6'-Sulfo Sialyl Le\textsuperscript{x} but Not 6-Sulfo Sialyl Le\textsuperscript{x} Expressed on the Cell Surface Supports L-selectin-mediated Adhesion\textsuperscript{*}

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In order to determine if a sulfated oligosaccharide on the cell surface can function as an L-selectin ligand, a novel approach for \textit{in vitro} transfer of oligosaccharides was utilized (Srivastava, G., Kaun, K. J., Hindskaul, O., and Palicic, M. M. (1992) J. Biol. Chem. 267, 22356–22361). CHO cells were incubated with synthetic 6'-sulfo sialyl Le\textsuperscript{x}, NeuNAco2−3(sulfate-6)Galβ1−4(Fucα1−3)GlcNAc or 6-sulfo sialyl Le\textsuperscript{x}, NeuNAco2−3Galβ1−4(Fucα1−3)sulfate−6GlcNAc oligosaccharide linked to C-6 of a fucose residue in GDP-fucose and a milk fucosyltransferase. The resultant CHO cells expressing 6'-sulfo sialyl Le\textsuperscript{x} or 6-sulfo sialyl Le\textsuperscript{x} on their cell surface were tested for adhesion to E-selectin and L-selectin chimeric proteins coated on plates. The results indicate that 6'-sulfo sialyl Le\textsuperscript{x} supports L-selectin-mediated adhesion much better than sialyl Le\textsuperscript{x} similarly tagged on the cell surface. In contrast, 6-sulfo sialyl Le\textsuperscript{x} containing a sulfate group on the N-acetylgalactosamine residue did not support adhesion with either selectin. These combined results suggest that 6'-sulfo sialyl Le\textsuperscript{x} is a much better ligand than sialyl Le\textsuperscript{x} oligosaccharide for L-selectin.

It has been suggested that among the members of the selectin family, E- and P-selectin bind to sialyl Le\textsuperscript{x}, NeuNAco2−3Galβ1−4(Fucα1−3)GlcNAc present on neutrophils and other leukocytes (1–4), although the identity of the real physiological epitope remains to be clarified (5). In trophils and other leukocytes (1–4), although the identity of the natural ligand for L-selectin, we thus elected in the present study to employ an novel method for transferring oligosaccharides to the cell surface of CHO cells by a combination of synthetic chemistry and enzymatic transfer (25). In this method, oligosaccharides can be acceptors for this reaction. We then tested the resulting CHO cells expressing 6'-sulfo sialyl Le\textsuperscript{x} or 6-sulfo sialyl Le\textsuperscript{x} for adhesion to E- or L-selectin chimeric protein. The results clearly demonstrate that 6'-sulfo Le\textsuperscript{x} is a better ligand for L-selectin than sialyl Le\textsuperscript{x}, and 6-sulfo sialyl Le\textsuperscript{x} does not support the adhesion to either L-selectin or E-selectin.

**EXPERIMENTAL PROCEDURES**

Establishment of CHO Cells Stably Expressing CD34 and C2GnT—CHO DG44 cells expressing C2GnT and leukosialin, CHO-leu2C2GnT, were established as described (16). CHO-leu2C2GnT cells were transfected by the LipofectAMINE method (28) with pCDM8-CD34 (29) and pHyg in a 10:1 molar ratio, as described (30). Clonal cell lines were prepared in part by the payment of page charges. This article must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{*} The abbreviations used are: Le\textsuperscript{x}, Lewis\textsuperscript{x}; CHO, Chinese hamster ovary; C2GnT, core 2 β,1-6-N-acetylgalactosaminyltransferase; MAA, Maackia amurensis agglutinin; BSA, bovine serum albumin; FTHIII, fucosyltransferase III.
obtained by limiting dilution, and each cell line was assessed for the expression of CD43 by immunofluorescence, using anti-CD34 antibody (HPLA-2, Becton Dickinson) (30). Cells which were positive for CD34 were further tested for the expression of C2GnT and leukosialin using T305 antibody that recognizes core 2 branched oligosaccharides attached to leukosialin (16). PCDM8-CD34 (29) was kindly provided by Dr. Brian Seed, Massachusetts General Hospital, Boston.

**Synthesis of GDP-fucose-Oligosaccharide Conjugates**—The 6-sulfo sialy Lex Le\(^a\) derivative, NeuNacα-2→3Galα-3Galβ-1→4Fucα-1→3Glcnacβ1→O(CH\(_2\))\(_8\)COOMe and 6-sulfo sialy Lex Le\(^a\) derivative, NeuNacα-2→3Galα-1→4(NaO\(_3\))Sβ-6)Galβ-1→4Fucα-1→3Glcnacβ1→O(CH\(_2\))\(_8\)COOMe were synthesized using modifications of the reported procedures described (25, 31). The detailed procedure for synthesizing these oligosaccharides will be published elsewhere. These oligosaccharides were attached to each molecule of BSA, this predicts that 0.509 pmol of oligosaccharides are present in each coated well. In parallel, the number of binding sites of MAA to sialyl Lex-Le\(^a\)-BSA conjugates was determined by addition of biotinylated MAA (Vector) followed by 125I-streptavidin (Amersham). At the saturation levels, the amount of bound MAA should be equivalent to 0.509 pmol/well. This measurement thus allowed us to build a calibration curve.

To estimate the amount of the sialy Le\(^a\) or sulfo sialy Le\(^a\) oligosaccharides on the cell surface, various amounts of biotinylated MAA were added at 4°C to various CHO cells tagged with oligosaccharides. The amount of bound MAA was determined by binding of 125I-streptavidin at 4°C.

**RESULTS AND DISCUSSION**

**Transfer of Oligosaccharides Conjugated to GDP-Fuc—CHO** cells were incubated with the fucosyltransferase and GDP-Fuc-sialyl Le\(^a\), GDP-Fuc-6-sulfo sialyl Le\(^a\), or GDP-Fuc-6-sulfo sialyl Le\(^a\). As shown in Fig. 2A, the resultant CHO-sialy Le\(^a\) cells express a substantial amount of sialyl Le\(^a\), detected by immunofluorescent staining using anti-sialyl Le\(^a\) antibody. Fig. 2B shows the corresponding immunostaining of CHO-FTIII cells, which were produced by gene transfer of Fuc-TIII (22, 31). The staining pattern of CHO-sialy Le\(^a\) is distinct from that of CHO-FTIII cells in that CHO-sialy Le\(^a\) cells show more clustered staining (Fig. 2A).

In order to estimate how much sialyl Le\(^a\), 6-sulfo sialyl Le\(^a\), or 6-sulfo sialyl Le\(^a\) oligosaccharides were transferred, the binding assays using MAA were performed on these cells. Control CHO cells were found to have 1.84 × 10\(^5\) binding sites of MAA (Fig. 3A), since they express 2,3,3-linked sialic acid (37, 38). Assuming that 0.23,3-linked sialic acid in sialyl Le\(^a\), 6-sulfo sialyl Le\(^a\), and 6-sulfo sialyl Le\(^a\) equally bind to MAA, the binding sites of MAA in these three CHO cells were determined. The results demonstrated almost identical numbers of binding sites of MAA (4.39 × 10\(^5\), 4.35 × 10\(^5\), and 4.64 × 10\(^5\)) in CHO-sialy Le\(^a\), CHO-6-sulfo sialy Le\(^a\), and CHO-6-sulfo sialy Le\(^a\). (Fig. 3, B, C, and D). By subtracting the amount of 2,3,3-linked sialic acid present in control CHO cells, it can be concluded that 2.51 to 2.80 × 10\(^5\)/cell of sialy Le\(^a\), 6-sulfo sialyl Le\(^a\), and 6-sulfo sialyl Le\(^a\) were transferred to the cell surface of CHO cells. These results also corroborated our hypothesis that 2,3,3-linked sialic acid in different oligosaccharides bind almost equally to MAA, providing that the efficiency...
CHO-6-sulfosialyl Le\(^x\) to E-selectinchimeric protein. Adhesion of CHO-sialyl Le\(^x\), CHO-6-sulfo sialyl Le\(^x\), and CHO-6-sulfo sialyl Le\(^x\) cells are shown by open bars (columns 2, 5, and 10). Inhibition of those adhesions by anti-sialyl Le\(^x\) antibody (column 4) and control mouse IgM antibodies (column 5) are shown. The control experiments were carried out in the absence of the fucosyltransferase (columns 3, 8, and 11). Data shown correspond to the mean ± S.D. from three replicate experiments.

Adhesion of CHO-sialyl Le\(^x\) and CHO-6-Sulfo Sialyl Le\(^x\) Cells to E-selectin—Fig. 4 demonstrated clearly that CHO-sialyl Le\(^x\) adhered well to E-selectin chimera (column 2), while the adhesion was minimum to control human IgG proteins (column 3). The adhesion was completely inhibited by preincubation of the cells with anti-sialyl Le\(^x\) antibody (column 4), anti-E-selectin antibody, or by the addition of 5 mM EDTA (data not shown), but not by control mouse IgM (column 5). Surprisingly, CHO-6-sulfo sialyl Le\(^x\) cells adhered well to E-selectin (column 7). However, almost no adhesion was detected for CHO-6-sulfo sialyl Le\(^x\) cells (column 10).

Adhesion of CHO-6-Sulfo Sialyl Le\(^x\) and CHO-Sialyl Le\(^x\) to L-selectin—As shown in Fig. 5, CHO cells expressing 6-sulfo sialyl Le\(^x\) adhered well to L-selectin chimera (column 5). This adhesion could be inhibited by preincubation with anti-L-selectin antibody (column 7), but not by control mouse IgG (column 8). In contrast, sialyl Le\(^x\) oligosaccharides on CHO cells modestly supported the adhesion to L-selectin (column 2). Apparently, the expression of CD34 is critical since CHO cells which were not transfected with CD34 cDNA did not support the adhesion to L-selectin (data not shown). More strikingly, CHO cells expressing 6-sulfo sialyl Le\(^x\) did not adhere to L-selectin at all (column 10). These combined results indicate that 6-sulfo sialyl Le\(^x\) is a much better ligand for L-selectin than sialyl Le\(^x\) while it is a slightly less efficient ligand for E-selectin than sialyl Le\(^x\). 6-Sulfo sialyl Le\(^x\) on the other hand is hardly bound to either E- or L-selectin.

Previous studies have demonstrated that GlyCAM-1, which carries L-selectin ligands, can have a sulfate group in C-6 of galactose, 6'-sulfo sialyl Le\(^x\) and C-6 of N-acetylgalactosamine, 6-sulfo sialyl Le\(^x\) (17, 19). The present study clearly indicates that 6'-sulfo sialyl Le\(^x\) is a better ligand for L-selectin than sialyl Le\(^x\). These results are consistent with the previous report that sulfation was required for efficient binding of L-selectin to GlyCAM-1 (10). However, we also found in the present study that sialyl Le\(^x\) is an inefficient yet moderate ligand for L-selectin. These results are consistent with the recent report on the inhibition of L-selectin-mediated adhesion using synthetic oligosaccharides; their results indicate that sialyl Le\(^x\) is as effective as sulfate→3→sulfate→6→Galβ1→4Glc for inhibiting the binding of L-selectin to GlyCAM-1 (39). The present study also demonstrated that 6-sulfo sialyl Le\(^x\) is not an efficient ligand for either E- or L-selectin. It is possible that the 6-sulfo sialyl Le\(^x\) structure present in GlyCAM-1 does not serve well as a ligand for L-selectin. It was also reported that 6-sulfo sialyl Le\(^x\) inhibits L-selectin binding to adressin (42). In those studies, however, only inhibition assay was employed, and the comparison with 6'-sulfo sialyl Le\(^x\) was not tested.

Recent crystallographic studies showed the structure of the carbohydrate-binding domain of E-selectin (40). In that study, sialyl Le\(^x\) tetrasaccharide was tentatively modeled based on...
the structure of the complex of mannosse oligosaccharide-mannose-binding protein (41) and NMR data on sialyl Le\(^x\) oligosaccharide (40). These results suggest that one of the critical amino acids involved in its binding may be glutamic acid 92 (40, 41). It is thus tempting to speculate that a sulfate group at C-6 of N-acetylgalactosamine in 6-sulfo sialyl Le\(^x\) causes steric hindrance as well as charge repulsion to glutamic acid 92. It is also noteworthy that E- and L-selectin have a strong homology in the carbohydrate-binding domain, including those amino acids critical for their binding to carbohydrate ligands shown for mannosse-binding protein (40, 41, 43). It is thus not surprising that only quantitative differences in efficiency can be found between sialyl Le\(^x\) and 6-sulfo sialyl Le\(^x\) for L-selectin- and E-selectin-mediated adhesion, as shown in the present study.

As described in the introduction, the chemical and enzymatic tagging is powerful when testing the activity of oligosaccharides that cannot be synthesized on cells by gene transfer. We expect that the method employed in the present study will be an important tool in determining the roles of oligosaccharides on the cell surface in various studies.

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