inactive serine protease domain) whose boundaries are clearly defined at the sequence level. The N-domain has a helix-extended-helix motif with a cluster of hydrophobic residues on the helical side of the loop and a cluster of positively charged residues on the extended strand. It has been shown that HGF/SF binds both heparan sulfate and DS, and its interaction with DS occurs through an iduronate-rich octasaccharide comprised of unsulfated iduronate residues in combination with 4-*O*-sulfated *N*-acetylgalactosamine [19,21] . Thus, our present study, using a neoglycolipid microarray not only corroborates their evidence that DS and heparin specifically interact with HGF/SF and the binding to DS is a 10-100-fold weaker interaction than heparin, but also shows it to be one of the useful tools for the detection of a carbohydrate-protein interaction.

In case of RANTES, it has been reported that according to the competition binding assay, heparin most strongly inhibited RANTES binding, but heparan sulfate, DS and chondroitin sulfates A and C had lower inhibition efficiency than heparin; the order of preference was heparin >> chondroitin sulfate C > heparan sulfate > DS > chondroitin sulfate A [22]. It has also been shown that the binding affinities of

RANTES are different among GAG; the affinity for heparin is 24-fold those of DS and chondroitin sulfate A; the order of preference was heparin > heparan sulfate > DS > chondroitin sulfates A or C [23]. In our microarray experiments, however, there was no difference in the order of preference among DS and chondroitin sulfates, whereas heparin was found to be the most effective ligand. Moreover, it appeared to us that the binding of RANTES to GAG might only be dependent on an electrostatic interaction, but not an intrinsic interaction, although the binding of RANTES to GAG is tolerant to 0.35 M NaCl.

KGF/FGF-7 is unique among the FGF family members since it has distinctive target cell specificity and can only bind to the FGF receptor isoform (FGFR2IIIb) on the surface of epithelial cells [24], and is known to promote wound healing through the enhanced proliferation of keratinocytes. Recent studies have shown that the wound fluid-derived DS, as well as a physiological concentration of commercially purified DS, can support KGF/FGF-7-mediated cell proliferation in vitro [1,16], implicating that the effect of DS on FGFR2IIIb receptor activation may be important for wound healing. However, an essential structure involved in DS as well as its minimal DS chain length required for DS-KGF/FGF-7 interaction still remains unknown. In the present studies, we were able to show that KGF/FGF-7 bound preferentially to skin DS oligosaccharides, especially to the spots that were more than 10-mers, even under high ionic strength. This result is in good agreement with a decasaccharides for the smallest size of the activation of KGF [25]. They have recently reported that the minimum size required for activation of FGF-7 was a 10-mer rich in monosulfated (primarily 4-O-sulfated) disaccharide and iduronic acid.

It has been established previously that DS and heparin increases the rate of inhibition of thrombin by HCII about 1000-fold [26]. At first, we did not expect much in the way of getting a distinct binding signal from our microarray, because Maimone and Tollefson [10] had reported that the smallest HCII-binding fragments obtained from pig skin DS were hexasaccharides, and also that hexasaccharides having clusters of disaccharides, such as IdoA(2S)-GalNAc(4S) had the highest affinity binding site for HCII; moreover, this disaccharide comprised only about 5% in intact DS. However, by our microarray oligosaccharide ligands for HCII could be detected even if the amount of oligosaccharides was less than 1 pmol, and also showed that there might be some structural differences in the degree of clustered sulfated iduronic acid residues between the skin and mucosal DSs.

In the present study, we could confirm that our microarray assay was feasible for identifying an intrinsic interaction from an electrostatic interaction, because the binding signals obtained were found to depend on the ionic strength. Particularly in RANTES, the binding depends only on the number of negative charges in ligand oligosaccharides, while the binding intensity towards oligosaccharides was somewhat stronger than any of HGF/SF, KGF/FGF-7 and HCII. As the interaction of RANTES and interferon-γ with their ligands was found to be electrostatic, and they also bound to several sulfated probes including such as sulfated Lewis a and x oligosaccharides and HNK-1 antigen, as well as chondroitin sulfates [5], the characteristic features of RANTES remind us of a mechanism of ligand oligosaccharides for increasing local concentration of the humoral mediators in tissues, protecting it from degradation. Although 0.35 M NaCl was sufficient to distinguish intrinsic interaction from nonspecific electrostatic interaction, the concentration of NaCl might be different depending on the situation.

Another point to emphasize is the importance of the elimination of a non-specific hydrophobic interaction between ligands and the lipid portion of NGLs. BSA, casein and Tween 20 alone did not always act as good blockers, however, in investigating the interaction of ADHP-NGLs with sulfated ligands, the use of OV plus Tween 20 was feasible as a blotting agent to eliminate background due to non-specific hydrophobic interactions.

The use of OV as a blocking agent was found to work well with the GAG recognition system, but we must say that OV is not a versatile blocking agent, and also needs to be evaluated with other recognition systems that recognize N-glycans, because it has been known that OV contains both high mannose-type sugar chains and a hybrid of high mannose-type and hetero-type sugar chains [27,28].

To compare the binding intensities of ligands to DS oligosaccharides with those of heparin oligosaccharides, we prepared a polyclonal antibody that was able to recognize heparin oligosaccharides. As described in the Material and Methods, the precise epitope structure remained unknown. However, Chai *et al*. [29] have recently shown that the tetrasaccharides obtained from partial digestion of porcine intestinal heparin have characteristic structures, such as ΔUA(2S)-GlcNS(6S)-GlcA-GlcNS(6S) and ΔUA(2S)-GlcNS(6S)-IdoA-GlcNS(6S), except for the tetrasaccharides having either GlcNS(6S)-IdoA(2S) or GlcNS-IdoA(2S) linkages in their inside, which are known to be specific cleavage sites of heparinase. As mentioned earlier, the epitope is tentatively assigned as ΔUA(2S)-GlcNS(6S)-IdoA- or ΔUA(2S)-GlcNS(6S)-GlcUA- at the non-reducing terminal.

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References

1. Trowbridge, J.M., Gallo, R.L.: Dermatan sulfate: New functions from old glycosaminoglycan. Glycobiolgy **12**, 117R–25R (2002)
2. Desnoyers, L., Arnott, D., Pennica, D.: WISP-1 binds to decorin and biglycan. J. Biol. Chem. **276**, 47599–607 (2001)
3. Stoll, M.S., Mizuochi, T., Child, R.A., Feizi, T.: Improved procedure for the construction of neoglycolipids having antigenic and lectin-binding acyivities from reducing oligosaccharides. Biochem. J. **256**, 661–4 (1988)
4. Feizi, T., Stoll, M.S., Yuen, C.-T., Chai, W., Lawson, A.M.: Neoglycolipids: probes of oligosaccharide structure, antigenicity and function. Methods Enzymol. **230**, 484–519 (1994)
5. Fukui, S., Feizi, T., Galustian, C., Lawson, A.M., Chai, W.: Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. Nature Biotechnol. **20**, 1011–7 (2002)
6. Feizi, T., Chai, W.: Oligosaccharide microarrays to decipher the glycol code, Nature Reviews. Mol. Cell. Biol. **5**, 582–8 (2004)
7. Feizi, T.: In: Crocker, P.R. (ed.) Mammalian Carbohydrate Recognition Systems, Results and Problems in Cell Differentiation, Vol. 33 pp. 2001–223. Springer-Verlag, Berlin Heidelberg (2001)
8. Fransson, L.-Å., Havsmark, B.: Structure of dermatan sulfate. VII. The copolymeric structure of dermatan sulfate from horse aorta. J. Biol. Chem. **245**, 4770–83 (1970)
9. Habuchi, H., Yamagata, T., Iwata, H., Suzuki, S.: The occurrence of a wide variety of dermatan sulfate-chondroitin sulfate copolymers in fibrous cartilage. J. Biol. Chem. **248**, 6019–28 (1973)
10. Maimone, M.M., Tollefsen, D.M.: Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity. J. Biol. Chem. **265**, 18263–71 (1990)
11. Stoll, M.S., Feizi, T., Loveless, R.W., Chai, W., Lawson, A.M., Yuen, C.-T.: Fluorescent neoglycolipids; Improved probes for oligosaccharide ligand discovery. Eur. J. Biochem. **267**, 1795–804 (2000)
12. Linker, A., Hovingh, P.: Structural studies on heparin. Tetrasaccharides obtained by heparinase degradation. Carbohyd. Res. **127**, 75–94 (1984)
13. Damle, S.P., Coster, L., Gregory, D.J.: Proteodermatan sulfate isolated from pig skin. J. Biol. Chem. **257**, 5523–7 (1982)
14. Sudo, M., Sato, K., Chaidedgumjorn, A., Toyoda, H., Toida, T., Imanari, T.: ^{1}H nuclear magnetic resonance spectroscopic analysis for determination of glucuronic and iduronic acids in dermatan sulfate, heparin and heparan sulfate. Analytical Biochem. **297**, 42–51 (2001)
15. Haycock, J.W.: Polyvinylpyrrolidone as a blocking agent in immunochemical studies. Analytical Biochem. **208**, 397–9 (1993)
16. Penc, S.F., Pomahac, B., Winkler, T., Dorschner, R.A., Eriksson, E., Herndoni, M., Gallo, R.L.: Dermatan Sulfate Released after Injury Is a Potent Promoter of Fibroblast Growth Factor-2 Function. J. Biol. Chem. **273**, 28116–21 (1998)
17. Trowbridge, J.M., Rudisill, J.A., Ron, D., Gallo, R.L.: Dermatan sulfate binds and potentiates activity of keratinocyte growth factor (FGF-7). J. Biol. Chem. **277**, 42815–20 (2002)
18. Liaw, P.C.Y., Becker, D.L., Stafford, A.R., Fredenburgh, J.C., Weitz, J.I.: Molecular basis for the susceptibility of fibrin-bound thrombin to inactivation by heparin cofactor II in the presence of dermatan sulfate but not heparin. J. Biol. Chem. **276**, 20959–65 (2001)
19. Lyon, M., Deakin, J.A., Rahmonune, H., Fernig, D.G., Nakamura, T. Gallagher: Hepatocyte growth factor/scatter factor binds with high affinity to dermatan sulfate. J. Biol. Chem. **273**, 271–8 (1998)
20. Bećhard, D., Gentina, T., Delehedde, M., Scherpereel, A., Lyon, M., Aumercier, M., Vazeux, R., Richet, C., Degand, P., Jude, B., Janin, A., Fernig, D.G., Tonnel, A.-B., Lassalle, P.: Endocan is a novel chondroitin sulfate/dermatan sulfate proteoglycan that promotes hepatocyte growth factor/scatter factor mitogenic activity. J. Biol. Chem. **276**, 48341–9 (2001)
21. Lyon, M., Deakin, J.A., Mizuno, K., Nakamura, T. Gallagher: Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. J. Biol. Chem. **269**, 11216–23 (1994)
22. Proudfoot, A.E.I., Fritchley, S., Borlat, F., Shaw, J.P., Vilbois, F., Zwahlen, C., Trkola, A., Marchant, D., Clapham, P.R., Wells, T.N.C.: The BBXB motif of RANTES is the principal site for heparin binding and controls receptor selectivity. J. Biol. Chem. **276**, 10620–6 (2001)
23. Martin, L., Blanpain, C., Garnier, P., Wittamer, V., Parmentier, M., Vita, C.: Structural and functional analysis of the RANTES-glycosaminoglycans interactions. Biochem. **40**, 6303–18 (2001)
24. Finch, P., Cunha, G., Rubin, J., Wong, J., Ron, D.: Pattern of keratinocyte growth factor and keratinocyte growth factor receptor expression during mouse fetal development suggests a role mediating morphogenetic mesenchymal-epithelial interactions. Develop. Dyn. **203**, 223–40 (1995)
25. Taylor, K.R., Rudisill, J.A., Gallo, R.L.: Structural and sequence motifs in dermatan sulfate for promoting fibroblast growth factor-2 (FGF-2) and FGF-7 activity. J. Biol. Chem. **280**, 5300–306 (2004)
26. Tollefson, D.M., Pestka, C.A., Monafo, W.J.: Activation of heparin cofactor II by dermatan sulfate. J. Biol. Chem. **258**, 6713–6 (1983)
27. Tai, T., Yamashita, K., Ito, S., Kobata, A.: Structures of the carbohydrate moiety of OV glycopeptide III and the difference in specificity of endo-β-N-acetylglucosaminidase CII and H. J. Biol. Chem. **252**, 6687–94 (1977)
28. Yamashita, K., Tachibana, Y., Kobata, A.: The structure of the galactose-containing sugar chains of OV. J. Biol. Chem. **253**, 3862–9 (1978)
29. Chai, W., Hounsell, E.F., Bauer, C.J., Lawson, A.M.: Characterization by LSI-MS and 1H NMR spectroscopy of tetra-, hexa-, and octa-saccharides of porcine intestinal heparin. Carbohydrate Res. **269**, 139–56 (1995)