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Carbohydrate Structures of Recombinant Soluble Lamp-1 and Leukosialin Containing Sialyl Le^x Terminus

Shinobu Sueyoshi, Ritsuko Sawada and Minoru Fukuda*

The Glycobiology Program, La Jolla Cancer Research Foundation, La Jolla, CA 92037 U.S.A. and Department of Biochemistry, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Abstract—Recombinant soluble lamp-1 and soluble leukosialin can be produced from CHO cells which express sialyl Le^x structures after stable transfection of fucosyltransferase-III. It was shown previously that those soluble lamp-1 and leukosialin are potent inhibitors for *E*-selectin-mediated adhesion of human colonic tumor cells (Sawada, R.; Tsuboi, S.; Fukuda, M. *J. Biol. Chem.*, **1994**, *269*, 1425). In the present study, we have determined the amount of the sialyl Le^x structure present in recombinant, soluble lamp-1 and soluble leukosialin. CHO cells were metabolically labeled with [^{3}H]-galactose and recombinant soluble lamp-1 and leukosialin were purified from the spent medium. Glycopeptides containing *N*-glycans derived from lamp-1 were fractionated by sequential lectin affinity chromatography. Similarly, *O*-glycans released from leukosialin were fractionated by Bio-Gel P-4 gel filtration. The terminal structures of carbohydrate chains were determined by sequential digestion with specific glycosidases. The results clearly indicate that soluble lamp-1 contains much more sialyl Le^x structure than soluble leukosialin. Considering that soluble leukosialin and lamp-1 are almost equally effective as inhibitors for *E*-selectin-mediated adhesion, the results strongly suggest that densely clustered *O*-glycans are better presenters for *E*-selectin ligands than *N*-glycans.

Introduction

Cell surface carbohydrates are often characteristic of cell types.[1,2] One of those structures, sialyl Le^x, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→R, was found in granulocytes and monocytes.[3,4] In addition, Le^x structure Galβ1→4(Fucα1→3)GlcNAcβ1→R was found to be enriched in these leukocytes.[5] These characteristic structures can be detected by monoclonal antibodies which specifically bind to each structure. It was also discovered that a unique structure, NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→R, is enriched in leukocytes of patients with chronic myelogenous leukemia.[6] This structure was later found to be recognized by VIM-2 monoclonal antibody.[7]

It has been shown recently that sialyl Le^x structure is the ligand for *E*- and *P*-selectin.[8–12] During inflammation, *E*- and *P*-selectin newly appear on the cell surface of endothelia and they bind sialyl Le^x structures present on granulocytes and monocytes. It was shown that this initial binding, 'rolling effect' leads into extravasation of leukocytes, establishing the recruitment of leukocytes at inflammatory sites.[13,14]

Tumor cells, in particular, carcinoma cells were shown to contain an increased amount of sialyl Le^x structures.[15–17] Carcinoma cells are also enriched with the sialyl Le^a terminus,[18] NeuNAcα2→3Galβ1→3(Fucα1→4)-GlcNAcβ1→R, which is also the ligand for *E*-selectin.[19,20] It was shown that these tumor cells bind to *E*-selectin.[21,22] Moreover, it was demonstrated recently that highly metastatic tumor cells adhere more strongly to *E*-selectin and such adhesion can be inhibited by soluble lamp-1 and leukosialin which were produced from Chinese hamster ovary cells expressing sialyl Le^x structures.[23,24]

CHO cells do not express a proper fucosyltransferase which forms sialyl Le^x structures.[8] In order to make CHO cells express sialyl Le^x structures, they were transfected with α1–3/4 fucosyl transferase, Fuc-TIII.[25] In this background, CHO cells were further transfected to stably express soluble lamp-1 or soluble leukosialin.[23,24]

In the present study, we have analyzed the terminal structures of carbohydrates attached to these soluble lamp-1 and leukosialin, which were found to be potent inhibitors for *E*-selectin-mediated adhesion. Based on the structures determined, possible roles of interaction between multiple ligands and *E*-selectin are discussed.

Results

Fractionation of glycopeptides obtained from soluble lamp-1

Soluble lamp-1 was purified from [^{3}H]-galactose labeled CHO cells which express sialyl Le^x (Fig. 1A). As a control, soluble lamp-1 was purified from control CHO cells which do not express sialyl Le^x, due to the absence of Fuc-TIII. The glycopeptides, prepared by pronase digestion of the purified proteins, were first fractionated by ConA-Sepharose (Figs 2A and 2C). The glycopeptides unbound to ConA–Sepharose (Fraction I) were then further fractionated by DSA–Sepharose as shown in Figure 2, B and D. The relative proportions of IA, IB and IC did not differ significantly between CHO cells expressing sialyl Le^x and those not expressing sialyl Le^x (Table 1).

*Correspondence should be addressed to: Dr Minoru Fukuda, Department of Biochemistry, Institute of Medical Science, University of Tokyo, 108 Tokyo, Japan.

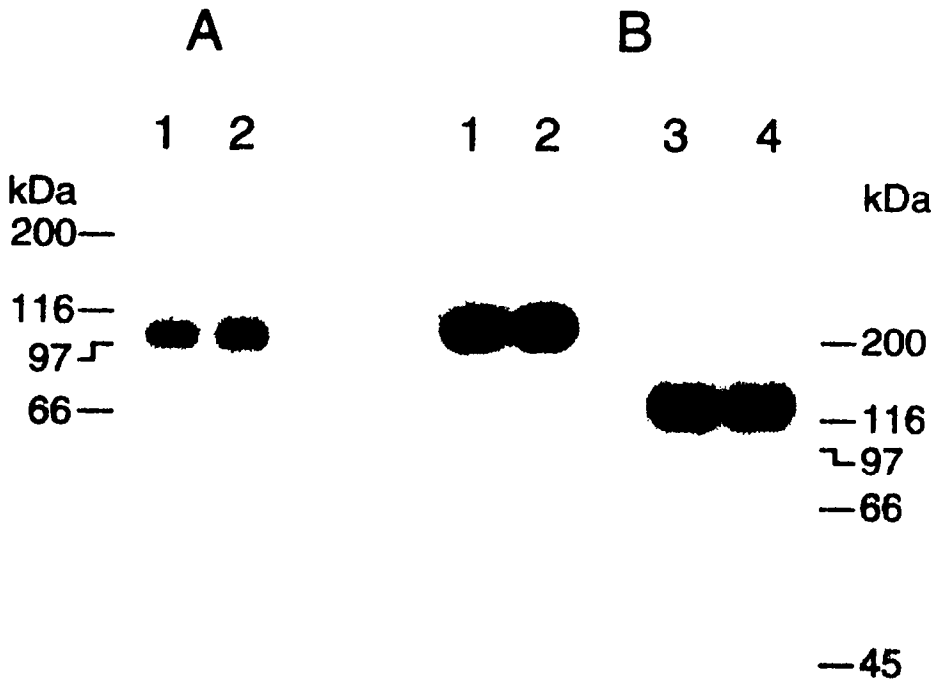

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified recombinant lamp-1 and leukosialin. A, Recombinant soluble lamp-1 was purified by wheat germ agglutinin–Sepharose followed by QAE–Sephadex column chromatography. The column was first eluted by 0.2 M NaCl, then by 0.4 M NaCl. Lanes 1 and 2 represent samples eluted in 0.4 M NaCl and 0.2 M NaCl, respectively. For carbohydrate analysis, the sample shown in lane 2 was used since the majority of the soluble lamp-1 was recovered after 0.2 M NaCl elution. B, Recombinant soluble leukosialin-IgG hinge-constant chimeric protein was purified from control CHO (lanes 1 and 3) and CHO/FT-III (lanes 2 and 4) cells, as described in the Experimental Procedures. The purified sample was subjected to 8 % polyacrylamide gel electrophoresis before (lanes 1 and 2) and after reduction (lanes 3 and 4) with 2-mercaptoethanol, followed by fluorography as described. [24]

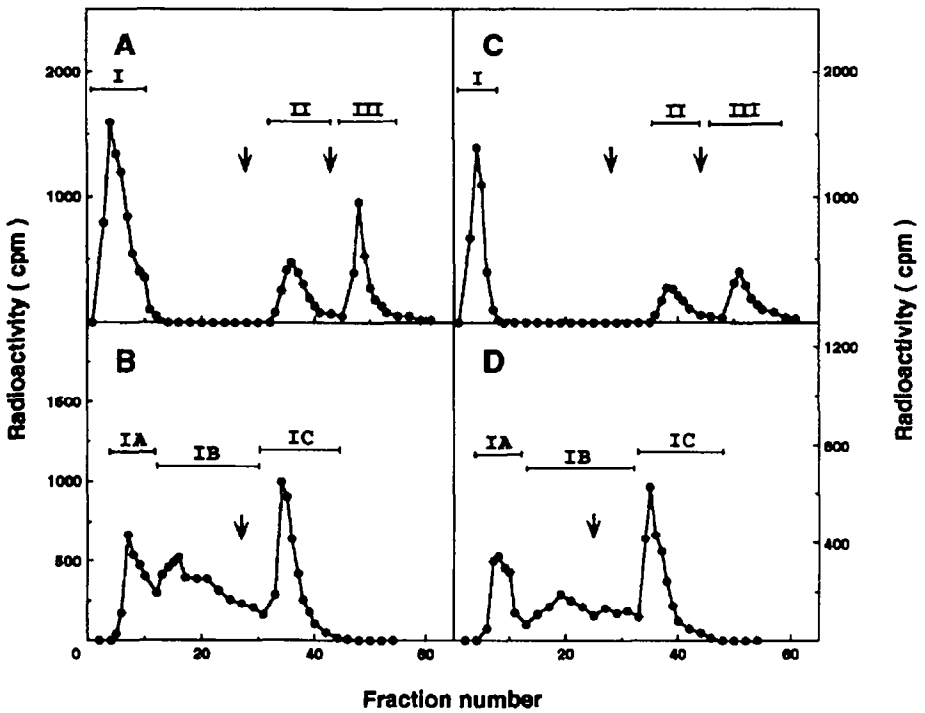

Figure 2. Lectin affinity chromatography of [^{3}H]-galactose-labeled glycopeptides from recombinant soluble lamp-1. Recombinant soluble lamp-1 was purified from the spent medium of control CHO and CHO/FT-III cells and glycopeptides were prepared by pronase digestion. The radiolabeled glycopeptides were sequentially applied to ConA–Sepharose (A and C) and DSA–Sepharose (B and D), as described in the Experimental Procedures. Haptenic sugars were added at the points indicated by arrows within graphs. In ConA–Sepharose chromatography, 5 mM followed by 0.2 M methyl α-mannoside was used as haptenic sugars. In DSA–Sepharose chromatography, a mixture of tri-*N*-acetylchitotriose and tetra-*N*-acetylchitotetrase (5 mg/mL) was used. A and B, soluble lamp-1 from control CHO cells; C and D, soluble lamp-1 from CHO/FT-III.

Table 1. Relative proportions of terminal structures present in recombinant soluble lamp-1

	Galβ1→4GlcNAcβ1→R	Galβ1→4GlcNAcβ1→R (3 ← Fucα1)	NeuNAcα2→3Galβ1→4GlcNAcβ1→R	NeuNAcα2→3Galβ1→4GlcNAcβ1→R (3 ← Fucα1)
CHO/FT-III				
IA (22.5%)[a]	7.9%	1.6%	5.8%	7.2%
IB (38.3%)	3.2%	0	30.1%	5.0%
IC (39.2%)	16.7%	0	22.5%	0
CHO				
IA (21.4%)	3.5%	0	17.9%	0
IB (46.9%)	2.9%	0	44.0%	0
IC (31.7%)	11.5%	0	20.2%	0

[a]The number denotes a relative proportion among 1A, 1B and 1C fractions.

Le^x and sialyl Le^x content of soluble lamp-1

It was shown previously that β-galactosidase can cleave the β-galactosyl linkage only after α-fucose is removed from Galβ1→4(Fucα1→3)GlcNAcβ1→R structure.[4,5,26] It was also shown that α-fucosidase can cleave Galβ1→4(Fucα1→3)GlcNAcβ1→R and Galβ1→3(Fucα1→4)GlcNAcβ1→R but not NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→R or NeuNAcα2→3Galβ1→3(Fucα1→4)GlcNAcβ1→R (Fig. 3).[27,28] It is thus possible to estimate the amount of Le^x structure by digestion with β-galactosidase before and after treatment of α-fucosidase. Moreover, it is possible to estimate the amount of sialyl Le^x since it can be digested only after sialic acid is removed. The results obtained by sequential digestion are summarized in Table 1. The results show that more than 12 % of the terminal structure is sialyl Le^x while Le^x is present in 1.6 % of the terminal structure. It is also noteworthy that the majority of sialyl Le^x structures are in the glycopeptides which were not bound to DSA–Sepharose (Fraction IA and IB).

Structure of O-glycans isolated from soluble leukosialin

Figure 1B shows the leukosialin samples examined by SDS–polyacrylamide gel electrophoresis. As expected, the recombinant proteins behaved as a dimer in the absence of reducing agent (Fig. 1B, lanes 1 and 2). These samples were first digested by pronase and the resulting glycopeptides were applied to a column of Sephadex G-50. The glycopeptides with high molecular weights were then subjected to alkaline-borohydride treatment and released *O*-linked oligosaccharides were separated on the same Sephadex G-50 gel filtration as described previously.[29] *O*-Glycans were then applied to QAE–Sephadex column chromatography and neutral and sialic-acid containing oligosaccharides were separated.

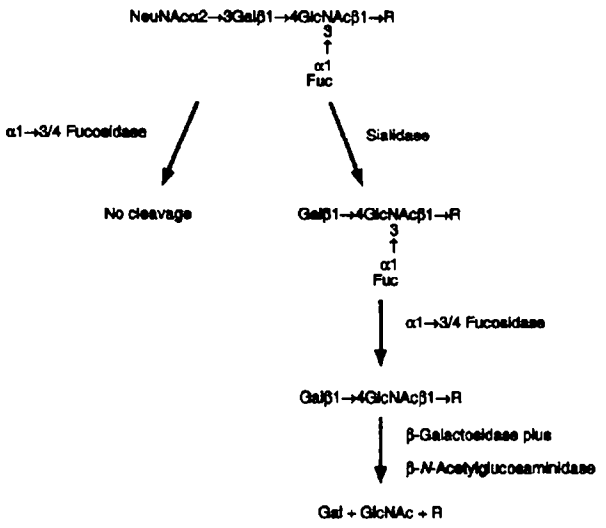

Figure 3. Scheme for sequential glycosidase treatment. α-Fucosidase scarcely cleaves intact, sialylated oligosaccharides while it can cleave efficiently after desialylation.[27,27] 8β-Galactosidase and β-*N*-acetylglucosaminidase can hydrolyze only after fucose is removed.

Figures 4A and D show the elution profiles of neutral sugars from both control CHO and CHO/FT-III cells. In both samples, Galβ1→3(Galβ1→4GlcNAcβ1→6)GalNAcOH and Galβ1→3GalNAcOH are the major oligosaccharides (peaks b and a).

Sialylated *O*-glycans were first desialylated by Newcastle disease virus sialidase and then resultant neutral oligosaccharides were subjected to Bio-Gel P-4 gel filtration (Fig. 4B, C, E and F). Sequential glycosidase treatment was carried out in a similar fashion and the amount of fucosylated and non-fucosylated *O*-glycans was determined. In order to determine if fucosylated oligosaccharides are also present in oligosaccharide fractions containing poly-*N*-acetyllactosaminyl *O*-glycans, sequential glycosidase digestion with or without fucosidase was carried out on oligosaccharides eluted in fractions 42–52 and fractions 53–60 (Fig. 4E). When the oligosaccharides eluted in fractions 42–52 were sequentially digested by α-fucosidase, β-galactosidase, and β-*N*-acetylglucosaminidase, the elution position of the resultant oligosaccharide shifted about one and a half *N*-acetylglucosamine units. The resultant oligosaccharides were converted to the oligosaccharide b after one more sequential digestion with β-galactosidase and β-*N*-acetylglucosaminidase. The oligosaccharide eluted at the oligosaccharide b was then converted to Galβ1→3GalNAcOH after digestion with the glycosidases. Considering that poly-*N*-acetyllactosamine extension can take place only in the presence of core 2 β-1,6-*N*-acetylglucosaminyltransferase,[29] the core structure of this oligosaccharide was judged to be Galβ1→3(Galβ1→4GlcNAcβ1→6)GalNAcOH. The oligosaccharides eluted in fractions 53–60 were similarly subjected to structural analysis. The results, summarized in Table 2, show that a very small proportion (at most 0.9 %) of the oligosaccharides contain sialyl Le^x termini. However, we could not detect polylactosaminyl *O*-glycans in the corresponding oligosaccharides from the other samples (see Table 2).

Discussion

In the present study, we determined the amount of sialyl Le^x, Le^x and *N*-acetyllactosaminyl terminal structures in both soluble lamp-1 and leukosialin. Soluble lamp-1 and leukosialin were produced in CHO cells which were transfected to stably express Fuc-TIII. In addition, soluble leukosialin was produced in the presence of core 2

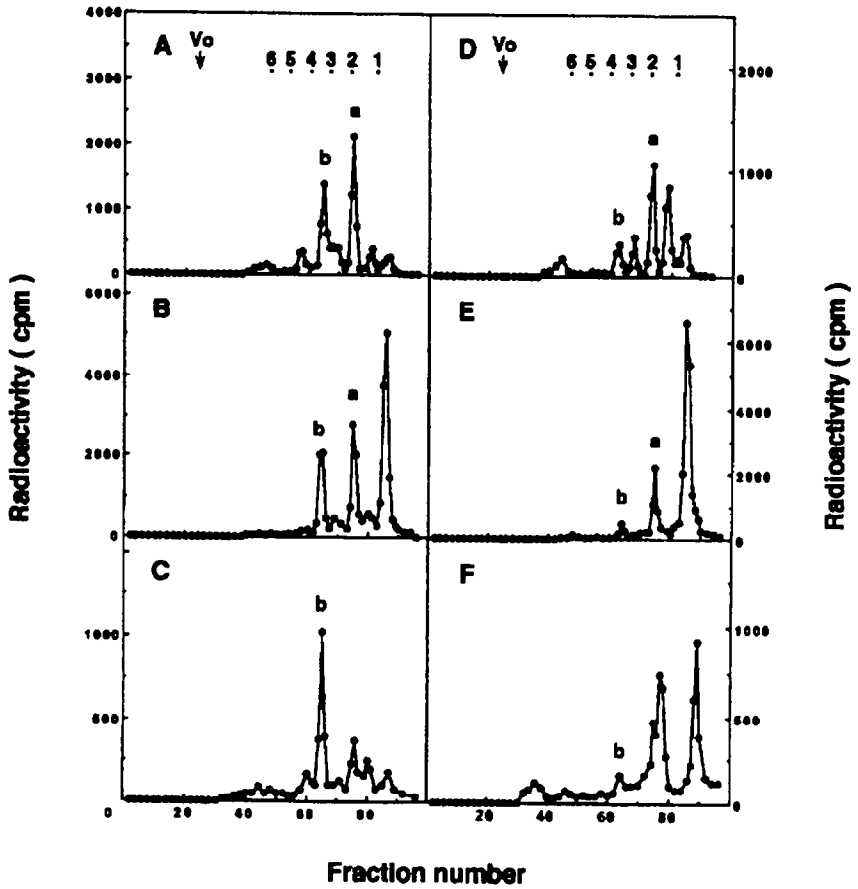

Figure 4. Gel permeation chromatography of *O*-linked oligosaccharides released from [^{3}H]-galactose-labeled recombinant leukosialin. Neutral *O*-glycans (A and D) from recombinant leukosialin synthesized in control CHO cells (A) and CHO/FT-III cells (D) were subjected to Bio-Gel P-4 gel permeation chromatography. Monosialosyl oligosaccharides (B and E) and disialosyl oligosaccharides (C and F) were treated with sialidase and then subjected to Bio-Gel P-4 gel permeation chromatography. A, B, C, *O*-glycans from control CHO cells; D, E, F, *O*-glycans from CHO/FT-III cells. The elution of standard oligosaccharides are: a, Galβ1→3GalNAcOH; b, Galβ1→3(Galβ1→4GlcNAcβ1→6)GalNAcOH. The majority of other peaks were due to contaminants. 1–6 denotes the elution positions of *N*-acetylglucosamine and its dimer (di-*N*-acetylchitobiose), trimer, tetramer, pentamer and hexamer.

Table 2. The relative proportion of O-linked oligosaccharides present in recombinant soluble leukosialin

Oligosaccharides	CHO/FT-III	CHO
NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1 3 ↑ Fucα1 ; 6 ↘ Galβ1→3GalNAcOH	0.4%	0
Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1 3 ↑ Fucα1 ; 6 ↘ NeuNAcα2→3Galβ1→3GalNAcOH		
NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1 6 ↘ Galβ1→3GalNAcOH	1.4%	0
Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1 6 ↘ NeuNAcα2→3Galβ1→3GalNAcOH		
NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1 3 ↑ Fucα1 ; 6 ↘ Galβ1→3GalNAcOH	0.5%	0
Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1 3 ↑ Fucα1 ; 6 ↘ NeuNAcα2→3Galβ1→3GalNAcOH		
NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GalNAcβ1 6 ↘ Galβ1→3GalNAcOH	0.9%	0.4%
Galβ1→4GlcNAcβ1→3Galβ1→4GalNAcβ1 6 ↘ NeuNAcα2→3Galβ1→3GalNAcOH		
NeuNAcα2→3Galβ1→4GlcNAcβ1 6 ↘ NeuNAcα2→3Galβ1→3GalNAcOH	2.5%	1.4%
NeuNAcα2→3Galβ1→4GalNAcβ1 6 ↘ Galβ1→3GalNAcOH	8.5%	11.4%
Galβ1→4GlcNAcβ1 6 ↘ NeuNAcα2→3Galβ1→3GalNacOH		
Galβ1→4GlcNAcβ1 6 ↘ Galβ1→3GalNacOH	11.2%	30.7%
NeuNAcα2 6 ↘ NeuNAcα2→3Galβ1→3GalNAcOH	0	0.4%
NeuNAcα2→3Galβ1→3GalNAcOH	41.0%	15.7%
Galβ1→3GalNAcOH	33.6%	40.0%

branchings, directed by stable expression of core 2 β-1,6-*N*-acetylglucosaminyltransferase. It was shown previously that CHO cells stably expressing Fuc-TIII are capable of expressing sialyl Le^x structures by demonstration of the reactivity with anti-sialyl Le^x antibody.[12,22] Moreover, it was demonstrated that core 2 branchings are necessary to express sialyl Le^x structures in *O*-glycans.[28,29] It was shown previously that human T-cell activation is associated with the appearance of core 2 branchings.[30] The recent results demonstrated that activated T-lymphocytes

acquire sialyl Le^x.[31] These combined results thus strongly suggest that activated T-lymphocytes acquire sialyl Le^x structure due to the expression of the core 2 branchings.

The present study revealed that the sialyl Le^x terminal structure is abundantly present in *N*-glycans of soluble lamp-1 and constitutes 12.2 % of the total terminal structures (Table 1). In contrast, the amount of sialyl Le^x structure present in *O*-glycans of soluble leukosialin was found to be extremely low (Table 2). This result is consistent with the fact that leukosialin from HL-60 contains only a small amount (less than 1 % of the total terminal structure) of sialyl Le^x structure.[28] This difference in the amount of sialyl Le^x structure between *N*-glycans and *O*-glycans is most likely due to the difference in the amount of poly-*N*-acetyllactosaminyl extension between *N*- and *O*-glycans. As shown previously, *N*-glycans from lamp-1 molecules contain a significant amount of poly-*N*-acetyllactosamines.[33] The present study revealed that in contrast only 3.2 % of leukosialin *O*-glycans contain poly-*N*-acetyllactosaminyl extension. The latter result is consistent with that obtained in HL-60 leukosialin, where only 3 % of the total *O*-glycans were found to contain poly-*N*-acetyllactosaminyl extension.[28]

It can be calculated that soluble leukosialin contains less than 0.7 moles of sialyl Le^x terminus while soluble lamp-1 contains about 3.5 moles of sialyl Le^x, considering that each leukosialin molecule contains approximately 80 *O*-glycans[32], and each lamp-1 molecule contains 12 *N*-glycans having three or four side chains.[33] However, the inhibitory capacity of soluble leukosialin and soluble lamp-1 in *E*-selectin-mediated adhesion is almost indistinguishable at high concentrations.[24] This suggests that *O*-glycans are better presenters for *E*-selectin ligands than *N*-glycans. *O*-Glycans attached to mucin-type glycoproteins such as leukosialin are present as densely-clustered oligosaccharides.[32] There are more chances when two neighboring *O*-glycans simultaneously contain the sialyl Le^x structure. In *N*-glycans, in contrast, there are only three or four side chains which are close to each other, suggesting that chances of having sialyl Le^x structures in the vicinity are rare. Once *E*-selectin binds to one oligosaccharide, it is likely that this binding is not so strong considering that *E*-selectin is monovalent. Binding of *E*-selectin to an oligosaccharide is dynamic and association and dissocation most likely take place in a short time. If additional ligand is present next to the ligand, there is more chance that *E*-selectin still binds to the oligosaccharide because this additional structure is close to the first one which was bound in the first place. This is most likely the reason why soluble leukosialin inhibits *E*-selectin-mediated binding as efficient as the soluble lamp-1. In fact, it was recently shown that the ligand for *P*-selectin is carried by a mucin-type glycoprotein with ~M_r 120,000.[34,35] Moreover, it was found that two major glycoproteins carrying L-selectin were found to be GlyCAM-1 and CD34, and both of these glycoproteins are mucin-like glycoproteins containing highly clustered *O*-glycans[36,37]. Further studies will be of significance to determine how much multivalent oligosaccharides bind stronger to selectins than monovalent oligosaccharides.

Experimental Procedures

Expression of soluble lamp-1 in CHO cells

To produce soluble lamp-1 molecules that display sialyl Le^x determinants, CHO cells were first co-transfected by the lipofectin procedure with pcDNAI–FucTIII and pHyg in a 10:1 molar ratio, as described previously.[23] CHO cell lines, obtained after limiting dilution, were tested for expression of sialyl Le^x by immunofluorescence and those positive cells were chosen as described.[23] pcDNAI–FucTIII[25] was kindly provided by Dr John Lowe, Howard Hughes Medical Institute, University of Michigan.

A clonal cell line stably expressing sialyl Le^x structures, termed CHO/FT-III was then cotransfected with pSV2dhfr and pSRα s-lamp-1. pSRα s-lamp-1 encodes soluble lamp-1 by introducing a stop codon right before the transmembrane domain.[23] The transfected cells were selected in α-MEM without nucleotides for 14 days, and the cells were then propagated for gene amplification by increasing concentrations of methotrexate (final concentration of 0.5 μM).

Expression of soluble lamp-1 was determined by immunoblotting of the conditioned medium as described.[23] One cell line that produced an abundant amount of soluble lamp-1 was chosen for further study. As control, parent CHO cells which were not transfected to express Fuc-T–III were made to express soluble lamp-1 in a similar way.

Expression of soluble leukosialin in CHO cells

A CHO cell line stably expressing core 2 β-1,6-*N*-acetylglucosaminyltransferase and the membrane form of leukosialin was established as described.[29] This cell line, CHO-leu•C2GnT was transfected with pSV2dhfr and pSRα•s-leu•IgG as described.[24] pSRα•s-leu•IgG encodes a chimeric protein of a soluble leukosialin and IgG1 hinge-constant region sequence and was prepared as described previously.[24] After the transfection, the cells were cultured in α-MEM without nucleotides and then propagated with increasing concentrations of methotrexate as described above.

The supernatant of each clonal cell line obtained by limiting dilution was tested for the presence of the leukosialin-IgG chimera using goat anti-human IgG-peroxidase conjugate. The cell line expressing the highest amount of soluble leukosialin-IgG chimera was chosen and this was used as the source for control leukosialin.

A clonal cell line stably expressing soluble leukosialin, established in the above experiments, was transfected by the lipofectin procedure with pcDNAI–FucTIII and pHyg and selected in the presence of hygromycin as described.[24] A cell line expressing both soluble leukosialin and sialyl Le^x antigen, assessed for its expression by immunofluorescence, was chosen for the production of the soluble leukosialin-IgG chimera.

Purification of soluble lamp-1 and soluble chimeric leukosialin-IgG protein from the conditioned medium of CHO cells

The CHO cells expressing soluble lamp-1 or chimeric leukosialin-IgG protein were cultured in α-MEM supplemented with 10 % fetal calf serum and 0.5 mM methotrexate (Bethesda Research Laboratories). After the cells reached subconfluence, they were metabolically labeled with [^{3}H]-galactose as described previously.[33]

The soluble lamp-1 was purified from the conditioned medium by wheat germ agglutinin–Sepharose followed by DEAE–Sephadex A-50, as described previously.[23] The soluble lamp-1 was bound and eluted from the columns in both chromatographies.

The soluble chimeric leukosialin-IgG protein was purified by directly applying the conditioned medium to a column of protein A–Sepharose. The bound protein was eluted with 0.1 M glycine–HCl buffer, pH 3.8, and the eluted fractions (1 mL) were immediately neutralized with 225 μL of 1.5 M tris, pH 8.8.[24]

Fractionation of glycopeptides derived from soluble lamp-1

In order to analyze the carbohydrate moieties attached to soluble lamp-1, the cells were metabolically labeled with [^{3}H]-galactose and the soluble lamp-1 was purified as described above. The purified lamp-1 was then digested with pronase and resultant glycopeptides were fractionated by sequential affinity chromatography employing concanavalin A (ConA)–Sepharose and *Datura stramonium* agglutinin (DSA)–Sepharose as described previously.[33] By this procedure, *N*-glycans enriched with Le^x or sialyl Le^x structures were not bound to ConA–Sepharose (Fraction I), separating from bi-antennary complex type *N*-glycans (Fraction II) and high-mannose type oligosaccharides (Fraction III). Fraction I was further separated into those unbound to DSA–Sepharose (IA), those retarded from DSA–Sepharose (IB), and those bound and eluted from DSA–Sepharose (IC).

Fractionation of O-glycans attached to leukosialin

[^{3}H]-galactose-labeled leukosialin was digested with pronase and glycopeptides were recovered after Sephadex G-50 gel filtration as described.[29,38] *O*-Linked oligosaccharides were released by alkaline borohydride treatment (0.1 M NaOH–1 M $NaBH_4$ at 45 °C for 20 h). Released *O*-glycans, separated on Sephadex G-50 gel filtration, were then fractionated by a column (0.5 × 4.0 cm) of QAE–Sephadex A-25 equilibriated in water.[39] Neutral oligosaccharides were then fractionated by Bio-Gel P-4 gel permeation chromatography. Acidic oligosaccharides, which were bound to the QAE–Sephadex column, were desialylated and then subjected to Bio-Gel P-4 gel permeation chromatography.

Bio-Gel P-4 (400 mesh) was packed in two water jacketed stainless steel columns (0.8 cm × 50 cm for each column) and two columns were consecutively connected to the HPLC apparatus of a Gilson 306 manometric module pump with Beckman 163 Variable Wavelength Detector. The columns were equilibrated with water warmed at 55 °C and the flow rate was 15 mL/h, collecting 0.5 mL in each fraction.[39] The column was calibrated by *N*-acetylglucosamine and its oligomers which were detected by the absorption at 210 nm as described.[39] Once we established the elution positions of the oligosaccharides, they were fractionated by the same column chromatography but having a minimum amount of *N*-acetylglucosamine oligomers. The fractions containing the oligosaccharide were pooled, desalted by gel filtration in a small column of Sephadex G-25, eluted with water, and then subjected to glycosidase treatment.

Glycosidase treatment

Radiolabeled oligosaccharides or glycopeptides were digested with 40 milliunits of Newcastle disease virus neuraminidase in 80 μL of 50 mM cacodylate buffer, pH 6.5 at 37 °C for 24 h.[28] It has been shown that Newcastle disease virus sialidase can cleave α-2,3-linked sialic acid but barely cleaves the other linkages.[40] The oligosaccharides or glycopeptides were digested sequentially by diplococcal 15 milliunits of β-galactosidase and 20 milliunits of β-*N*-acetylglucos-aminidase[28] before or after treatment of 20 milliunits of bovine kidney α-fucosidase in 100 μL of 50 mM sodium acetate–phosphate buffer, pH 5.5 for 24 h. As shown previously, diplococcal β-galactosidase specifically cleaves Galβ1→4 GlcNAc linkage.[41] In order to inhibit potentially contaminating glycosidase activities, the digestion was carried out in the presence of either fucose (10 mM) plus *N*-acetylglucosamine (20 mM) or fucose (10 mM) plus γ-galactonolactone (20 mM) as inhibitors. γ-Galactonolactose was purchased from Sigma.

After desalting by applying to a column (0.5 × 4 cm) of AG50W-X8/AG1-X8, the sample was applied to the same Bio-Gel P-4 columns as described above. In order to confirm that fucose is linked through α1→3 or α1→4, α1→3/4 specific fucosidase from *Streptomyces* sp. 142[27] was used in some of the experiments as described previously.[28] Newcastle disease virus sialidase was prepared in our laboratory while the other glycosidases were purchased from Boehringer–Mannheim except for *Streptomyces* fucosidase, which was purchased from Takara.

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