Biochemical and Biophysical Research Communications **278,** 90–96 (2000)
doi:10.1006/bbrc.2000.3768, available online at http://www.idealibrary.com on IDEAL®

P- and E-Selectins Recognize Sialyl 6-Sulfo Lewis X, the Recently Identified L-Selectin Ligand[1]

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Received October 4, 2000

Recently we identified sialyl 6-sulfo Le^x as a major L-selectin ligand on high endothelial venules of human peripheral lymph nodes. In this study we investigated the ligand activity of sialyl 6-sulfo Le^x to E- and P-selectins and compared it with the binding activity of conventional sialyl Le^x, by using cultured human lymphoid cells expressing both carbohydrate determinants. The results of the recombinant selectin binding studies and the nonstatic monolayer cell adhesion assays indicated that both sialyl 6-sulfo Le^x and conventional sialyl Le^x served as ligand for E- and P-selectins, while L-selectin was quite specific to sialyl 6-sulfo Le^x. Anti-PSGL-1 antibodies as well as *O*-sialoglycoprotein endopeptidase treatment almost completely abrogated the binding of P-selectin but barely affected the binding of E-selectin, indicating that these carbohydrate determinants carried by *O*-glycans of PSGL-1 selectively serves as a ligand for P-selectin, while the ligand for E-selectin is not restricted to PSGL-1 nor to *O*-sialoglycoprotein endopeptidase-sensitive glycans. The binding of L-selectin was markedly reduced by *O*-sialoglycoprotein endopeptidase treatment but only minimally affected by anti-PSGL-1 antibodies, indicating that *O*-glycans carrying sialyl 6-sulfo Le^x were the major L-selectin ligands, while PSGL-1 was only a minor core protein for L-selectin in these cells. These results indicated that each member of the selectin family has a distinct ligand binding specificity.

Key Words: **cell adhesion; selectin; sialyl 6-sulfo Lewis X; sialyl Lewis X; PSGL-1; fucosyltransferase; sulfotransferase.**

Abbreviations used: BSA, bovine serum albumin; Fuc-T, fucosyltransferase; GlcNAc, *N*-acetyl-glucosamine; PBS, phosphate-buffered saline; OSGE, *O*-sialoglycoprotein endopeptidase; PSGL-1, P-selectin glycoprotein ligand-1; sialyl Le^x, sialyl Lewis X, NeuAcα2 → 3Galβ1 → 4[Fucα1 → 3]GlcNAcβ1 → R; sialyl 6-sulfo Le^x, sialyl 6-sulfo Lewis X, NeuAcα2 → 3Galβ1 → 4[Fucα1 → 3][SO_3^--6]GlcNAcβ1 → R.

[1] This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports, and Culture, Japan (11680648 and on priority areas 10178104, 12036227, 12217174), grants-in-aid for the Second Term Comprehensive Ten-Year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan, and a grant from the Princess Takamatsu Foundation for the Promotion of Cancer Research.

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Selectin is a family of cell adhesion molecules, which recognize carbohydrate determinants expressed on the cell surface (1–3). Since C-type lectin domains of the three members of the selectin family bear a high homology, it had been assumed that selectins would bind to a common carbohydrate determinant such as sialyl Le^x or sialyl Le^a (4–6). Recently we identified sialyl 6-sulfo Le^x as a major L-selectin ligand (7–9), whose activity in relation to E- and P-selectin had not been thoroughly studied previously. Sialyl 6-sulfo Le^x is expressed on certain subsets of peripheral leukocytes and epithelial cancer cells as well as high endothelial venules of lymph nodes (10, 11), and it is important to see whether or not it also serves as a ligand for E- or P-selectin. If sialyl 6-sulfo Le^x can serve as a ligand for P-selectin, for example, is the core protein PSGL-1 still required for the sulfated carbohydrate determinant in the binding to P-selectin, as was the case with the non-sulfated carbohydrate determinant, sialyl Le^x? In this study we investigated the ligand activity of sialyl 6-sulfo Le^x for E- and P-selectins, and compared it with the binding activity of conventional sialyl Le^x, using human lymphoid cells expressing both carbohydrate determinants.

0006-291X/00 $35.00

MATERIALS AND METHODS

Monoclonal antibodies directed to sialyl 6-sulfo Le^x-related carbohydrate determinants. The antibodies G152 directed to sialyl 6-sulfo Le^x and G72 directed to sialyl 6-sulfo *N*-acetyl lactosamine were prepared as described previously (8, 12). G152 is specific to sialyl 6-sulfo Le^x, and does not react to conventional sialyl Le^x. A classical anti-sialyl Le^x antibody, CSLEX-1, was obtained from the American Type Culture Collection (Manassas, VA). This antibody is reactive to conventional sialyl Le^x, but not to sialyl 6-sulfo Le^x (7). The anti-sialyl Le^x antibody 2F3, which was established against a synthetic sialyl Le^x-active glycolipid and recognizes both sialyl Le^x and sialyl 6-sulfo Le^x (7), was prepared as described previously (13). Anti-PSGL-1 blocking antibody PL1 (3E2-25-5, IgG_1) (14) was obtained from Immunotech (Marseilles, France), and KPL1 (IgG_1) from PharMingen (San Diego, CA) (15).

Transfection of cultured human lymphoid cell line Namalwa KJM-1 with Fuc-T VII. The cell line Namalwa KJM-1, a subline of the human Burkitt lymphoma cell line Namalwa, was chosen as a host for transfection of Fuc-T VII. Vectors containing the replication origin of Epstein–Barr virus, such as pAMo, were shown to replicate stably in an episomal state in this cell line (16). The structure of the expression vector, pAMo, in which cDNAs are expressed under the control of the long terminal repeat promoter of Moloney murine leukemia virus, was described previously (16). Namalwa KJM-1 cells were transfected with pAMo-Fuc-T VII or parental vector pAMo as described previously (17). After the transfection, cells grown in the selection medium containing G418 (0.5 mg/ml) were used in the experiments without further cloning procedures.

Binding analysis of recombinant selectins by flow cytometry and nonstatic monolayer cell adhesion assay. Namalwa KJM-1 transfectant cells were cultured in RPMI1640 supplemented with 10% FCS and were harvested at a semi-confluent stage. Recombinant human E-, P-, and L-selectin-IgG chimera were kindly provided by Drs. H. Kondo and Y. Inoue of Department of Chemistry, R&D Laboratories, Nippon Organon K.K., Osaka, Japan. Recombinant selectins were preincubated with affinity-purified rabbit anti-human IgG (Dako, Glostrup, Denmark) followed by incubation with phycoerythrin–streptavidin (Dako), before application to the staining of Namalwa KJM-1 transfectant cells. The cells were preincubated with blocking antibodies (10 μg/ml) for 30 min at 37°C if indicated. Treatment with *O*-sialoglycoprotien endopeptidase (Cedarlane, Hornby, Ontario, Canada) was performed with 60 μg of the enzyme per 5×10^6 cells at 37°C for 1 h. For analysis of expression of sialyl 6-sulfo Le^x and related carbohydrate determinants, the cells were mixed and incubated with the anti-carbohydrate monoclonal antibody (purified antibody at 1 μg/ml or culture supernatant at a dilution of 1:10) at 4°C for 30 min. The cells were then washed three times with PBS containing 0.5% BSA and stained with 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Silenus Laboratories, Australia) at 4°C for 30 min. Nonstatic monolayer cell adhesion experiments were performed as described previously (18, 19). The cells were labeled with BCECF-AM, and were incubated in the presence or absence of the inhibitor anti-carbohydrate antibodies (25 μg/ml) for 30 min at 37°C if indicated. BCECF-AM-labeled transfectant cells were added to the E- or P-selectin transfected CHO cells, which were cultured in monolayer in 24-well plates. The plates were incubated with rotation at 90 rpm for 20 min at room temperature. After non-adherent cells were washed out three times with PBS, the cells were lysed with 0.5% NP-40 and the attached cells were counted by measuring fluorescence using an Arvo 1420 multi-label counter (Wallac, Gaithersburg, MD).

RESULTS

Expression of sialyl 6-sulfo Le^x and conventional sialyl Le^x determinants on cultured human lymphoid cells transfected with Fuc-T VII. A lymphoid leukemia cell line Namalwa KJM-1, a clone of the cultured human lymphocytic leukemia cells Namalwa, expressed 6-sulfo *N*-acetyllactosamine-related determinants, indicating they contain a significant endogenous 6-sulfotransferase activity. This indicated that the cell line has substrates necessary for the synthesis of sialyl 6-sulfo Le^x determinants and is suitable for this study. The cells did not express sialyl Le^x-related carbohydrate determinants, and the transfection of human lymphoid cells with Fuc-T VII cDNA resulted in significant expression of sialyl 6-sulfo Le^x and conventional sialyl Le^x as ascertained by flow cytometric analysis (Fig. 1). Sialyl 6-sulfo Le^x on the transfectant cells was thought to be synthesized through the action of transfected Fuc-T VII and endogenous 6-sulfotransferase. The cells contained a significant amount of mRNA for HEC-GlcNAc-6ST reported by Bistrup *et al.* (20), and a small amount of mRNA for GlcNAc-6ST reported by Uchimura *et al.* (12, 21) (Kanamori, A., and Kannagi, R., unpublished results). The mock-transfected cells did not express any determinants including sialyl 6-sulfo Le^x and conventional sialyl Le^x (Fig. 1).

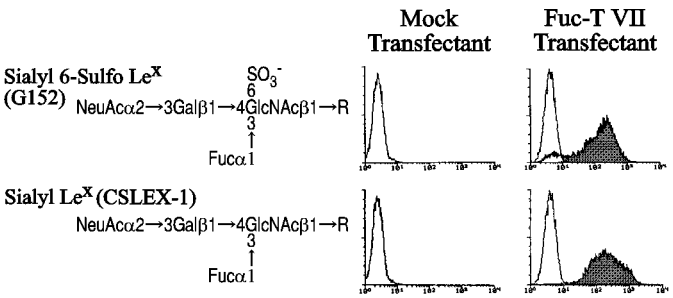

FIG. 1. Expression of sialyl 6-sulfo Le^x and conventional sialyl Le^x on cultured human lymphoid cells transfected with Fuc-T VII cDNA or mock-transfected cells as ascertained by flow cytometry using anti-carbohydrate monoclonal antibodies with well-defined specificity. Abscissa, relative fluorescence intensity; ordinate, cell number. The carbohydrate structure specifically recognized by the respective antibody, G152 and CSLEX-1, is shown in the left panel.

Binding of recombinant human selectins to Fuc-T VII transfectant cells. The binding of recombinant human selectin-Igs to the Fuc-T VII transfectant cells were studied to assess and differentiate the respective role played by sialyl 6-sulfo Le^x and conventional sialyl Le^x in the selectin-mediated cell adhesion (Fig. 2). All three recombinant selectins showed significant binding to the Fuc-T VII transfectant cells (Fig. 2A). Binding of recombinant E- and P-selectin-Ig to the cells was moderately inhibited by either the sialyl 6-sulfo Le^x antibody G152 or anti-conventional sialyl Le^x antibody CSLEX-1, and combination of the two antibodies resulted in almost complete inhibition of the binding (Fig. 2). This suggested that sialyl 6-sulfo Le^x and conventional sialyl Le^x are both involved in the binding. The binding of recombinant E-selectin tended to be inhibited more strongly by CSLEX-1 than by G152, whereas that of recombinant P-selectin was more

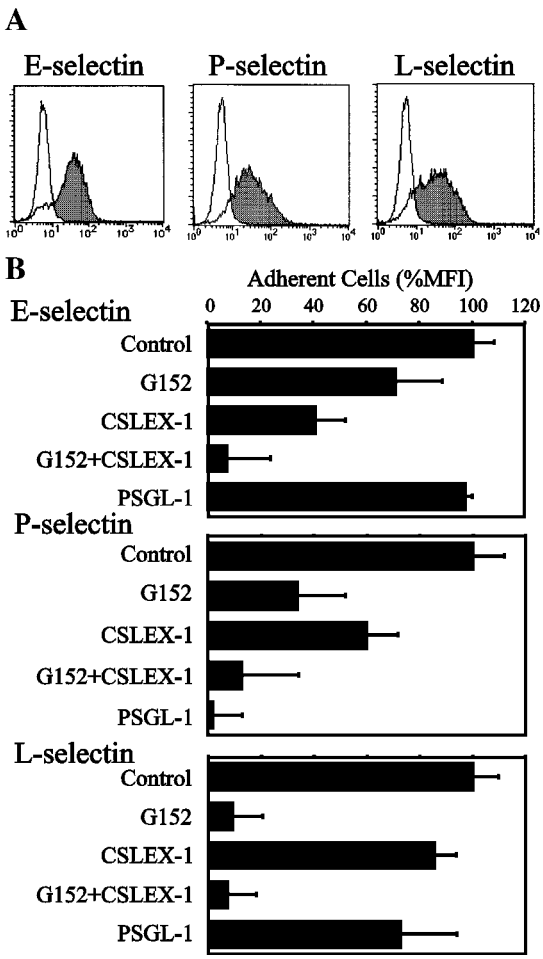

FIG. 2. Binding of recombinant human E-, P-, or L-selectin-Ig to the human lymphoid cells transfected with Fuc-T VII cDNA and contribution of carbohydrate determinants or core protein PSGL-1 in the adhesion. (A) Results of flow cytometric analyses; (B) results of inhibition studies using blocking antibodies. Antibodies used are G152 specific for sialyl 6-sulfo Le^x and CSLEX-1 specific for conventional sialyl Le^x. KPL-1 was used as the blocking antibody for PSGL-1. Representative results obtained in three independent experiments are shown.

prominently affected by G152 than by CSLEX-1. This tendency was consistently observed in three independent experiments. The binding of recombinant L-selectin-Ig was nearly completely inhibited by G152, but only minimally affected by CSLEX-1, confirming our previous findings that sialyl 6-sulfo Le^x is a much preferred ligand for L-selectin compared to conventional sialyl Le^x (8, 9).

Pretreatment of the cells with the anti-PSGL-1 antibody almost completely abrogated the binding of P-selectin-Ig, whereas it had a only minimal effect on the binding of E-selectin-Ig, confirming the importance of PSGL-1 in the binding of P-selectin, but not E-selectin (22). It is well known that conventional sialyl Le^x must be carried on PSGL-1 for the binding to P-selectin. The current findings indicate that the same applies to sialyl 6-sulfo Le^x. On the other hand, binding of recombinant L-selectin-Ig to these cells was significantly but only weakly inhibited by the treatment with the anti-PSGL-1 antibody, KPL-1. This was reproducible in three independent experiments in which 10–20% inhibition of L-selectin-Ig binding was consistently observed, whereas the binding of P-selectin-Ig was nearly 90% inhibited by the same treatment in every experiment.

Adhesion of Fuc-T VII-transfected cells to E- and P-selectin-CHO cells. To further assess the specificity of binding of selectins to sialyl 6-sulfo Le^x and conventional sialyl Le^x, we have performed non-static monolayer cell adhesion experiments (Fig. 3). The cells transfected with Fuc-T VII strongly adhered to E- and P-selectin-CHO cells (Fig. 3A). Adhesion of the transfectant cells to P-selectin-CHO cells was weaker than to E-selectin-CHO cells, probably because of the weak expression of PSGL-1, the core protein required for the binding to P-selectin (23) on the Namalwa KJM-1 cells.

The adhesion of the cells transfected with Fuc-T VII cDNA to E-selectin- or P-selectin-CHO cells was again significantly inhibited by the addition of either anti-sialyl 6-sulfo Le^x antibody G152 or anti-sialyl Le^x antibody CSLEX-1, as shown in Fig. 3B. This further confirmed that sialyl 6-sulfo Le^x and conventional sialyl Le^x are both involved in the binding. Again, the adhesion to E-selectin-CHO cells tended to be inhibited more strongly by CSLEX-1 than by G152, while that to

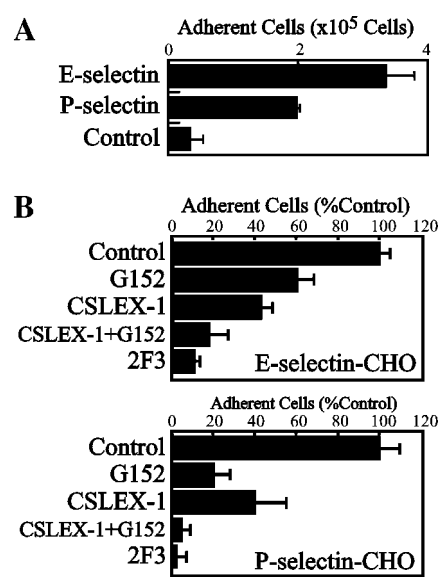

FIG. 3. Adhesion of human lymphoid cells transfected with Fuc-T VII cDNA to E- and P-selectin-expressing CHO cells and contribution of carbohydrate determinants in the adhesion. (A) Adhesion to E-selectin-CHO cells, P-selectin-CHO cells, and parental CHO cells. (B) Results of inhibition studies using blocking antibodies. Antibodies used are G152 specific for sialyl 6-sulfo Le^x, CSLEX-1 specific for conventional sialyl Le^x, and 2F3 reactive to both sialyl 6-sulfo Le^x and conventional sialyl Le^x.

P-selectin-CHO cells was more prominently affected by G152 than by CSLEX-1. The inhibitory effects of these antibodies were additive, and the combination of the two antibodies further inhibited the binding, as well as the addition of the 2F3 antibody, which recognizes both determinants (Fig. 3B).

O-Sialoglycoprotein endopeptidase susceptibility of sialyl 6-sulfo Le^x determinant on cultured human lymphoid cells transfected with Fuc-T VII cDNA. As shown in Fig. 4A, the sialyl 6-sulfo Le^x determinant on the cells transfected with Fuc-T VII was highly susceptible to the treatment of the cells with OSGE, indicating the sialyl 6-sulfo Le^x determinant was predominantly carried by the *O*-glycans of membrane glycoproteins in these cells. The expression of sialyl 6-sulfo *N*-acetyl lactosamine was also highly susceptible to the OSGE treatment to a similar degree as sialyl 6-sulfo Le^x. On the other hand, conventional sialyl Le^x, which has no sulfate residue, was not effectively cleaved by the enzyme; its expression showed a mean decrease of only about 27% by the enzymatic treatment in terms of mean fluorescence intensity (Fig. 4A). This suggested that *O*-glycans were preferentially 6-O-sulfated in these cells. The enzymatic treatment completely abrogated expression of the PL-1- or KPL-1-defined epitope on PSGL-1 in these cells (Fig. 4A).

Binding of recombinant E-selectin-Ig to the cells was only minimally impaired by the OSGE treatment (Fig. 4B), confirming that E-selectin could recognize sialyl Le^x or sialyl 6-sulfo Le^x determinants carried by molecules other than *O*-glycans. In contrast, binding of recombinant P-selectin-Ig was almost completely abrogated by the enzymatic treatment (Fig. 4B), which is compatible with the nearly complete degradation of PSGL-1 by the treatment. The L-selectin-Ig binding was considerably reduced by the treatment, yet a weak but significant reactivity was nevertheless retained (Fig. 4B). This may reflect an extensive but incomplete hydrolysis of sialyl 6-sulfo Le^x on the surface of cells (Fig. 4A).

When the same cells treated with OSGE were subjected to non-static monolayer cell adhesion assays, adhesion of the treated cells to E-selectin-CHO cells was again only minimally affected, whereas strong inhibition of adhesion to P-selectin-CHO cells was noted (Fig. 4C), confirming the preference of P-selectin for ligands carried by *O*-glycans.

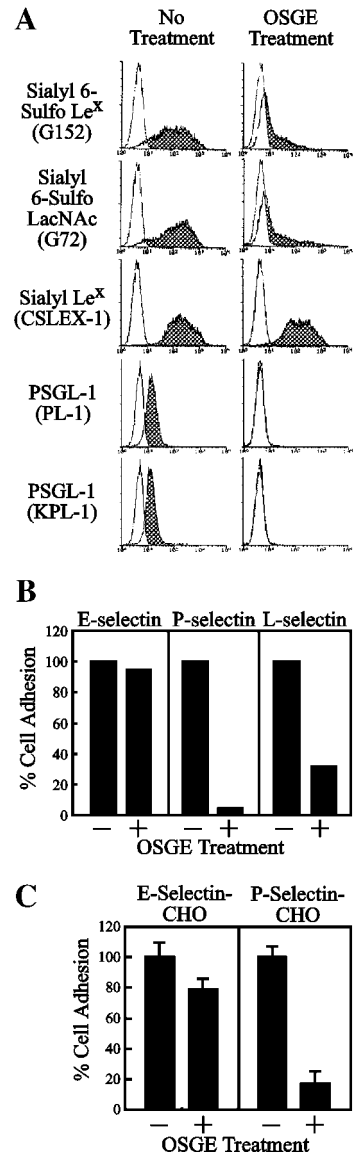

FIG. 4. Effect of *O*-sialoglycoprotein endopeptidase treatment on carbohydrate determinant expression and selectin binding activity of human lymphoid cells transfected with Fuc-T VII cDNA. (A) Effect of *O*-sialoglycoprotein endopeptidase treatment on the expression of sialyl 6-sulfo Le^x and related determinants. Antibodies used are G152 for sialyl 6-sulfo Le^x, G72 for sialyl 6-sulfo *N*-acetyl lactosamine, CSLEX-1 for conventional sialyl Le^x, and PL-1 for PSGL-1. Abscissa, relative fluorescence intensity; ordinate, cell number. (B) Effect of *O*-sialoglycoprotein endopeptidase treatment on the binding of recombinant E-, P-, or L-selectin-Ig to human lymphoid cells transfected with Fuc-T VII cDNA. (C) Effect of *O*-sialoglycoprotein endopeptidase treatment on the adhesion of human lymphoid cells transfected with Fuc-T VII cDNA to E- or P-selectin-expressing CHO cells. OSGE treatment, *O*-sialoglycoprotein endopeptidase treatment.

DISCUSSION

Previously we succeeded in reconstituting functional L-selectin ligand on cultured human cell lines such as ECV304 or COS-7 cells by transfecting Fuc-T VII and 6-sulfotransferase genes (9). Significant L-selectin-mediated cell adhesion was observed only with the cells doubly transfected with Fuc-T VII and 6-sulfotransferase genes which expressed sialyl 6-sulfo Le^x (9). On the other hand, E-selectin-mediated adhesion was supported by the cells transfected with Fuc-T VII gene alone expressing sialyl Le^x, as well as the doubly transfected cells expressing sialyl 6-sulfo Le^x

TABLE 1

Summary of Ligand Specificity of Selectins Based on Findings Obtained in This Study

	E-Selectin	P-Selectin	L-Selectin
Carbohydrate determinant	Sialyl Le^x or sialyl 6-sulfo Le^x Sialyl Le^x > sialyl 6-sulfo Le^x	Sialyl 6-sulfo Le^x or sialyl Le^x Sialyl 6-sulfo Le^x > sialyl Le^x	Sialyl 6-sulfo Le^x
PSGL-1 as core protein	Minimum contribution	Indispensable	Minor contribution

(9). This indicated that L-selectin is quite specific to sialyl 6-sulfo Le^x, while E-selectin can bind to either sialyl Le^x or sialyl 6-sulfo Le^x. However, P-selectin-mediated cell adhesion was not observed with these cells, and this was putatively ascribed to the absence of PSGL-1, the core protein assumed to be specifically required for P-selectin-mediated cell adhesion, on the cells applied for the previous cell adhesion experiments (9). Whether or not sialyl 6-sulfo Le^x is really recognized by P-selectin remains to be seen. Another point was the importance of PSGL-1 in the L-selectin-mediated cell adhesion. Several previously published studies concluded that conventional sialyl Le^x carried by PSGL-1 is a good ligand for L-selectin (24, 25) as well as P-selectin, whereas our previous study indicated that the cells expressing sialyl 6-sulfo Le^x but not PSGL-1 significantly adhered to L-selectin (9).

The characteristics of the cell adhesion experimental system applied in the present study are different in several aspects from those of the previously applied system. Both Fuc-T VII and 6-sulfotransferase were transfected to the cells used in the previous experimental system (9), which resulted in very strong expression of sialyl 6-sulfo Le^x and elimination of conventional sialyl Le^x expression from the surface of cells. The cells applied in the present study were not transfected with 6-sulfotransferase cDNA, and expressed both determinants probably because of the moderate activity of endogenous 6-sulfotransferases. This enabled direct comparison of the relative contribution of the two determinants expressed on the very same cells in the cell adhesion. The most important difference is that the cells applied in the current study express PSGL-1, while the epithelioid cells applied in the previous studies did not. The presence of PSGL-1 on the cells applied in the present experiments enabled us to study the significance of sialyl 6-sulfo Le^x in P-selectin-mediated cell adhesion.

The results of the present study clearly indicated that the sialyl 6-sulfo Le^x determinant serves as a ligand for all selectins. Sialyl 6-sulfo Le^x, but not conventional sialyl Le^x, served as a good ligand for L-selectin, while E-selectin recognized sialyl 6-sulfo Le^x as well as conventional sialyl Le^x. P-selectin was also found to be reactive to both sialyl 6-sulfo Le^x and conventional sialyl Le^x provided these determinants were carried on the PSGL-1 molecule.

The examination of binding specificities of selectins using inhibitory antibodies also indicated that each selectin member has its own distinct ligand specificity as summarized in Table 1. The ligand specificities of E- and P-selectins are similar in that they recognized both sialyl 6-sulfo Le^x and conventional sialyl Le^x. The results of OSGE treatment indicated that P-selectin exclusively recognized the ligands carried on *O*-glycans, while E-selectin mainly recognized OSGE-resistant glycans. The binding of cells expressing sialyl 6-sulfo Le^x to P-selectin was strongly dependent on PSGL-1, while E-selectin-mediated adhesion was not dependent on PSGL-1. This is in line with the previous notion that sulfated tyrosine residue in PSGL-1 is specifically required for P-selectin, but not for E-selectin (23, 26, 27). Our current results suggested that the 6-sulfation in the carbohydrate portion did not replace the role played by sulfated tyrosine residues in the PSGL-1 molecule in the binding to P-selectin.

These ligand specificities of E- and P-selectins are in a clear contrast to the specificity of L-selectin, which has a marked preference to sialyl 6-sulfo Le^x compared to conventional sialyl Le^x. Previously we have shown that the cells expressing conventional sialyl Le^x did not bind L-selectin, while those expressing sialyl 6-sulfo Le^x significantly bound to L-selectin (9). This was confirmed in this study by showing that the binding of the human lymphoid cells expressing both determinants to L-selectin was inhibited only by anti-sialyl 6-sulfo Le^x, but not by conventional anti-sialyl Le^x antibody, even the cells expressing both determinants were used in the experiments.

The present investigation further indicated that PSGL-1 has a relatively minor contribution in L-selectin binding compared to that P-selectin. The leukocyte-leukocyte interaction mediated by L-selectin has been suggested to be mediated by the binding of L-selectin to specific carbohydrate determinants carried by PSGL-1 (24, 25). The modest inhibitory effect of anti-PSGL-1 antibody observed in the binding to L-selectin indicated that sialyl 6-sulfo Le^x carried by the core molecules other than PSGL-1 played a significant role in the binding of L-selectin in the current experimental system. This could be related to the weak expression of PSGL-1 on the cells applied in the current study. This, however, was in a clear contrast to the binding of P-selectin, which was strictly PSGL-1-

dependent as indicated by the nearly complete inhibition of binding by anti-PSGL-1 antibody, even when such a low-expressor cell line of PSGL-1 was used in the experiments.

ACKNOWLEDGMENTS

We thank Drs. Yoshimasa Inoue and Hirosato Kondo, Department of Chemistry, R&D Laboratories, Nippon Organon K.K., Osaka, Japan, for the gifts of recombinant selectins.

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