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# Novel glycoproteins inhibiting the binding of colorectal cancer cells to E-selectin

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**Novel glycoproteins carrying sialyl-LeA (SLeA) antigens (SL-GP) were isolated from ascites fluid from a patient with colorectal cancer by immunoaffinity chromatography. Their characteristics, including binding capacity to E-selectin, were investigated. SL-GP showed a typical mucin type amino acid composition in which Ser, Thr and Pro together accounted for greater than 50% of the total amino acid residues. A large amount of carbohydrate (about 80%) was present in SL-GP. The number of O-glycans carrying SLeA antigens comprised about 9% of the total number of O-glycosidic chains. SL-GP could bind to IL-1$\beta$ treated HUVEC, and the binding was inhibited by anti-E-selectin and anti-SLeA monoclonal antibodies. The binding of colorectal cancer cells, LS 180, to HUVEC was assayed in the presence of SL-GP, oligosaccharides prepared from SL-GP and human milk SLeA hexasaccharide. SL-GP inhibited the binding most effectively, whereas equivalent amounts of the SL-GP oligosaccharides and milk SLeA hexasaccharide inhibited it only slightly. These results constitute direct evidence that a unique arrangement of SLeA antigens on the polypeptide chain, probably a cluster, is essential for the binding to E-selectin.**

***Keywords*: E-selectin, sialyl-LeA, mucin-glycoprotein, HUVEC, ascites fluid**

## Introduction

E-selectin is a member of the family of adhesion molecules classified as the selectins. Although many reports have suggested that carbohydrate moieties, SLeX and its isomer, sialyl-LeA (SLeA), can function as ligands for E-selectin, their physiological relevance remains to be established [1–3]. It is generally agreed that monovalent carbohydrate ligands do not exhibit high affinity in carbohydrate-protein interactions [4]. We have also demonstrated that some monoclonal antibodies are strongly reactive only with clustered antigenic oligosaccharides. For instance, the epitopic structure for the anti-Tn antibody comprises three or four consecutive sequences of GalNAc-Ser/Thr. The clustering of relatively common GalNAc-Ser/Thr could lead to the formation of an uncommon structure exhibiting antigenicity [5, 6]. Similar observations were made for the interaction of selectins with SLeX tetrasaccharides, considerably high concentrations (μM–mM) being required for even partial inhibition of the binding compared to the effective inhibition by a clustered carbohydrate patch [7–11].

In the present work, we isolated and characterized a mucin carrying SLeA antigens (SL-GP), from ascites fluid from a cancer patient. Adhesion of colorectal cancer cells to HUVEC was examined in the presence of SL-GP, and equivalent amounts of oligosaccharides prepared from SL-GP by alkaline treatment and SLeA hexasaccharide isolated from human milk. The adhesion was inhibited by a much smaller amount of SL-GP compared to the oligosaccharides. This suggests that a cluster arrangement of SLeA oligosaccharides on the polypeptide chain is essential for the adhesion.

## Materials and methods

### Materials

Ascites fluid from a patient with colorectal cancer was kindly provided by Dr S. Fushiki, Kyoto Prefectural University of Medicine. Murine monoclonal antibodies, MSW 113, MLS 102 and MLS 128, which recognize the SLeA, sialyl-Tn and Tn antigens, respectively, were established as described previously [12–14]. Anti-E-selectin antibody used was a monoclonal antibody which was produced by a H 18/7 clone established by Bevilacqua *et al.* [15] and which was purchased from Nippon Becton Dickinson, Tokyo. Anti-P-selectin antibody used was a monoclonal antibody which was established by Katayama *et al.* [16] and which was purchased from Takara Shuzo, Otsu. HUVEC and IL-1$\beta$ were purchased from, Kurabo, Tokyo, and Genzyme, Cambridge, respectively.

#### *Purification of SL-GP from ascites fluid*

Lyophilized ascites fluid was dissolved in 25 mM Tris-HCl buffer (pH 7.5) 0.1 M NaCl, 5 mM EDTA and 0.5 mM PMSF, and then centrifuged at 15 000 × g for 20 min. The

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supernatant was subjected to Sepharose 6B column chromatography. The excluded fraction was collected and the proteins were precipitated with acetone. The precipitate, after being dissolved in 25 mM phosphate buffer (pH 7.5) was applied to an immunoaffinity column of MSW 113 $F(ab')_2$ coupled to Sepharose CL-4B. After extensive washing with the same buffer, glycoproteins expressing SLeA were eluted with 50 mM diethylamine (pH 11.5). The eluate was dialysed against water, reduced and then carboxymethylated according to Klug *et al.* [17]. The glycoprotein was further purified by gel filtration on TSK gel G-4000$SW_{XL}$ in the presence of 4 M guanidine-HCl.

*Dot blot analysis*

SL-GP was loaded on a nylon membrane and then left standing at room temperature for 15 min. After blocking with 5% BSA-PBS, the membrane was incubated with a monoclonal antibody (10 $\mu g\,ml^{-1}$) at 4 °C for 20 h and then with $^{125}$I-labelled protein A at room temperature for 2 h, successively. After the incubation, the membrane was washed with PBS, followed by autoradiography.

*Analysis of amino acids and amino sugars*

SL-GP was hydrolyzed in 6 M HCl at 110 °C for 24 h, and in 6 M HCl at 100 °C for 16 h, for amino acids and amino sugars, respectively. The hydrolysates were analysed with a Hitachi L-8500 amino acid analyser.

*Carbohydrate analysis*

SL-GP was hydrolyzed in 2.5 M trifluoroacetic acid at 100 °C for 16 h. After re-*N*-acetylation, the sugars were pyridylaminated according to the method of Suzuki *et al.* [18], and then analysed with TSK gel Sugar AXI. Sialic acid was determined by the method of Hara *et al.* [19].

*Alkaline borohydride treatment of SL-GP and fractionation of the released O-glycans*

SL-GP was treated with 0.05 M NaOH-$^3$H-labelled $NaBH_4$ at 45 °C for 16 h according to Carlson [20]. The released O-glycans were fractionated on Sephadex G-50 ($1.0 \times 107$ cm) and then the radioactivities were determined. The elution positions of SLeA antigens were determined by measuring the radioactivity immunoprecipitated from each fraction with MSW 113.

*Binding of $^{125}$I-labelled SL-GP to HUVEC in the presence of various inhibitors*

HUVEC ($\sim 1 \times 10^4$ cells), which had been stimulated with IL-1$\beta$ (1 $ng\,ml^{-1}$) for 4 h, and non-stimulated HUVEC were washed with 25 mM Hepes buffer (pH 7.3) 0.15 M NaCl, 5 mM EDTA, and then with 25 mM Hepes buffer (pH 7.3) 0.15 M NaCl, successively. Then the cells were incubated with $^{125}$I-labelled SL-GP (10 ng protein) at 4 °C for 40 min in 50 µl of the same buffer plus 5 mM $CaCl_2$. After the incubation, the cells were extensively washed with the incubation solution and then the bound radioactivities were determined. As antibodies, MSW 113, anti-E-selectin and anti-P-selectin, were used.

It should be noted that under the conditions used P-selectin was not detected on HUVEC.

*Binding of LS 180 cells, a human colorectal carcinoma cell line, to HUVEC in the presence of various inhibitors*

IL-1$\beta$ stimulated HUVEC ( $\sim 3 \times 10^3$ cells) were incubated with various amounts of inhibitors at 4 °C for 30 min, and then LS 180 cells ($\sim 3 \times 10^5$ cells) were added. The final volume was adjusted to 50 µl per well. After incubation at 4 °C for 30 min, the wells were sealed, turned upside down and centrifuged at 150 rpm for 5 min, and then the number of bound cells was determined.

*Other methods*

SDS-PAGE was carried out according to the method of Laemmli [21]. $F(ab')_2$ fragments were prepared by treatment of the native antibodies with pepsin and purified by gel filtration on Sephadex G-50. The fragments were coupled to BrCN-activated Sepharose CL-4B. SL-GP was labelled with $^{125}$I-NaI according to Hunter [22].

Oligosaccharides were prepared from SL-GP by alkaline-borohydride treatment according to the method of Carlson [20]. SLeA hexasaccharide was isolated from human milk as described previously [12].

## Results

### Isolation of Mucin-type Glycoproteins carrying SL-GP from Cancerous Ascites Fluid

Gel filtration on Sepharose 6B was carried out to separate mucin-type glycoproteins of high molecular weight from many serum glycoproteins and proteins. The bulk of the glycoproteins and proteins was eluted in the included fractions. We examined the distribution of the SLeA antigens in each fraction by dot blot analysis. Although the included fractions contained considerable amounts of antigens, we did not examine these fractions further because of the difficulty in excluding non-antigenic proteins completely. Thus, we used the excluded fraction for further purification. The key step in the purification was immunoaffinity column chromatography. Since mucins having multiantigenic sites on their polypeptide chains bind strongly to antibodies due to avidity, they are eluted with difficulty from an immunoaffinity column containing native immunoglobulins with a commonly used elution buffer such as diethylamine (pH 11.5) or glycine-HCl buffer (pH 2.3) and the yield is usually very low. By using $F(ab')_2$ fragments instead of native monoclonal antibodies as the immunoadsorbent, mucin-type glycoproteins carrying SLeA antigens, designated as SL-GP, could be isolated in relatively high yields. The eluate was reduced and carboxymethylated as described under Materials and Methods. To exclude some copurified contaminating proteins and monoclonal antibody fragments,

which had leaked out from the affinity column, the reduced and carboxymethylated eluate was subjected to TSK gel G4000SW$_{XL}$ column chromatography. The molecular mass of SL-GP eluted close to the void fraction was estimated roughly to be 3000 kDa. The purity was examined by SDS-PAGE, followed by both carbohydrate and protein staining (Fig. 1, lanes A, B). SL-GP apparently remained near the top of a 5% running gel. No protein could be detected on Coomassie Brilliant Blue (CBB) staining, even when an excess amount of protein was applied. The purity was further assessed by autoradiography by running the radioiodinated SL-GP (Fig. 1, lane C). In general, mucins are iodinated so poorly that if there are any contaminating non-mucin proteins, they are readily iodinated and detected on autoradiography. SL-GP gave diffuse bands on SDS-PAGE corresponding to different molecular masses, probably due to their polymorphism, but no non-antigenic glycoproteins were apparently detected. Specific activity was determined based on the radioactivity of $^{125}$I-protein A bound to the antigen-antibody complex per μg protein on a dot blot. The purified SL-GP showed about 8000-fold purification over the ascites fluid, and about 60 μg protein of SL-GP was obtained from 1 litre of the ascites fluid.

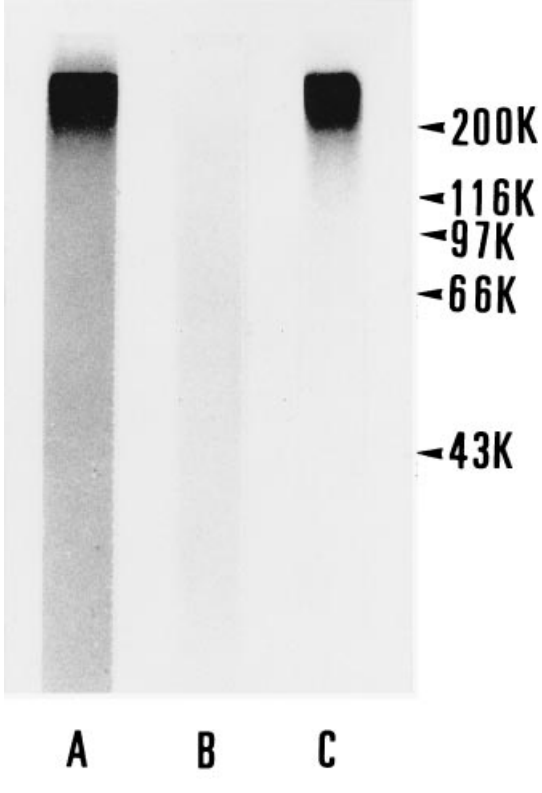


**Figure 1.** SDS-PAGE of SL-GP. SL-GP was subjected to SDS-PAGE, followed by staining with PAS (A) and CBB (B). SL-GP was radioiodinated by the chloramine T method and then subjected to SDS-PAGE, followed by autoradiography (C).

*Amino acid and carbohydrate compositions*

As shown in Table 1, the amino acid composition of SL-GP was typical of mucins, containing large amounts of Ser, Thr and Pro. These amino acids together accounted for more than 50% of the amino acids. These characteristics and the amino acid composition closely resembled those of mucins from LS 174T colon cancer cells [23] and SW 1990 pancreatic cancer cells [24], which are coded for by the MUC 2 gene [25]. The carbohydrate composition, expressed as the molar ratio of sugar residues versus GalNAc, is shown in Table 1. The presence of both Man and GalNAc suggests that both N- and O-linked oligosaccharides occur on the mucin. SL-GP contained as much as 80% carbohydrate by weight.

## O-glycans of SL-GP

The O-glycans of SL-GP were released and labelled by alkaline-$^{3}$H-borohydride treatment. The released O-glycans were fractionated on Sephadex G-50. Most of the radioactivity was recovered in relatively low molecular weight fractions, suggesting the predominant presence of incompletely synthesized carbohydrate chains. The SLeA oligosaccharides were immunoprecipitated from each fraction and the radioactivities were determined. As shown in Fig. 2, the SLeA oligosaccharides were distributed in a wide molecular size range, including in a fraction of considerably high molecular weight. Dot blot analysis showed that SL-GP carried sialyl Tn and Tn antigens in addition to SLeA antigens (Fig. 3).

## Binding of $^{125}$I-labelled SL-GP to HUVEC

IL-1$\beta$ treated or non-treated HUVEC were incubated with $^{125}$I-labelled SL-GP in the presence of various inhibitors. As shown in Fig. 4, the binding was effectively inhibited by

**Table 1.** Amino acid and carbohydrate compositions of SL-GP

| | | | |
|---|---|---|---|
| Asp | 4.4 | GalNAc | 1.0 |
| Thr | 25.8 | GlcNAc | 1.2 |
| Ser | 14.3 | Man | 0.3 |
| Glu | 6.0 | Fuc | 0.6 |
| Gly | 6.5 | Gal | 1.5 |
| Ala | 6.7 | SA | 1.4 |
| Val | 4.8 | | (mol/mol GalNAc) |
| Met | 0.7 | | |
| Ile | 3.5 | | |
| Leu | 4.3 | | |
| Tyr | 1.2 | | |
| Phe | 2.3 | | |
| Lys | 1.8 | | |
| His | 2.4 | | |
| Arg | 1.5 | | |
| Pro | 12.7 | | |
| | (mol/100 mol) | | |

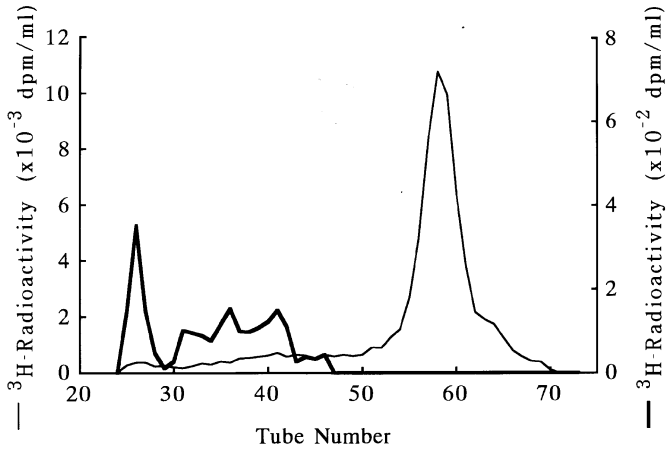


**Figure 2.** Gel filtration of O-glycans prepared from SL-GP on Sephadex G-50. SL-GP was treated with 0.05 M NaOH-$^{3}$H-$NaBH_4$ as described under Materials and Methods. The released O-glycans were applied to Sephadex G-50 (1.0 × 107 cm) and fractions of 1 ml were collected. The SLeA antigens were immunoprecipitated with MSW 113 from each fraction and the radioactivities were determined. —— total radioactivity, ▬ radioactivity of the SLeA antigen.

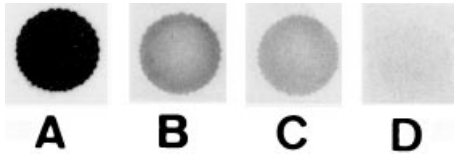


**Figure 3.** Coexpression of carbohydrate antigens on SL-GP. The carbohydrate antigens of SL-GP were examined by dot blot analysis. Three antigens were detected on successive incubations with a monoclonal antibody and $^{125}$I-protein A, followed by autoradiography. (A) SLeA antigen; (B) sialyl-Tn antigen; (C) Tn antigen; (D) control.

the MSW 113 and anti-E-selectin antibody, but not by the anti-P-selectin antibody, indicating that E-selectin expressed on HUVEC and SLeA antigens on SL-GP were involved in the binding. Binding of SL-GP to untreated HUVEC was about 10% of the maximum binding to treated HUVEC.

### Binding of colorectal cancer cells, LS 180, to HUVEC in the presence of SL-GP, oligosaccharides from SL-GP and SLeA hexasaccharide from human milk

The binding was E-selectin dependent since more than 90% of the binding was inhibited by anti E-selectin antibody, whereas antibodies against VCAM-1 and ICAM-1 had no significant effect.

More than 50% of the binding was inhibited by SL-GP at an amount corresponding to 10 ng of sialic acid (Figs 5 and 6). In contrast, only slight inhibition was observed with equivalent amounts of oligosaccharides prepared from SL-GP and human milk SLeA hexasaccharide (Figs 7

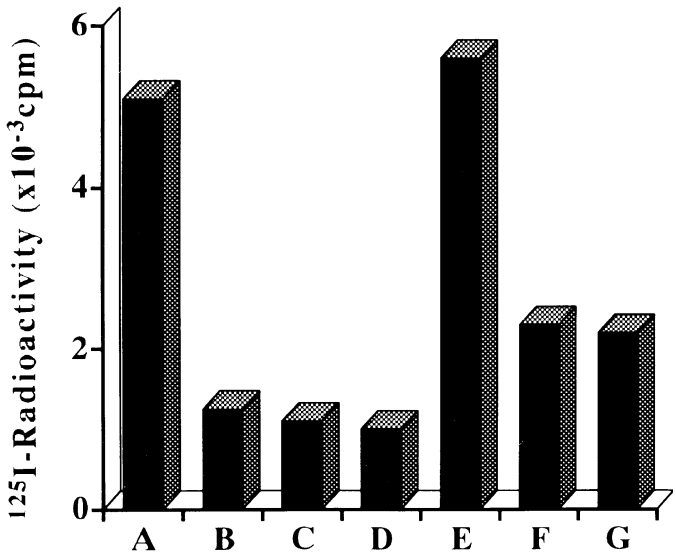


**Figure 4.** Binding of $^{125}$I-labelled SL-GP to HUVEC. IL-1$\beta$-treated and non-treated HUVEC were incubated with $^{125}$I-labelled SL-GP in the presence of various inhibitors at 4 °C for 40 min. After extensive washing with 25 mM HePES, (pH 7.3) – 0.15 M NaCl – 5 mM $CaCl_2$, the bound radio-activities were determined. The volume of the incubation mixture was 50 µl. Inhibitors, (A) none, (B) EDTA (5 mM), (C) MSW 113 (2 µg), (D) MSW 113 (5 µg), (E) anti P-selectin (5 µg), (F) anti E-selectin (2 µg), (G) anti E-selectin (5 µg).

and 8). It appeared that a cluster arrangement of SLeA antigens on the polypeptide chain of SL-GP is required for the inhibition. Treatment of SL-GP with N-glycanase had no effect on the binding of SL-GP to HUVEC.

## Discussion

It has been shown that the ligands for the selectin family are oligosaccharides bearing terminal SLeA or SLeX structures or ones carrying sulfate groups instead of sialic acids [2, 3]. However, not all glycoproteins carrying these antigens bind to selectins. It has been argued that clustered O-linked carbohydrates may be involved in the interaction of glycoproteins with selectins, mainly based on the fact that treatment with a mucin-specific protease abolished the ligand activity [10, 11, 26].

To characterize a mucin with ligand activity for selectins in detail, we have tried to isolate mucin-type glycoproteins carrying SLeA antigens. The key step was purification on an immunoaffinity column. Generally, carbohydrate antigens on mucins form multiantigenic sites which seem to remarkably strengthen the antigen-antibody reaction due to avidity. Therefore, it is difficult to elute a mucin from an immunoaffinity column with commonly used elution media. We tried to use a $F(ab')_2$ fragment prepared from MSW 113 as an immunoadsorbent with success. Since the fragment exhibited modest affinity for mucins carrying SLeA

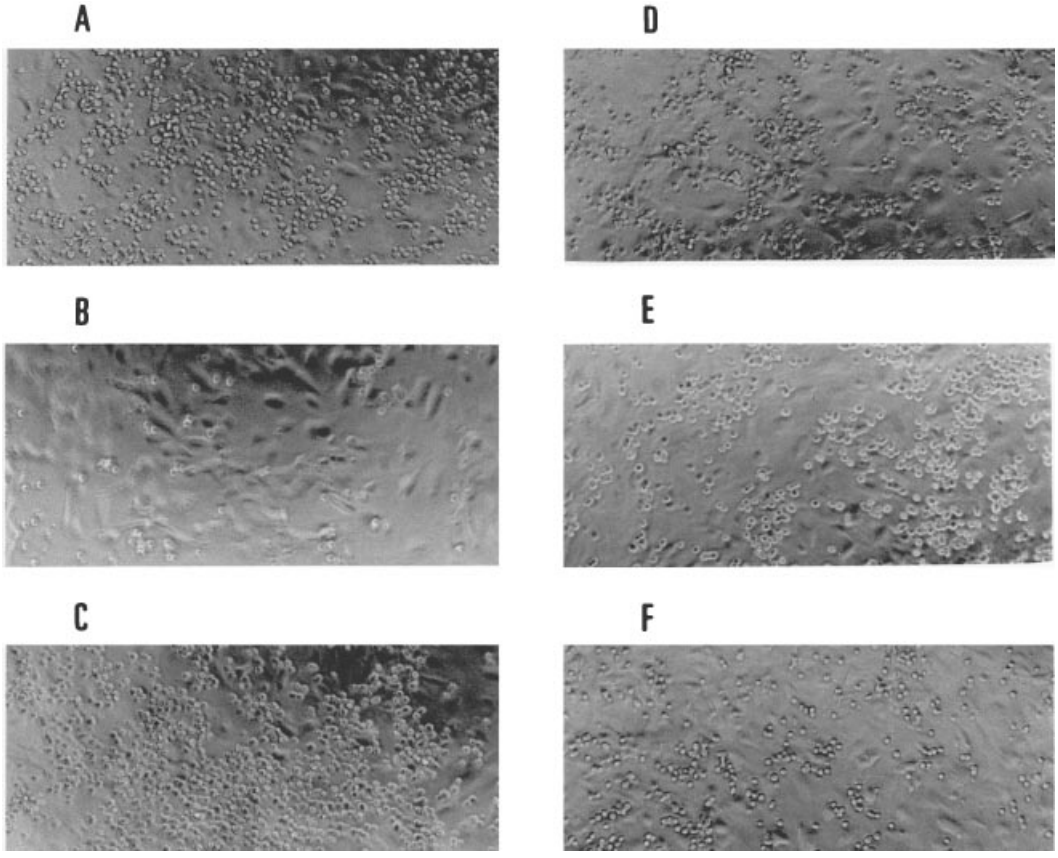


**Figure 5.** Binding of LS 180 cells to HUVEC in the presence of SL-GP. IL-1$\beta$ treated or non-treated HUVEC were incubated with LS 180 cells in the presence of SL-GP as described under Materials and Methods. The amounts of inhibitors are expressed as those of sialic acid present in SL-GP. Unbound cells were removed by centrifugation. HUVEC, except for in B, were treated with IL-1$\beta$ for 4 h. (A) no inhibitor; (B) no inhibitor; (C–F) SL-GP corresponding to 1, 5, 10 and 30 ng sialic acid, respectively.

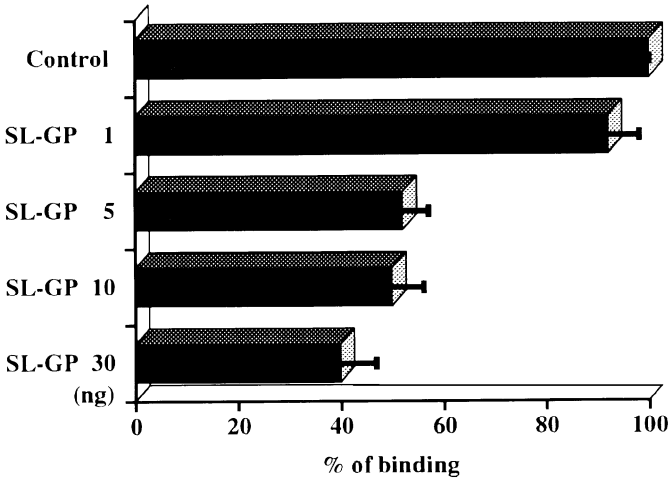


**Figure 6.** Inhibition of binding of LS 180 cells to HUVEC. The data in Fig. 5 were converted into histograms of per cent binding (mean $\pm$ SEM), defined as (experimental binding – background)/(maximum binding – background). The background binding (to non-treated HUVEC) was about 10% of the maximum binding.

antigens, the mucin was readily eluted from the column in a high yield.

In general, the purity of a mucin is tricky to assess. The purity of our preparation was shown by staining with CBB and a periodate-Schiff reagent (PAS) after SDS-PAGE. It was further confirmed by autoradiography of $^{125}$I-labelled SL-GP. From the results, it was inferred that proteins without SLeA antigens were excluded from our preparation.

In an attempt to characterize the polypeptide moiety of SL-GP, $^{125}$I-labelled SL-GP was treated with trimethane sulfonic acid under various conditions, and the products were subjected to SDS-PAGE. However, no discrete band attributable to the polypeptide moiety was detected.

The amino acid composition was characterized by high contents ($\sim$ 50%) of Ser, Thr and Pro. The ratio of Ser/Thr (1.8) was similar to that of the products of the MUC 2 gene, rather than to that of MUC 1 (1.1) [27]. The O-glycans of SL-GP were composed of many short carbohydrate chains and there were relatively small amounts of SLeA antigens varying in molecular size. The molecular sizes of some of the

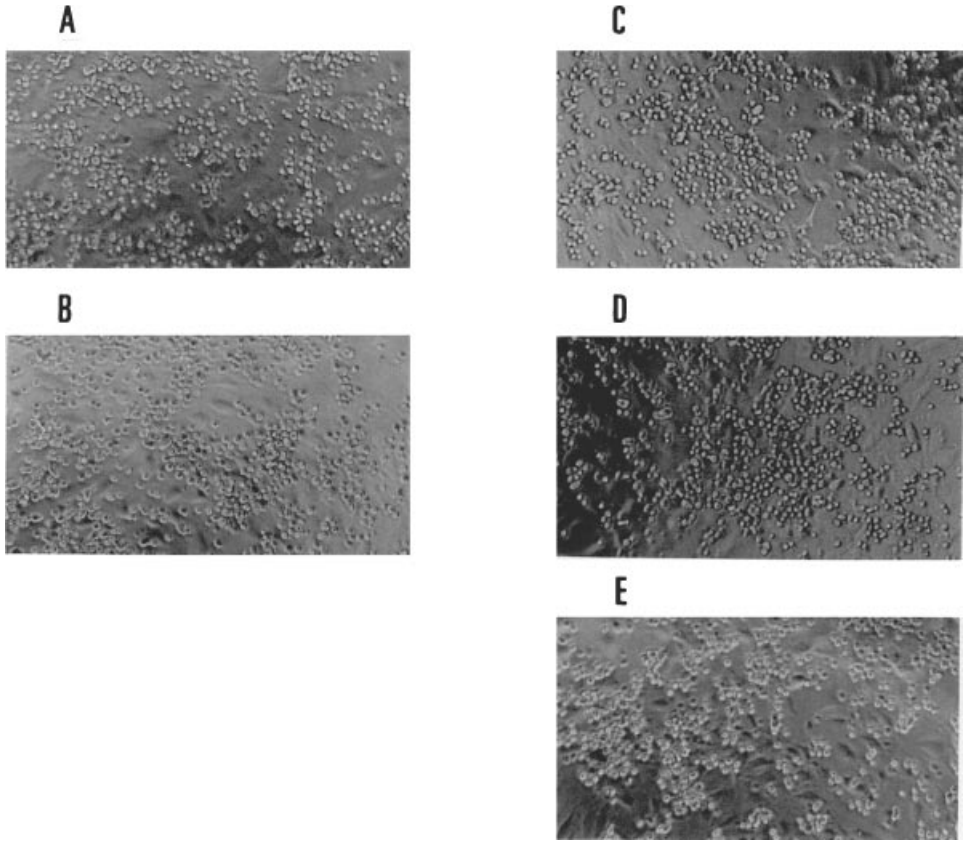


**Figure 7.** Binding of LS 180 cells to HUVEC in the presence of oligosaccharides prepared from SL-GP and human milk SLeA hexasaccharide. HUVEC were treated with IL-1$\beta$ for 4 h. The amounts of inhibitors are expressed as those of sialic acid present in the oligosaccharides. The inhibitors were as follows. (A, B) oligosaccharides corresponding to 10 and 100 ng sialic acid, respectively; (C, D, E) SLeA hexasaccharide corresponding to 10, 100 and 1000 ng sialic acid, respectively.

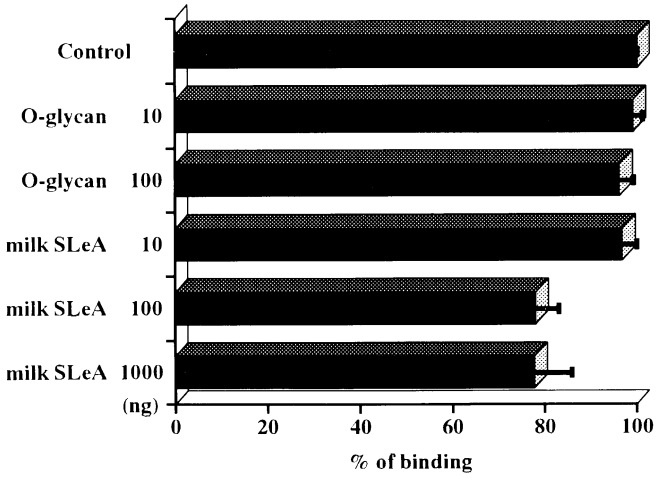


**Figure 8.** Inhibition of binding of LS 180 cells to HUVEC. Data in Fig. 7 were converted into histograms as in Fig. 6.

SLeA antigens were much higher than that of the SLeA hexasaccharide. Endo-$\beta$-galactosidase treatment did not reduce the molecular size, indicating the absence of a polylactosamine structure. The oligosaccharides thus obtained may have complicated and highly branched structures, a part of which should bear the SLeA structure at the non-reducing end, being consistent with our previous report [28]. The details remain to be elucidated.

The low molecular weight O-glycans may comprise oligosaccharides produced on incomplete synthesis and their sialylated counterparts. Expression of the Tn and sialyl-Tn antigens was clearly shown on dot blotting. It would be interesting to determine how both long and incomplete carbohydrate chains are produced on the same polypeptide chain.

Binding experiments clearly indicated that E-selectin expressed on HUVEC and SLeA antigens on SL-GP were involved in the binding. This is also supported by the observation that maximum binding of $^{125}$I-labelled SL-GP to HUVEC was obtained 4 h after the addition of IL-1$\beta$ to the culture medium of HUVEC, being consistent with the time schedule of E-selectin induction [1].

The residual ligand activity observed in the presence of MSW 113 may be due to other antigens. In fact, SL-GP contained a small amount of SLeX antigens (data not shown).

The inhibitory effect of SL-GP on the binding was far greater than those of the milk SLeA hexasaccharide and

oligosaccharides released from SL-GP. When the amount of SL-GP is expressed in terms of sialic acid, 50% inhibition was obtained with ~10 ng sialic acid of SL-GP, whereas 20% inhibition and less than 10% inhibition were obtained with 1 μg of the milk SLeA hexasaccharide and 100 ng of the oligosaccharides from SL-GP, respectively. This is the first direct evidence that when the arrangement of SLeA antigens on the polypeptide chain is disrupted, individual oligosaccharide chains have little affinity for E-selectin. To fully understand selectin-ligand interactions, elucidation of the detailed structure of clustered SLeA antigens including the polypeptide portion are essential.

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