Expression Patterns of α2,3-Sialyltransferases and α1,3-Fucosyltransferases Determine the Mode of Sialyl Lewis X Inhibition by Disaccharide Decoys*

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A variety of human adenocarcinomas express sialylated, fucosylated Lewis blood group antigens on cell surface and secreted mucins. Binding of these antigens to P-selectin on platelets is thought to facilitate formation of platelet-tumor emboli in the circulation, which in turn allows sequestration of the tumor cells in the microvasculature. Here we report a pharmacologic approach for blocking these interactions through metabolic inhibition of sialylation. Peracetylated forms of Galβ1,4GlcNAcβ-O-naphthalenemethanol and GlcNAcβ1,3Galβ-O-naphthalenemethanol were taken up by LS180 human colon carcinoma cells, O-deacetylated, and utilized as biosynthetic intermediates, resulting in heterogeneous oligosaccharides. The primed oligosaccharides included sialylated, sulfated, and fucosylated products based on mass spectrometry. Assembly of free oligosaccharides on the glycosides decoyed glycosylation of cellular glycoproteins, as assessed by altered binding of lectins and carbohydrate-specific antibodies. Expression of α2,3-sialylated oligosaccharides on the cell surface was diminished specifically, whereas α2,6-sialylation and fucosylation were not. In U937 lymphoma cells, the glycosides decreased fucosylation without affecting sialylation. The differential inhibitory activities correlated inversely with fucosyltransferase and sialyltransferase activity based on enzyme assays and microarray analysis. Regardless of the mechanism, the disaccharides blocked the cells from forming selectin ligands and inhibited adhesion to immobilized selectins, suggesting that the glycosides might prove useful for interfering with tumor cell adhesion and metastasis.

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Oligosaccharides containing sialyl Lewis X (sLeα,1 NeuAcα2,3Galβ1,4[Fucα1,3GlcNAcβ]) and sialyl Lewis A (sLeα, NeuAcα2,3Galβ1,3[Fucα1,4GlcNAcβ]) are commonly associated with glycoproteins on the surface of tumor cells (for review, see Ref. 1). Clustered arrangements of these antigens can mediate binding of tumor cells to selectin adhesion receptors on activated endothelia, platelets, or leukocytes, which is thought to increase the probability that tumor cells lodge as emboli in the microvasculature of distant organs during metastasis (2–7). Postmortem survival studies of patients bearing colon and lung adenocarcinomas indicate higher mortality for individuals whose tumors express sLeα or sLeα (e.g. Refs. 8–10), suggesting that inhibition of the formation of selectin carbohydrate ligands on tumor cells might reduce tumor metastasis. Reduced metastasis occurs after the removal of selectin ligands from tumor cells genetically (11–13) or enzymatically (2, 3), by blockade of selectin interactions with heparin (14), or by knock-out of P-selectin in the host (3). Thus, a pharmacologic strategy for metabolically altering selectin ligand expression would be desirable, since it might lead to novel therapeutic agents for treating metastatic disease.

Attempts to design inhibitors of glycosylation have focused on alkaloids that alter processing of Asn-linked oligosaccharides (15), glycosides of N-acetylgalactosamine (16), various fluorinated analogs of common sugars (17, 18), and substrate analogs (19–21). Several compounds partially inhibit O-linked (mucin-like) oligosaccharide synthesis when added to cells at millimolar concentrations, such as N-acetylgalactosaminides (e.g. GalNAcα-O-benzyl) (22). This agent resembles naturally occurring glycoprotein intermediates (GalNAcα-O-Thr/Ser present in glycoproteins) and diverts oligosaccharide synthesis from endogenous glycoconjugates to the exogenous glycoside. Thus, the compound acts as a “primer” and a metabolic decoy. Suitably conjugated disaccharides also act as primers, such as per-O-acetylated Galβ1,4GlcNAcβ-O-naphthalenemethanol (AcGgN-NM), GlcNAcβ1,3Galβ-O-naphthalenemethanol (AcGnG-NM), and Galβ1,3GalNAcα-O-naphthalenemethanol, since these compounds resemble intermediates en route to mature O-linked chains of glycoproteins. Disaccharide-based primers act at much lower concentrations than N-acetylgalactosaminides.
tosaminides (10–50 μM versus 1–2 mM, respectively) (23–25) and have proven effective in blocking leukocyte adhesion to immobilized selectins in vitro (24). This finding encouraged us to examine their effect on human carcinoma cells, since positive results might suggest their use as antymetastatic agents.

EXPERIMENTAL PROCEDURES

Disaccharides, Cell Lines, and Radiolabeling—AcGn-NM, AcGnG-NM, and Galβ3Galβ1-N-acetylhexosaminitol methanethiosulfinate (AcGn-NM) were prepared as described (25, 26). [6-3H]GlcN-HCl (21.6 Ci/mmol), [6-3H]Gal (29.5 Ci/mmol), [6-3H]GlcN-HCl (21.6 Ci/mmol), and H2SO4 (1325 Ci/mmol) were purchased from PerkinElmer Life Sciences. Hal-8, MCF-7, and MV-522 cells were gifts from Dr. Osamu Matsuo (Kinki University School of Medicine, Osaka), Dr. Renate Pfliz (University of California, San Diego), and Dr. Nissi Varki (University of California, San Diego), respectively. All other cell lines were purchased from the American Type Culture Collection (Manassas, VA) (LS180, CCL 187; WiDr, CCL 218; A549, CCL 185; H460, HTB 177; A427, HTB 53; BT-20, HTB 19; SK-BR-3, HTB 30; PC-3, CRL 435; and U937, CRL 1593). Radiolabeling experiments were done in Dulbecco’s modified Eagle’s medium with low glucose (1 mM) supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.4% albumin (Gibco). Human umbilical cord vein endothelial cells were purchased from Cambrex Bio Science, Walkersville, MD. CSLEX-1, P-selectin, E-selectin, and E-rayta lectin-stained cells were incubated with and incubated with Streptavidin-R-phycocerythrin (1.85 μg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). In some experiments, cells were incubated with the monoclonal antibodies CSLEX-1 (anti-sLex, 5 μg/ml) or CD15 (anti-Lea; 8 μg/ml) (BD Biosciences) followed by fluorescein isothiocyanate-conjugated goat antibodies against mouse or human IgM (30 μg/ml).

Analysis of Primed Oligosaccharides—AcGn-NM and AcGnG-NM were dissolved in MeSO and added to growth medium to achieve the concentrations indicated in the table and figures. The final concentration of MeSO was adjusted to 0.5% (v/v). Cells were added to the supplemented medium at a density of ~2 × 10⁶ cells/ml. LS180 cells were incubated with the disaccharides and 50 μCi/ml [6-3H]fucose, [6-3H]glucosamine, [6-3H]GlcN, or H218O (2.5 ml). The conditioned medium was adjusted to 0.5 M NaCl and centrifuged to remove any particulate material, and the supernatants were applied to C18 Sep-Pak cartridges (Waters, Milford, MA) that had been prewashed sequentially with methanol, water, and 0.5 M NaCl. After application of the sample, the columns were washed with 0.5 M NaCl (2.5 ml) and water (25 ml). Radioactive oligosaccharides were eluted with 40% methanol. The products were analyzed by anion-exchange chromatography, (QAE-Septadex; Sigma) to separate the charged and neutral radiolabeled products (27). A sample of charged radiolabeled oligosaccharides was treated with Arthrobacter ureafaciens sialidase (20 milliunits/10⁶ cells (Calbiochem)) and analyzed by reverse phase HPLC (TosoHaas RP18, 4.6 mm × 25 cm, Tosoh Biosep LLC, Montgomeryville, PA) connected to a HPX 60H solvent delivery system (Rainin Instrument Company, Oakland, CA). The column was first washed with water (flow rate 0.5 ml/min) and then with increasing amounts of acetonitrile in water. Radioactivity in the eluant was monitored by the Radiomatic Flo-one Beta detector (PerkinElmer Life Sciences) connected in-line to the column.

Mass spectrometric data were recorded on a Thermo Finnigan LCQ

| Table I | Expression of sLeα and selectin ligands in tumor cells |
| --- | --- |
| Cancer cell lines | Tissue | Binding | Adhesion |
| LS180 | Colon | + | + | + | + |
| WiDr | Colon | + | + | + | + |
| A549 | Lung | + | + | + | + |
| H460 | Lung | + | + | + | + |
| A427 | Lung | + | + | + | + |
| Hal-8 | Lung | /+ | + | + | + |
| MV-222 | Lung | /+ | + | + | + |
| BT-20 | Breast | + | + | + | + |
| PC-3 | Prostate | + | + | + | + |
| U937 | Lymphoma | + | + | + | + |

| TABLE II | Mass spectrometry of oligosaccharides formed on GGN-NM and GgG-NM in LS180 colon adenocarcinoma cells |
| --- | --- |
| Oligosaccharidesa | Calculated | [M+H]+ | Found (m/z) |
| GgG-NM-neutral products | [HexNAc]3GgG-NM | 726.3 | 727.4 | 749.3 |
| [Hex]3[HexNAc]3GgG-NM | 888.3 | 889.8 | 911.4 |
| [Hex]2[HexNAc]3GgG-NM | 1091.4 | 1092.3 | 1115.0 |
| [Fuc]GgG-NM | 669.6 | 670.3 | 692.9 |
| [HexNAc]3[Fuc]3GgG-NM | 872.3 | 873.3 | 895.8 |
| [Hex]3[HexNAc]2[Fuc]GgG-NM | 1034.4 | 1035.3 | 1057.2 |
| [Hex]2[HexNAc]1[Fuc]2GgG-NM | 1180.5 | 1181.8 | 1209.8 |
| [Hex]1[HexNAc]1[Fuc]3GgG-NM | 1383.5 | 1384.9 | 1407.3 |
| [Hex]1[HexNAc]2[Fuc]2GgG-NM | 1691.6 | 1692.3 | 1715.0 |
| GgG-NM-charged products | [SO3H]1[Fuc]3GgG-NM | 749.2 | 750.2 | 772.4 |
| [SO3H]2[Fuc]2GgG-NM | 828.2 | 830.3 | 852.1 |
| [Neu5Ac]1[Fuc]3GgG-NM | 960.4 | 961.9 | 983.1 |
| GgG-NM-neutral products | [Hex]3[GgG-NM] | 685.3 | 686.5 | 709.0 |
| [Hex]2[Fuc]GgG-NM | 831.3 | 832.3 | 854.9 |
| GgG-NM-charged products | [Neu5Ac]1[Hex]3[GgG-NM] | 976.4 | 977.7 | 999.9 |
| [Neu5Ac]1[Hex]2[Fuc]2GgG-NM | 1122.4 | 1123.1 | 1145.9 |
| [SO3H]1[Hex]3[Fuc]2GgG-NM | 911.3 | 912.8 | 934.8 |
| [SO3H]1[Hex]2[Fuc]3GgG-NM | 991.2 | 992.7 | 1014.1 |

a Hex, most likely galactose; Fuc, fucose; Neu5Ac, N-acetylneuramic acid.
prior to analysis. Another portion of the sample ([6-3H]Fuc-labeled oligosaccharides. *, location of charged oligosaccharides containing sialic acid and sulfate.

LS180 colon adenocarcinoma cells (~2 × 10^5) were incubated with 50 μCi AcGnG-NM for 4–7 days in the presence of 50 μg/ml [6-3H]Fuc. The radiolabeled products were isolated by Sep-Pak C18 chromatography, and the neutral and charged oligosaccharides were separated by chromatography on a column of QAE-Sephadex (see "Experimental Procedures"). A sample (~5,000 cpm) was dissolved in 0.2 ml of water and injected into the C18 reverse phase HPLC column, and the products were eluted using a gradient of acetonitrile in water (dashed line). Another portion of the sample (~5,000 cpm) was treated with sialidase prior to analysis. A, before treatment with sialidase. B, after treatment with sialidase. The arrows indicate the elution positions of neutral [6-3H]Fuc-labeled oligosaccharides.

Cell Adhesion to Immobilized Selectins—96-Well plates were coated overnight at 4°C with recombiant E- or P-selectin (2–16 μg/ml) and blocked with 1% bovine serum albumin in phosphate-buffered saline. The plates were coated with E-selectin at 2 μg/ml for U937, LS180, BT-20, WiDr, and A549; 4 μg/ml for Hal-8 and A427; and 16 μg/ml for H460. For P-selectin, the optimal concentrations were 1 μg/ml for A427; 2 μg/ml for LS180, A549, and Hal-8; and 6 μg/ml for U937. Tumor cells were harvested with EDTA (5 mM, 20 min), resuspended in Dulbecco’s modified Eagle’s medium containing 1% fetal bovine calf serum, labeled with Calcein AM (5 μM; Molecular Probes, Inc., Eugene, OR), and added to the selectin-coated wells (5 × 10^4 cells/well). The cells were allowed to settle for 25 min at room temperature, and the plates were then stirred at 75 rpm on an orbital shaker for 15 min. Nonadherent cells were removed at unit gravity by inverting the plates for 15 min in a vessel filled with phosphate-buffered saline (29). For Hal-8, A427, A549, H460, and MV522, the plates were inverted for only 2–5 min, since the cells were much less adherent. After inversion, the wells were aspirated, and the amount of fluorescence was measured using a computerized fluorimeter (CytoFluor II, Promega, Madison, WI), averaging the

classic mass spectrometer (San Jose, CA). Samples containing primed products in water (10 μl) were introduced into the electrospray ionization ion trap using an autosampler connected to a TSP P4000 quaternary pump using 50% acetonitrile containing 0.1% formic acid. The data were fit to compositions of known oligosaccharides present in animal cells.

Binding of CSLEX-1 and Selectin Chimeras to Tumor Cells—To measure the presence of selectin ligands, cells were grown to confluence for 4–7 days in six-well plates. Binding of CSLEX-1 and selectin/IgG chimeras (Ps-Ig and Es-Ig) (28) was performed as described (24), except the enzyme-linked immunosorbent assay incubation buffer consisted of Dulbecco’s phosphate-buffered saline with calcium (Invitrogen), 1% bovine serum albumin, 0.01% NaN₃, and 1% goat serum. U937 cells were incubated with CSLEX-1 for 30 min, PC-3 cells for 3–4 h, and all other cell lines for 2 h in order to maximize binding. Incubations with Es-Ig (0.125–0.625 μg/ml) and Ps-Ig (0.1–0.5 μg/ml) were done for 1 h. In some experiments, cells were first treated for 1 h at 37°C with A. ureafaciens sialidase in 100 μl of 0.05 M HEPES (pH 6.9) or 2 mM EDTA in phosphate-buffered saline.
values of four wells for each condition. Controls included treating cells for 1 h at 37 °C with A. versicolor siastadiase (200 milliunits/1 × 10^6 cells) in 0.05 M HEPES buffer (pH 6.9), pretreating wells with anti-E-selectin or anti-P-selectin monoclonal antibody (1 µg/mL), or growth of cells in a 50 µM concentration of the inactive disaccharide, AcGalβ1,3Galβ-O-NM. Cell viability was judged to be >90% by trypan blue exclusion at the completion of each experiment.

**Sialyltransferase and Fucosyltransferase Assays**—Total sialyltransferase and fucosyltransferase (FucT) activities were assayed in cell lysates prepared from U937 and LS180 cells. The cells were scraped from the culture dish in 50 µL of a solution containing 0.25% sucrose, 20 mM Tris (pH 7.5) with protease inhibitor mixture (1:100; Sigma catalog no. P8340), and frozen at −80 °C. The protein content of each cell lysate was assayed by the Bradford assay. Total sialyltransferase assays (25 µl) contained 50 mM sodium cacodylate (pH 7.4), 0.25% Triton X-100, 15 mM MnCl2, 1 µCi of CMP-[9-3H]Neu5Ac (33.8 Ci/mmol; PerkinElmer Life Sciences), 0.1 µM CMP-Neu5Ac, 1 mM Galβ1,4GlcNAcβ-O-NM, and 50 µg of cell lysate. After incubation at 37 °C for 2 h, the reaction products were diluted with 0.5 µL of 0.5 M NaCl and applied to a Sep-Pak C18 cartridge. After washing the cartridge with 25 µL of water, the products were eluted with 50% methanol, dried, and counted by liquid scintillation. FucT assays (20 µL) contained 25 mM sodium cacodylate (pH 6.2), 0.5% Triton X-100, 10 mM MnCl2, 0.5 µCi of GDP-[2-3H]Fuc (17.3 Ci/mmol; PerkinElmer Life Sciences), 0.1 mM GDP-Fuc, 1 mM Galβ1,4GlcNAc-NM or 1 mM SiaβGal1,4GlcNAc (Sigma), and 50 µg of cell lysate. Reactions were incubated at 37 °C for 2 h (Galβ1,4GlcNAcβ-O-NM, FucT-a) and 3 h (Siaβ2,Galβ1,4GlcNAc, FucT-b). To separate the reaction products from GDP-[2-3H]Fuc, samples containing Galβ1,4GlcNAcβ-O-NM were diluted with 0.5 ml of 0.5 M NaCl and applied to a Sep-Pak C18 cartridge. After washing the cartridge with 25 mL of water, the products were eluted with 50% methanol, dried, and counted by liquid scintillation. Reactions containing Siaβ2,Galβ1,4GlcNAc were diluted with 0.5 ml of 5 mM PO4 (pH 6.8) and applied to a small column of Dowex 1 (X8–400, phosphate form). Reaction products eluted in the flow-through and the wash (2 ml of water) (30).

**Sample Preparation and Processing for Microarray Analysis**—U937 and LS180 cells were grown in triplicate in 150-mm diameter culture dishes until confluent. RNA from each dish was isolated using TRIzol (Life Technologies, Valencia, CA) and kept frozen at −80 °C. Total RNA was prepared using standard Affymetrix (Santa Clara, CA) GeneChip protocols for all labeling, staining, and scanning procedures (available on the World Wide Web at affymetrix.com) (31). Each labeled sample was hybridized to individual glyco-v1 chips, an oligonucleotide designed with a mismatch-Ig and E-selectin-Ig chimeras, but not in all cases (e.g. A427 and A549 lung carcinomas, SK-BR-3 breast carcinoma, and PC-3 prostate carcinoma), consistent with the heterogeneity of carbohydrate ligands for these selectins. The LS180 human colon carcinoma was chosen for further study, since it expresses ligands that bound to CSLEX-1 and the selectin chimeras (Table I).

**RESULTS AND DISCUSSION**

The carbohydrate antigens sLeα and sLeα are expressed on leukocytes, epithelial cells, and many carcinomas in configurations that can be recognized by one or more selectin receptors. A survey of established tumor lines using a monoclonal antibody to sLeα (CSLEX-1) showed that many express sLeα-containing antigens, with the exception of the breast carcinomas, MCF-7 and SK-BR-3 (Table I). The pattern of reactivity correlated in most cell lines with binding of recombinant P-selectin-Ig and E-selectin-Ig chimeras, but not in all cases (e.g. A427 and A549 lung carcinomas, SK-BR-3 breast carcinoma, and PC-3 prostate carcinoma), consistent with the heterogeneity of carbohydrate ligands for these selectins. The LS180 human colon carcinoma was chosen for further study, since it expresses ligands that bound to CSLEX-1 and the selectin chimeras (Table I).

Peracetylated disaccharides, such as AcGnNM and AcGn-NM, metabolically inhibit the expression of sLeα in U937 lymphoma cells. Cells take up the glycosides by passive diffusion, deacetylate them with endogenous carboxyesterases, and use them as substrates for glycosyltransferases, resulting in the generation of oligosaccharides containing Lewis type antigens or precursor structures (23–25). Both compounds are nontoxic up to 100 µM based on trypan blue exclusion and normal growth rates. To determine whether the disaccharides had similar properties in LS180 colon carcinoma cells, they were added to the culture medium along with radioactive fucose, galactose, glucosamine, or sulfate to label newly made oligosaccharides. Both compounds stimulated the incorporation of radiolabeled sugars and sulfate into glycans assembled on the exogenous primers, confirming that they were taken up, deacetylated, and made available to the glycosyltransferases in the cell (data not shown).

![Fig. 3. Total sialyltransferase and fucosyltransferase activity in LS180 and U937 cells.](image-url)
The acetylated disaccharides resemble in behavior N-acetylgalactosaminides (e.g. GalNAc-O-Bn), which without prior acetylation are taken up by most types of cells. However, GalNAc-O-Bn impacts the first committed step in biosynthetic pathways at later stages of oligosaccharide assembly. Furthermore, the disaccharide-based compounds are active at much lower concentrations (10 μM versus 0.5 mM, respectively). As shown below, the acetylated disaccharides show more selective effects than GalNAc-O-Bn.

To examine the full array of oligosaccharides generated on the disaccharide primers, samples were analyzed by electrospray mass spectrometry (Table II). Several patterns were present, such as one or more Galβ1,4GlcNAcβ1,3 units on GGn-NM and the addition of a Gal residue to GgG-NM, consistent with the presence of β1,4Gal and β1,3GlcNAc transferases. Fucose was added to several oligosaccharides primed on GGn-NM and GgG-NM, presumably in the latter case after the addition of a Gal residue (24). The disaccharide-generated on GGn-NM and GgG-NM consisted of both neutral and charged species, and the charged products contained sialic acid and/or sulfate residues. Most of the charged species consisted of sialylated oligosaccharides based on their conversion to neutral oligosaccharides by treatment with a nonspecific sialidase. Fig. 1 shows that the charged fucosylated oligosaccharides were mostly sialylated (the arrows indicate the position of neutral oligosaccharides derived from the charged compounds after treatment with sialidase). The resistant material contained sulfate as assessed by labeling studies with 35SO4 (data not shown). Both primers generated oligosaccharides that resembled Lewis and sialylated Lewis type antigens (Table II).

**Disaccharide Treatment Alters Glycosylation**—To assess whether priming of oligosaccharides on the glycosides altered glycosylation of endogenous glycoconjugates, treated cells were analyzed by flow cytometry after reaction with fluorescent plant lectins and monoclonal antibodies that bind to specific carbohydrates. The signal intensity of untreated cells was set to 100% for each lectin or antibody, and the relative value for treated cells was determined (Fig. 2). In LS180 cells, staining by *M. amurensis* hemagglutinin, which recognizes terminal α2,3-linked sialic acid residues and 3-O-sulfated galactose (36), was reduced by both disaccharides. Binding was completely abolished by sialidase treatment (data not shown), indicating that the major determinant expressed by LS180 cells was a sialylated component. Binding of the monoclonal antibody CD15, which recognizes Lea (Galβ1,4(Fucα1,3)GlcNAc−), increased or did not change, suggesting that fucosylation was not inhibited. Peanut agglutinin, which recognizes Galβ1,3GalNAc−, strongly stained disaccharide-treated cells, suggesting a parallel decrease in sialylation of core 1 O-glycans. In contrast, staining by *S. nigra* agglutinin increased. This lectin recognizes α2,6-linked sialic acid-containing structures, suggesting that the disaccharides selectively decreased α2,3-sialylation.

Malignant transformation is often accompanied by increased β1,6 branching of Asn-linked glycans in glycoproteins, which has been correlated with tumor growth (37). No difference between control and disaccharide-treated cells was observed by flow cytometry after reaction with fluorescent plant lectins and monoclonal antibodies that bind to specific carbohydrates. The signal intensity of untreated cells was set to 100% for each lectin or antibody, and the relative value for treated cells was determined (Fig. 2). In LS180 cells, staining by *E. cristagalli* lectin, and *R. communis* agglutinin I (terminal β-linkaged galactose) revealed modest reduction in reactivity (Fig. 2). In general, treatment of LS180 cells with AcGGn-NM had similar but less profound effects on lectin staining than AcGnG-NM. On the other hand,
GalNAc/H9251-O-Bn had very different effects, profoundly reducing both H9251 2,3-sialylation (M. amurensis hemagglutinin) and fucosylation (CD-15) and increasing the reactivity of cells to P. vulgaris leukoagglutinin, L. esculentum lectin, S. tuberosum lectin, E. cristagalli lectin, and in particular S. nigra agglutinin and peanut agglutinin.

U937 cells primed many of the same oligosaccharides as in LS180 cells (Table II and Ref. 24), but they differed significantly in lectin reactivity after disaccharide treatment (Fig. 2). In U937 cells, both disaccharides significantly increased MAH staining and only modestly affected S. nigra agglutinin binding. Fucosylation decreased, based on reaction with A. auranti a lectin, which recognizes α1,3-linked fucose residues, in agreement with previous studies (24). GalNAc-O-Bn decreased both sialylation and fucosylation in these cells.

Inhibitory Activity Correlates Inversely with Sialyltransferase and Fucosyltransferase Expression—To investigate why the disaccharides differentially affected sialylation and fucosylation in LS180 and U937 cells, respectively, total sialyltransferase and fucosyltransferase levels were measured by enzyme assays of cell homogenates. The total sialyltransferase activity was significantly lower in LS180 cells compared with U937 cells (34 ± 3.2 pmol/mg/h versus 168 ± 16 pmol/mg/h), using Gal1,4GlcNAc-OM as the acceptor (Fig. 3). In contrast, the total fucosyltransferase activity using either Gal1,4GlcNAc-OM (FucT-a; 43 ± 4.7 pmol/mg/h) or NeuAc2,3Gal1,4GlcNAc (FucT-b; 12.2 ± 2.2 pmol/mg/h) as the acceptor was much lower in U937 cells compared with LS180 cells (FucT-a, 168 ± 16 pmol/mg/h; FucT-b, 240 ± 35 pmol/mg/h). Thus, an inverse correlation exists between overall enzyme activity and whether the disaccharides inhibit fucosylation or sialylation.

In order to examine whether specific transferases were affected, mRNA samples were isolated from U937 and LS180 cells and analyzed using the Glyco-v1 GeneChip microarray. Expression signals were calculated using the Perfect Match-only model of dChip (33). Microarray expression signals were analyzed by cluster analysis in order to reveal similarities among microarray data sets, and changes in gene expression were tested statistically across the two cell types. Affymetrix MA5.0 was used to make present, absent, and marginal calls for each gene expression measurement.

Hierarchical cluster analysis of expression data showed that a number of genes can be identified with high confidence as differentially expressed between the two cell types (data not shown). Interestingly, only ST3GalIV was detected in LS180 cells (Table III), suggesting that this isozyme was the target for the disaccharides. Recent genetic studies of ST3GalIV-deficient mice demonstrate the accumulation of the core 1 unit of O-
linked oligosaccharides (Galβ1,3GalNAcβ-) and oligosaccharides that terminate in Gal residues in a tissue- and glycoprotein-specific manner (38, 39). Absence of the enzyme results in enhanced binding of peanut agglutinin, like that seen in disaccharide-treated cells (2). ST3GalIV also appeared to be expressed at comparable levels in U937 cells, and ST3GalIV and ST3GalVI were also present (40, 41). These isozymes are thought to be involved in ganglioside biosynthesis. The lack of inhibition of sialylation in these cells suggests that the individual isozyme is less important than the overall level of activity (Fig. 3).

The expression data for the α1,3-fucosyltransferases also showed striking differences (Table IV). U937 cells expressed FucTIV and FucTVII, in agreement with previous studies of myeloid cells (42–44). The disaccharides affected fucosylation in this cell line (Fig. 2), suggesting that one or both isozymes utilize the disaccharides in vitro. LS180 cells expressed FucTIII and FucTVI (Table IV). These enzymes also apparently utilize the disaccharides, since fucosylated products were generated (Table II), but priming was not sufficient to block fucosylation of glycoconjugates on the cell surface (Fig. 2). The high overall level of fucosyltransferase activity in LS180 cells compared with U937 cells may explain the apparent lack of inhibition by the disaccharides.

**Inhibition of Selectin Ligand Presentation**—To test whether the decrease in α2,3-sialylation in LS180 cells led to depression of sLeα expression, cells were reacted with CSLEX-1, a monoclonal antibody that reacts with sLeα-containing oligosaccharides, and chimeras of E-selectin-Ig and P-selectin-Ig. The amount of bound reagent was assessed by enzyme-linked immunosorbent assay (Fig. 4). Both AcGnG-NM and AcGnG-NM decreased binding in a dose-dependent fashion. In general, AcGnG-NM was more effective than AcGnG-NM, with inhibition occurring at doses as low as −15 μM. Control experiments showed that treatment of the cells with sialidase or 2 mM EDTA abrogated binding of CSLEX-1 and E-selectin and P-selectin chimeras, as expected for selectin-dependent processes. Treatment of cells with 50 μM AcGnG-NM, which is not related in structure to oligosaccharides bearing sLeα, did not affect binding of CSLEX-1 (data not shown).

Many of the tumor lines expressing P-selectin and E-selectin ligands bound to plates coated with recombinant selectins (Table I). Cell adhesion to immobilized selectins is a multivalent process and therefore should be very sensitive to alterations in ligand presentation due to decreased sialylation. When treated with AcGnG-NM or AcGnG-NM, adhesion of LS180 cells was diminished in a dose-dependent manner, reaching ~80% inhibition at the highest dose tested (Fig. 5). Inhibition was similar to that caused by pretreatment of the cells with sialidase or blocking antibodies. Cells treated with acetylated Galβ1,3Galβ-0-NM, which did not diminish sLeα expression, did not affect cell adhesion, indicating the specificity of the effects.

**Summary and Perspective**—Acetylated disaccharides that resemble the core structure of Lewis antigens prime oligosaccharides in LS180 colon carcinoma cells. Priming in this way inhibits α2,3-sialylation and inhibits the formation of sialylated Lewis antigens recognized by selectins. In U937 cells, the disaccharides also caused an inhibition of selectin ligands, but in this case, the effect is due to altered fucosylation. The inhibition of sialylation and fucosylation by disaccharides validates the relevant α2,3-sialyltransferase (ST3GalIV) and α1,3-fucosyltransferases (FucTIV and FucTVII) expressed in sensitive tumor lines as a target for anti-selectin-based therapies.

Previous studies have shown that GalNAc-O-Bn can inhibit sialylation or fucosylation dependent on cell type (45–48). GalNAc-O-Bn in LS180 and U937 cells suppressed both α2,3-sialylation and α1,3-fucosylation and dramatically increased α2,6-sialylation, indicating a lack of specificity. Thus, acetylated disaccharides have several distinct advantages over monosaccharide primers such as GalNAc-O-Bn, including efficacy at much lower concentrations and greater specificity. Furthermore, the presence of two monosaccharide residues in the disaccharide primers provides a larger framework for making functional group analogs, which may prove successful as active site-directed inhibitors of the relevant α2,3-sialyltransferase and α1,3-fucosyltransferases.

The differential sensitivity of sialylation and fucosylation in LS180 and U937 cells appears to reflect differences in the level of expression of the relevant fucosyltransferases and sialyltransferases, with an inverse correlation to their overall activity. The microarray data provided additional insight by providing evidence for the selective effects on subsets of enzymes expressed in each cell type. Together, the findings suggest that one might be able to predict how pharmacological agents will affect glycosylation by simply measuring overall transferase activities and by profiling the enzyme mRNA levels. Since expression of the transferases may be regulated both transcriptionally and translationally, deviations from the expected patterns could occur. Other factors may play a role as well, including availability of nucleotide sugars and flux of endogenous substrates coming through the system. Nevertheless, the combination of priming data, lectin binding, enzyme assays, and microarray data provides a way to evaluate the potential efficacy of at least primer-based decoys and perhaps other inhibitory agents that act on glycosylation. Application of this information to particular tumor cell types may optimize drug discovery and development for treating cancer and other disorders, such as inflammation, in which selectin-carbohydrate interactions play a pathophysiological role.

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