Loss of Disialyl Lewis[a], the Ligand for Lymphocyte Inhibitory Receptor Sialic Acid-Binding Immunoglobulin-Like Lectin-7 (Siglec-7) Associated with Increased Sialyl Lewis[a] Expression on Human Colon Cancers

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ABSTRACT

Expression of sialyl Lewis[a] is known to be increased in cancers of the digestive organs. The determinant serves as a ligand for E-selectin and mediates hematogenous metastasis of cancers. In contrast, disialyl Lewis[a], which has an extra sialic acid attached at the C6-position of penultimate GlcNAc in sialyl Lewis[a], is expressed preferentially on nonmalignant colonic epithelial cells, and its expression decreases significantly on malignant transformation. Introduction of the gene for an $\alpha2\rightarrow6$ sialyltransferase responsible for disialyl Lewis[a] synthesis to colon cancer cells resulted in a marked increase in disialyl Lewis[a] expression and corresponding decrease in sialyl Lewis[a] expression. This was accompanied by the complete loss of E-selectin binding activity of the cells. In contrast, the transfected cells acquired significant binding activity to sialic acid-binding immunoglobulin-like lectin-7 (Siglec-7)/p75/adhesion inhibitory receptor molecule-1, an inhibitory receptor expressed on lymphoid cells. These results indicate that the transition of carbohydrate determinants from disialyl Lewis[a]-dominant status to sialyl Lewis[a]-dominant status on malignant transformation has a dual functional consequence: the loss of normal cell-cell recognition between mucosal epithelial cells and lymphoid cells on one hand and the gain of E-selectin binding activity on the other. The transcription of a gene encoding the $\alpha2\rightarrow6$ sialyltransferase was markedly down-regulated in cancer cells compared with nonmalignant epithelial cells, which is in line with the decreased expression of disialyl Lewis[a] and increased expression of sialyl Lewis[a] in cancers. Treatment of cancer cells with butyrate or 5-azacytidine induced strongly disialyl Lewis[a] expression, suggesting that histone deacetylation and/or DNA methylation may be involved in the silencing of the gene in cancers.

INTRODUCTION

The carbohydrate determinant sialyl Lewis[a] is known to be expressed strongly on cancers of the digestive organs and to serve as a ligand for vascular E-selectin in hematogenous metastasis of cancers (1–5). However, the molecular mechanism underlying accelerated expression of sialyl Lewis[a] determinant in cancers is not well understood. The levels of many glycosyltransferases involved in the synthesis of the determinant and also the levels of their mRNA expression have been studied, but no conclusive results have been obtained to date (4, 6–9).

It has long been known that cell surface carbohydrate determinants undergo drastic alteration during malignant transformation. The major mechanism that leads to the altered expression of carbohydrate determinant in cancer, in general, was called previously an "incomplete synthesis" in the early 1980s (10–12). The synthesis of complex carbohydrate determinants, well-developed on normal epithelial cells, tends to be impaired on malignant transformation, predisposing the cells to express less complicated carbohydrate determinants.

In line with this notion, we showed previously that the $2\rightarrow3$, $2\rightarrow6$ disialyl Lewis[a] determinant, which has an extra sialic acid residue attached at the C6 position of penultimate GlcNAc through $\alpha2\rightarrow6$ linkage to the well-known sialyl Lewis[a] determinant, is expressed preferentially on nonmalignant epithelial cells of the digestive organs, and its expression decreases on malignant transformation (13–15). In most specimens from patients with cancers of the pancreas, biliary tract, stomach, and colon, the $2\rightarrow3$ sialyl Lewis[a] determinant was expressed strongly in cancer cells, whereas disialyl Lewis[a] determinant having $2\rightarrow6$ sialyl modification was expressed preferentially on nonmalignant epithelial cells and expressed less frequently in cancer cells (13–15). In these three preceding studies, we predicted that a disturbance of $2\rightarrow6$ sialylation of the determinants on malignant transformation of epithelial cells could be one of the major mechanisms leading to the increased expression of sialyl Lewis[a], the mono-sialylated determinant, in cancers. This could well be a good example of the induction of cancer-associated carbohydrate determinants because of the above-mentioned incomplete synthesis.

We identified biochemically ST6GalNAc6 recently as a sialyltransferase responsible for the synthesis of $2\rightarrow3/2\rightarrow6$ disialyl Lewis[a] determinant (16). In the present study, we investigated the possible role of the $2\rightarrow3$, $2\rightarrow6$ disialyl Lewis[a] determinant in the enhancement of sialyl Lewis[a] expression in human colon cancers to see whether the classical concept of incomplete synthesis is applicable for the induction of sialyl Lewis[a] expression in human cancers. We attempted also to elucidate the biological function of $2\rightarrow3$, $2\rightarrow6$ disialyl Lewis[a] determinant using the cells transfected with ST6GalNAc6 cDNA.

MATERIALS AND METHODS

Cells, Antibodies, and Transfection of Cells with ST6GalNAc6 cDNA. Human colon cancer cell lines SW1083 and DLD-1, were maintained in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (BioWhittaker, Gaithersburg, MD) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. The ST6GalNAc6 transfectant cell lines, SW1083/ST6GalNAc6 clones 1–9, 1–5, 2–8, and 2–5, were established by the transfection of expression vector pcDNA3.1-hyg(+), containing cDNA for human ST6GalNAc6 (GenBank accession no. Ab035173) inserted at its *Nhe*I/*Xho*I site into SW1083 cells using the LipofectAMINE Plus Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. For analysis of the expression of carbohydrate determinants, the cells were mixed and incubated with the anticarbohydrate monoclonal antibody (purified antibody at

1 μg/ml or culture supernatant at a dilution of 1:10) at 4°C for 30 min. The anti-2→3 sialyl Lewis[a] antibody N19-9 (murine IgG1) was obtained from Alexis Biochemicals Corporation (Lausen, Switzerland). The anti-2→3, 2→6 disialyl Lewis[a] (FH7; murine IgG3) and anti-2→3, 2→6 disialyl Lewis[c] (FH9; murine IgG2a) were prepared as described previously (13–15). The cells were then washed three times with PBS containing 0.5% BSA and stained with 1:200 dilution of FITC-conjugated goat antimouse immunoglobulin (Silenus Laboratories, Hawthorn, Victoria, Australia) at 4°C for 30 min. Binding of the antibodies to the cells was evaluated by flow cytometry performed with FACScan (Becton Dickinson, Mountain View, CA).

For preparation of the monoclonal antibodies to Siglec-7, DA rats (the Shizuoka Agriculture Cooperative Association for Laboratory Animals, Shizuoka, Japan) were immunized once with Siglec-7-Chinese hamster ovary cells (1.3×10^7 cells; Ref. 17), and iliac lymph node cells were fused with the PAI myeloma in 18 days. Supernatants that reacted with Siglec-7-Chinese hamster ovary cells but not parent Chinese hamster ovary cells were identified by immunostaining. Positive hybridomas were cultured in ASF104 nonserum medium (Ajinomoto, Tokyo, Japan), and antibodies in the supernatants were purified with protein G-Sepharose (Amersham). A neutralizing anti-Siglec-7 antibody (13-3-D and rat IgG2b) was used in this study.

Binding Studies of Recombinant E-Selectin and Siglec-7. Recombinant human E-selectin-IgG chimera were kindly provided by Drs. Hirosato Kondo and Yoshimasa Inoue of the Department of Chemistry, R&D Laboratories, Nippon Organon K.K. (Osaka, Japan). For preparation of recombinant Siglec-7-immunoglobulin chimera and mutant Siglec-7-immunoglobulin chimera, the DNA fragment of extracellular domain of Siglec-7 and its mutant (R124K) were amplified by PCR using the following primers: 5′-CCAACC*GTCGA-CAT*GCTGCTGCTGCTGCTG-3′ (underline shows initiation codon, and an italic portion shows a SalI site); and 5′-CCAACC*ACTAGT*ACTCACCCTGT-TGCAGGGAGAGGTTCA-3′ (underline shows splicing donor, and an italic portion shows a SpeI site). The PCR products were digested with SalI and SpeI and then ligated to SalI and SpeI sites of pEF-Fc (a generous gift from Dr. Yoshihara, RIKEN, Saitama, Japan), to produce a fusion construct of Siglec-7 extracellular domain and human IgG Fc domain. The construct was then digested with SalI and NotI and ligated to XhoI and NotI sites of a modified pcXN2. The pcXN2-Siglec-7-Fc or -mutant Fc was transfected to Chinese hamster ovary cells to obtain a clone that secreted Siglec-7-Fc. The transfectants were cultured in ASF104 nonserum media, and Siglec-7-Fc in the supernatants was purified with protein G-Sepharose.

Recombinant E-selectin and Siglec-7 were preincubated with affinity-purified rabbit antihuman IgG (Dako, Glostrup, Denmark) followed by incubation with phycoerythrin-streptavidin (Dako) before application to the binding analyses with SW1083 or transfectant clones. Binding of recombinant molecules to the cells was evaluated by flow cytometry. When indicated, the cells were incubated with blocking antibodies (10 μg/ml) for 30 min at 37°C. For the inhibition experiments, rat monoclonal anti-Siglec-7 neutralizing antibody

(clone 13-3-D) was used at 10 μg/ml. In some experiments, the binding specificity of recombinant Siglec-7-immunoglobulin was ascertained by ELISA using pure synthetic carbohydrate determinants (18). The structures of carbohydrate determinants used in this study are summarized in Table 1.

Cell Adhesion Experiments Mediated by E-Selectin and Siglec-7. Nonstatic monolayer cell adhesion experiments were performed as described previously (5, 19). Parental SW1083 cells and SW1083/ST6GalNAc6 clones were cultured in monolayer in 24-well plates. To this, 2′7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester-labeled 300.19/E-selectin cells (1×10^6 cells/0.5 ml/well and murine B-lymphoma 300.19 cells transfected with human E-selectin cDNA; Ref. 20) were added, and the plate was placed on a rotating platform for incubation under shear (90 rpm) for 20 min at room temperature. The 300.19/E-selectin cells were kindly supplied by Dr. Geoffrey S. Kansas, Department of Microbiology-Immunology, Northwestern University Medical School (Chicago, IL). With Siglec-7-mediated cell adhesion, U937 cells transfected with cDNA for human Siglec-7 or mutant Siglec-7 were sialidase treated and labeled with 2′7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester. The U937 cells expressing mutant Siglec-7 were used as control for background binding. After nonadherent cells were washed out three times with PBS, the cells were lysed with 0.5% NP40, and the attached cells were counted by measuring fluorescence intensity using an Arvo 1420 multilabel counter (Wallac, Gaithersburg, MD). For generation of U937 cells expressing Siglec-7, pcDNA3.1-Siglec-7-*myc* (17) was digested with NheI and AflII. The fragment was then ligated to NheI-AflII sites of a modified pcXN$_c$ in which a multicloning site is inserted. A point mutant (R124K) was introduced using primers (sense, 5′-AGATACTTCTTTAA-GATGGAGAAAGG-3′; and antisense, 5′-CCTTTCTCCATCTTAAA-GAAGTATCT-3′) by the same method as described previously. The constructs were transfected into U937 cells by electroporation in a GenePulser at 240V with 950 μF capacitance (Bio-Rad, Richmond, CA). After 48 h of culture, G418 (600 μg/ml) was added to the cells, and the clones were then isolated and analyzed by flow cytometry.

Real-Time Reverse Transcription-PCR (RT-PCR) Analysis of ST6GalNAc6 mRNA Expression in Colon Cancers. Surgical specimens were obtained from 21 patients with colorectal cancer at surgical operation and processed as described previously (6, 9). The median age of patients was 59.8 years. The carcinomas were staged according to the Astler-Coller modification of Dukes' classification (21). Malignant and nonmalignant portions of each specimen were used for RNA extraction. Nonmalignant mucosa was scraped off using slide glasses, and tissue specimens of cancer were carefully excised to eliminate noncancerous tissue components. This was done with reference to histological findings of tissue sections prepared from the same specimens. The disialyl Lewis[a] determinant, the product of the ST6GalNAc6 gene, was expressed only on colonic epithelial cells and was not expressed in endothelial cells, fibroblasts, or mucosal leukocytes in the tissue sections. Samples were frozen rapidly and stored at −80°C until RNA extraction. Specimens were

Table 1 *Structures of carbohydrate determinants used in this study*

The disialyl Lewis[a] and disialyl Lewis[c] determinants were shown previously to be preferentially expressed on nonmalignant epithelial cells of digestive organs, whereas (mono-) sialyl Lewis[a] determinant was known to be expressed predominantly on cancer cells, suggesting that a decrease in 2 → 6 sialylation on malignant transformation is involved in the abnormal accumulation of sialyl Lewis[a] in cancer tissues (13–15). "R" stands for "→ 3Galβ1 → 4Glaβ1 → ceramide" in the synthetic determinants used in the enzyme-linked immunosorbent assays in experiments described in Fig. 4.

Determinant	Structure	Specific antibody
Disialyl Lewis[a] (2 → 3, 2 → 6 disialyl Lewis[a])	NeuAcα2 NeuAcα2 ↓ ↓ 3 6 Galβ1→3GlcNAcβ1→R 4 ↑ Fucα1	FH7
Sialyl Lewis[a] (2 → 3 sialyl Lewis[a], monosialyl Lewis[a])	NeuAcα2 ↓ 3 Galβ1→3GlcNAcβ1→R 4 ↑ Fucα1	N19-9
Disialyl Lewis[c] (2 → 3, 2 → 6 disialyl Lewis[c])	NeuAcα2 NeuAcα2 ↓ ↓ 3 6 Galβ1→3GlcNAcβ1→R	FH9

powdered in liquid N_2, and total cellular RNA was extracted with guanidine isothiocyanate and purified by cesium chloride gradient centrifugation. Real-time RT-PCR analysis was performed using ABI prism 7000 (Perkin-Elmer) with a TaqMan probe for S6GalNAc6 provided by the manufacturer (assay Id Hs00203739_m1). The results were normalized as relative values using glyceraldehyde 3-phosphate dehydrogenase as a reference to compare mRNA expression. The results of real-time RT-PCR were ascertained by conventional RT-PCR using the set of primers described below.

Induction of 2→3, 2→6 Disialyl Lewis[a] Determinant and ST6GalNAc6 mRNA Expression in Colon Cancer Cells. A cultured human colon cancer cell line, DLD-1, was cultured in the presence of butyrate (4 mM) or 5-aza-cytidine (20 μM) for 3 days and subjected to flow cytometric analysis for expression of 2→3, 2→6 disialyl Lewis[a]. Total RNA of cultured cells was isolated from 1×10^6 cells according to the acid guanidinium thiocyanate-chloroform extraction method using an Isogen kit (Nippon-Gene, Tokyo, Japan) and analyzed for ST6GalNAc6 mRNA by RT-PCR using ST6GalNAc6-specific primers (upper, CTCATCACCATCCTCATCCT; and lower, GAGACGGCATATGCTTCCAT) and human glyceraldehyde 3-phosphate dehydrogenase specific primers (upper, TGAAGGTCGGAGTCAACG-GATTTGGT; and lower, CATGTGGGCCATGAGGTCCACCAC).

Confocal Microscopic Analysis. Frozen sections of 10-μm thickness were prepared from a surgical specimen and studied for confocal microscopic observation. Polyclonal rabbit anti-Siglec-7 antibody (rabbit IgG raised against recombinant Siglec-7 and affinity purified) and monoclonal anti 2→3, 2→6 disialyl Lewis[a] (murine IgG) were used as primary antibodies. Alexa Fluor 488-labeled antirabbit IgG (green) and Alexa Fluor 594-labeled antimurine IgG (red) antibodies (Molecular Probes Inc., Eugene, OR) were used as secondary antibodies. A Bio-Rad Radiance 2100 inverted confocal laser scanning microscope equipped with LaserSharp 2000 software was used for observation.

RESULTS

Transfection of Cells with ST6GalNAc6 sDNA. The human cultured colon cancer cell line SW1083 expressed moderately 2→3 sialyl Lewis[a] determinant and essentially no 2→3, 2→6 disialyl Lewis[a] determinant under usual culture conditions. A significant induction of 2→3, 2→6 disialyl Lewis[a] determinant, as defined by the specific monoclonal antibody FH7, was observed when the cells were transfected with ST6GalNAc6 sDNA. Fig. 1A shows the results of flow cytometric analysis of the four clones, clones 1–9, 1–5, 2–8, and 2–5, where clones 1–9 and 1–5 were weak expressors, and clones 2–8 and 2–5 were strong expressors of 2→3, 2→6 disialyl Lewis[a] determinant. Expression of the 2→3, 2→6 disialyl Lewis[a] determinant in these clones was well correlated with the amount of ST6GalNAc6 mRNA as ascertained by real-time RT-PCR analyses (Fig. 1B). The mRNA levels in terms of relative expression coefficient (the number of amplicons/10^3 glyceraldehyde 3-phosphate dehydrogenase amplicons) were 15.7 ± 1.2 and 26.7 ± 1.2 in low expressor clones 1–9 and 1–5, respectively, and 60.5 ± 5.0 and 65.8 ± 8.6 in high expressor clones 2–8 and 2–5, respectively, whereas that for parental SW1083 cells were 0.58 ± 0.06 (mean $\pm$ SD; all of the clones showed statistical significance in comparison with parental cells at $P < 0.001$).

The expression of sialyl Lewis[a], the cancer-associated monosialy-lated determinant defined by N19-9 antibody, showed an inverse correlation with that of 2→3, 2→6 disialyl Lewis[a] determinant among these clones (Fig. 1A), suggesting substrate competition for the synthesis of these determinants. The correlation coefficient between the sialyl Lewis[a] expression and the 2→3, 2→6 disialyl Lewis[a] expression was $r = -0.988$ (Pearson's correlation coefficient), showing a good inverse correlation with statistical significance at $P < 0.002$. Because it has been shown previously that ST6GalNAc6 can transfer sialic acid only to the synthetic precursors of these determinants having no fucose residues, the substrate competition is predicted to
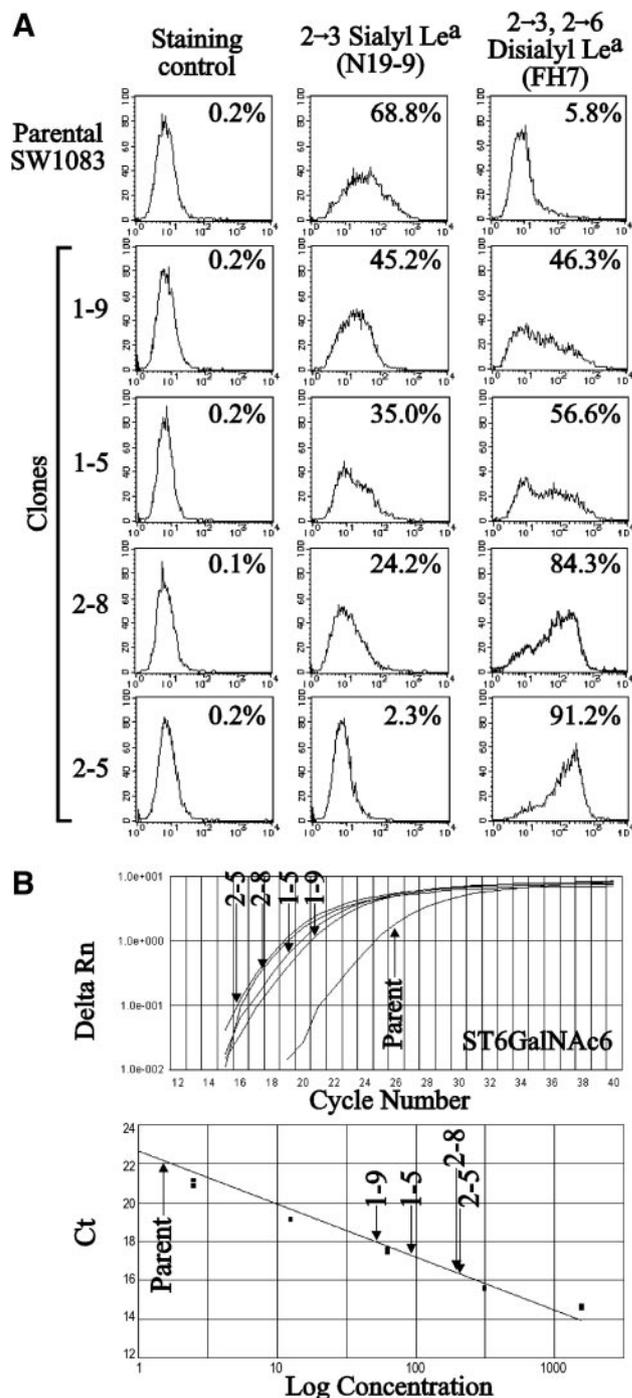

Fig. 1. Flow cytometric analysis of disialyl Lewis[a] and monosialyl Lewis[a] determinants on human colon cancer SW1083 cells and the clones transfected with ST6GalNAc6 cDNA. Results on four representative transfectant clones (1–9, 1–5, 2–8, and 2–5) are shown. A, ordinate, number of cells; abscissa, fluorescence intensity. Monoclonal antibody FH7 was used for detection of the 2→3, 2→6 disialyl Lewis[a] determinant and N19-9 for the 2→3 monosialyl Lewis[a] determinant. Percentage of positive cells is shown in the flow profiles. B, expression of ST6GalNAc6 mRNA in these clones analyzed by real-time reverse transcription-PCR. Delta Rn *versus* cycle number plot and *Ct versus* log concentration plot are shown. *Rn*, randon.

occur at the level of conversion of 2→3 sialylated Lewis[c] to 2→3, 2→6 disialylated Lewis[c] as suggested previously (16).

Significance of 2→3, 2→6 Disialyl Lewis[a] Determinant in E-Selectin-Mediated Cell Adhesion. Although we have shown previously that sialyl Lewis[a], the cancer-associated determinant, serves as a ligand for E-selectin and is capable of mediating E-selectin-medi-

ated cell adhesion (4, 5, 22), we tested next whether the transfectant cells, which have decreased sialyl Lewis[a] expression but an increased $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] expression, have any binding activity to E-selectin. As shown in Fig. 2A, the transfectant clones showed a significantly decreased adhesion to E-selectin-expressing 300.19 cells compared with parental SW1083 cells, and the degree of decrease correlated well with the decrease in sialyl Lewis[a] expression, whereas no correlation was observed with the expression of $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] on the clones.

The results of cell-cell adhesion experiments were clearly reproduced and supported by the binding studies of recombinant E-selectin in flow-cytometry, as shown in Fig. 2B, which indicated a good correlation of recombinant E-selectin-immunoglobulin binding to the expression of sialyl Lewis[a] determinant but not to $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] expression. The correlation coefficient between the sialyl Lewis[a] expression and E-selectin binding was $r = 0.998$ (statistically significant at $P < 0.0005$) in these clones. Addition of EDTA in the incubation medium almost suppressed completely the binding, thereby confirming that the binding is Ca^{2+}-dependent, which is

compatible with E-selectin-mediated binding. Addition of the N19-9 antibody specific to sialyl Lewis[a] abrogated completely the binding of recombinant E-selectin-immunoglobulin, whereas that of FH7 antibody specific to $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] had essentially no effect (Fig. 2B), indicating that only sialyl Lewis[a] served as a ligand for E-selectin, whereas $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] did not.

Significance of $2{\rightarrow}3$, $2{\rightarrow}6$ Disialyl Lewis[a] Determinant in Siglec-7-Mediated Cell Adhesion. Because the $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[c] determinant, with its structure closely related to $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] determinant, was reported earlier to have a binding activity to a sialic-acid-dependent cell adhesion molecule, Siglec-7 (23), we tested next the binding activity of the transfectant cells to recombinant Siglec-7-immunoglobulin. As shown in Fig. 3A, the parental SW1083 cells, which are devoid of $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] expression, did not show any appreciable binding of recombinant Siglec-7-immunoglobulin, whereas high-expresser clones showed strong binding. The binding was blocked completely by the addition of anti-Siglec-7 antibody to the incubation medium, indicating that the observed binding was Siglec-7 specific. The mutant recombinant Siglec-7, which lacks the essential amino acid residue for binding, showed no binding. In inhibition studies, the binding of Siglec-7 was not affected by the addition of anti-$2{\rightarrow}3$ sialyl Lewis[a] antibody N19–9 and inhibited strongly by the anti-$2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] antibody FH7, suggesting that most of the binding was mediated by the latter determinant (Fig. 3A).

Another antibody, FH9, which is known to be specific to $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[c] determinant (15, 24), showed also a significant inhibition of the Siglec-7 binding to the clones. Combination of FH7 and FH9 led to almost complete inhibition of Siglec-7 binding to the cells (Fig. 3A).

These results were reproduced in the results of nonstatic monolayer cell adhesion assays, which indicate the interaction of these molecules at the cell-to-cell level, as shown in Fig. 3B. The transfectant clones showed strong adhesion to Siglec-7-expressing U937 cells, whereas parental SW1083 cells showed no appreciable adhesion. The adhesion was abrogated almost completely by the addition of antisiglec-7 neutralizing antibody 13-3-D and inhibited strongly by the addition of anti-anti-$2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] antibody FH7. Combination of FH7 and FH9 conferred almost complete inhibition of adhesion.

These results suggest strongly that $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] served as a ligand for Siglec-7 on the clones transfected with ST6GalNAc6 cDNA, with an additional contribution of the $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[c] determinant on the clones. This was in line with the weak or moderate expression of $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[c] determinant on the clones but not on the parental SW1083 cells (Fig. 3C).

Confirmation of Binding of Recombinant Siglec-7 to Pure $2{\rightarrow}3$, $2{\rightarrow}6$ Disialyl Determinants. It is well known that $2{\rightarrow}3$ sialyl Lewis[a] determinant serves as a suitable ligand for E-selectin (5, 22, 25), and the binding activity of Siglec-7 to $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[c] has been reported also (23). Because the binding activity of Siglec-7 to $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] has not been known before and first reported in the present study, we assured next the direct binding activity using pure carbohydrate determinant and recombinant Siglec-7. As shown in Fig. 4, recombinant Siglec-7 showed significant binding activity to synthetic $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a], which was comparable with its binding to $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[c], indicating that the addition of fucose residue to $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[c] had no apparent effect on the binding of Siglec-7 to its ligands.

Expression of ST6GalNAc6 mRNA in Human Colon Cancer Tissues. As we have shown previously, the $2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a] determinant is expressed preferentially on nonmalignant epithelial cells of the digestive organs, and its expression tends to
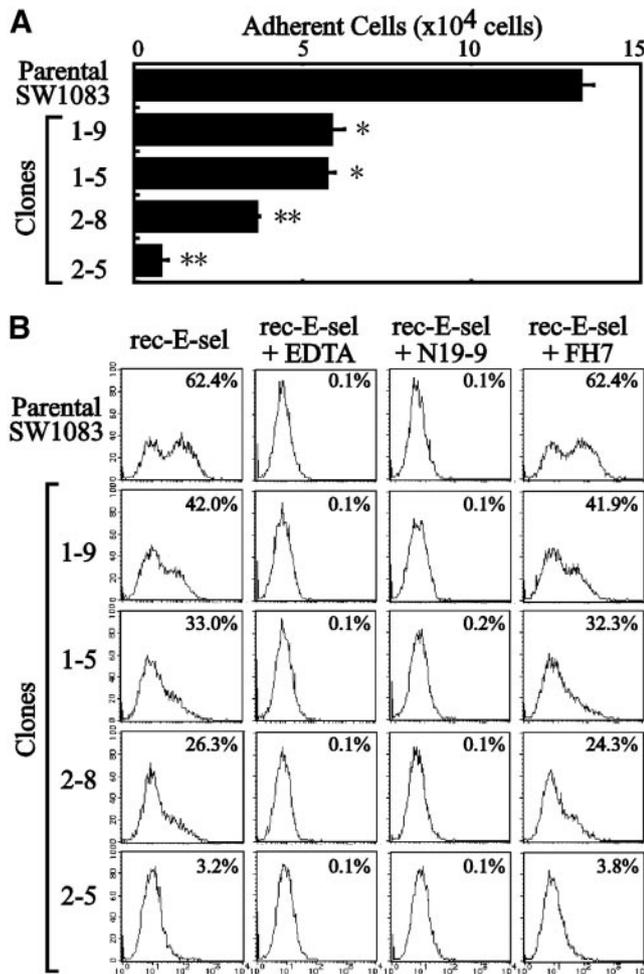

Fig. 2. Adhesion of cells transfected with ST6GalNAc6 cDNA to E-selectin. A, adhesion of 300.19/E-selectin cells to the parental SW1083 cells, and the transfectant clones as ascertained by the nonstatic monolayer cell adhesion experiments. *, difference from parental SW1083 cells was statistically significant at $P < 0.001$; **, $P < 0.0001$. B indicates binding of recombinant E-selectin-immunoglobulin (rec-E-sel) to the parental SW1083 cells, and the transfectant clones as ascertained by flow cytometric analysis. Percentage of positive cells are shown in the flow profiles. Recombinant E-selectin-immunoglobulin was polymerized and labeled using the secondary antibody phycoerythrin-streptavidin before addition to the cells (see "Materials and Methods"). EDTA and monoclonal antibodies N19-9 (anti-$2{\rightarrow}3$ monosialyl Lewis[a]) and FH7 (anti-$2{\rightarrow}3$, $2{\rightarrow}6$ disialyl Lewis[a]) were used for inhibition of binding at 10 μg/ml; bars, $\pm$SD.
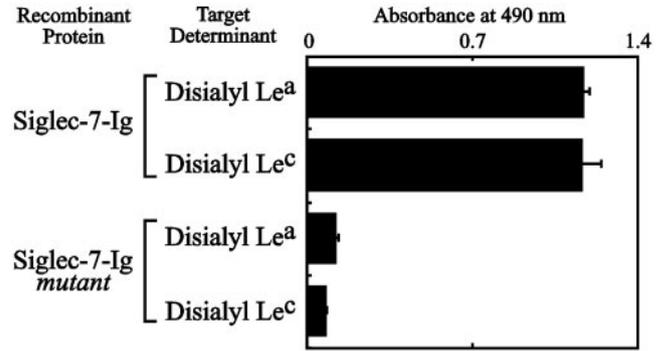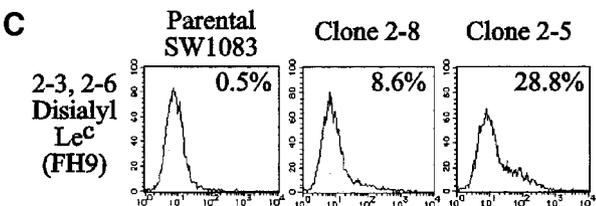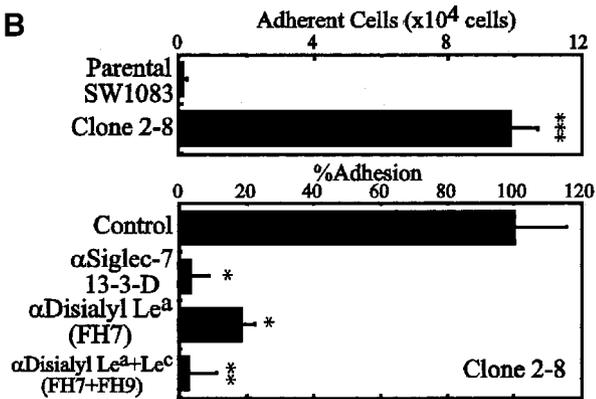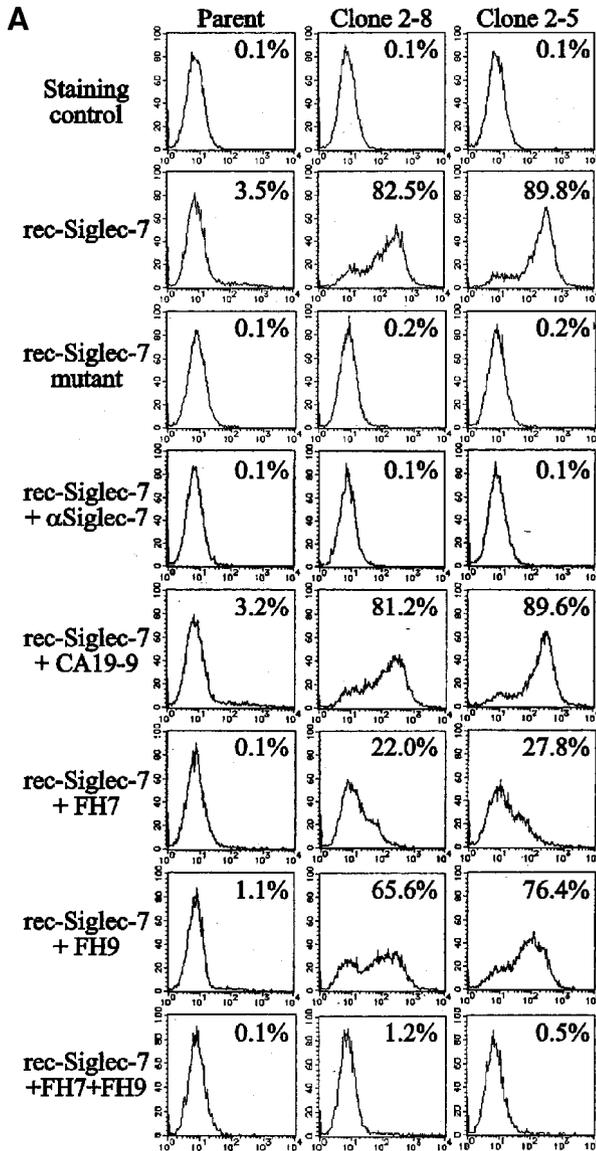
Fig. 4. Direct binding of recombinant Siglec-7-immunoglobulin to the pure synthetic carbohydrate determinants ascertained by ELISA. The synthetic carbohydrate determinants 2→3, 2→6 disialyl Lewis[a] and 2→3, 2→6 disialyl Lewis[c] were immobilized at the bottom of 96-well plates with phosphatidylcholine and cholesterol and reacted with a mixture of the recombinant Siglec-7-immunoglobulin or its mutant lacking the binding activity, affinity-purified biotinylated goat antihuman IgG antibody (20 μg/ml); avidin-peroxidase (20 μg/ml) was overlaid and incubated for 60 min at 4°C. The reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride followed by photometry at 490 nm (39); bars, ±SD.

decrease on cancer cells (13–15). We looked next at the expression of mRNA for ST6GalNAc6, the putative enzyme responsible for synthesis of the determinant in human colon cancer tissues. As shown in Table 2, real-time RT-PCR analysis indicated that its mRNA expression is decreased significantly in cancer tissues compared with nonmalignant epithelial cells, something quite compatible with our previous findings that the 2→3, 2→6 disialyl Lewis[a] determinant is expressed preferentially on nonmalignant epithelial cells. A significant decrease in the expression of ST6GalNAc6 was confirmed by conventional RT-PCR (data not shown). This tendency was observed more prominently in patients in the relatively early stages of cancers (Dukes' A and B) than in patients in the advanced stages (Dukes' C and D; Table 2). The difference between the Dukes' A and B group and the C and D group was statistically significant at $P < 0.02$.

Induction of Disialyl Lewis[a] and ST6GalNAc6 mRNA Expression in Cultured Human Colon Cancer Cells. Most cultured human colon cancer cells strongly expressed sialyl Lewis[a] but did not express or only weakly expressed disialyl Lewis[a]. When such cells were treated with butyrate, a histone deacetylase inhibitor, or 5-azacytidine, an inhibitor of DNA methylation, significant disialyl Lewis[a] expression was induced, accompanied by significant induction of ST6GalNAc6 mRNA as shown in Fig. 5. The time course and dose response studies indicated that the optimum concentration for butyrate was 2–4 mM and that for 5-aza-C was ~20 μM. Incubation of 3–5 days was necessary to detect a significant increase of ST6GalNAc6 mRNA in RT-PCR analysis, and incubation of 5–7 days was required to obtain maximum expression of the enzymatic product, disialyl Lewis[a], in flow cytometric analyses. These results suggested collectively that the down-regulation of ST6GalNAc6 gene expression that

Fig. 3. Adhesion of cells transfected with ST6GalNAc6 cDNA to Siglec-7. A, binding of recombinant Siglec-7-immunoglobulin to the parental SW1083 cells and the transfectant clones as ascertained by flow cytometric analysis. Recombinant Siglec-7-immunoglobulin was polymerized and labeled using the secondary antibody and phycoerythrin-streptavidin before addition to the cells (see "Materials and Methods"). Monoclonal antibodies 13-3-D (anti-Siglec-7), N19-9 (anti-2→3 monosialyl Lewis[a]), FH7 (anti-2→3, 2→6 disialyl Lewis[a]), and FH9 (anti-2→3, 2→6 disialyl Lewis[c]) were used for inhibition of binding at 10 μg/ml. B, adhesion of U937 cells expressing Siglec-7 to the parental SW1083 cells, and a transfectant clone ascertained by the nonstatic monolayer cell adhesion experiments. Percentage of positive cells are shown in flow profiles. For inhibition experiments, representative results using clone 2–8 are shown. *, difference from control was statistically significant at $P < 0.005$; **, at $P < 0.002$; and ***, at $P < 0.0001$. C shows expression of 2→3, 2→6 disialyl Lewis[c] determinant on the parental SW1083 cells, and the transfectant clones ascertained by flow cytometry. Parent, parental SW1083 cells. Ordinate, number of cells; abscissa and fluorescence intensity; bars, ±SD.

Table 2 *Quantitative RT-PCR^a analysis of mRNA levels of ST6GalNAc6 in human colon cancer tissues and nonmalignant mucosa*

Results of real-time RT-PCR analysis using specific TaqMan probe are shown. Sample RNA was prepared from the cancer tissue and nonmalignant colonic mucosa of the same patient ($n = 21$) except two cases where only cancer tissues were obtained.

| | Cancer^b | | Nonmalignant epithelia^b | | | Cancer: nonmalignant epithelia ratio^c | | |
	Mean ± SD	n	Mean ± SD	n	P^b	Mean ± SD	n	P^c
Total	10.39 ± 7.63	21	20.35 ± 8.49	19	P < 0.0002	0.65 ± 0.64	19	
Dukes' A and B	4.79 ± 4.87	6	22.60 ± 10.78	6	P < 0.005	0.28 ± 0.37	6	P < 0.02
Dukes' C and D	12.63 ± 7.47	15	19.31 ± 7.49	13	P < 0.02	0.82 ± 0.68	13	

^{a} RT-PCR, reverse transcription-PCR.

^{b} The amount of mRNA for ST6GalNAc6 in each sample was normalized to the housekeeping gene, *G3PDH*, by dividing by the amount of *G3PDH* mRNA in the same specimen and presented as a relative expression coefficient (number of amplicons per 10^3 *G3PDH* amplicons). *P* indicates the significance of the difference between the ST6GalNAc6 mRNA expression level in cancer tissues and that in nonmalignant epithelia in every group of patients as ascertained by Students' *t* test.

^{c} After normalization to the expression level of mRNA, the ratio of the ST6GalNAc6 mRNA amount in cancer tissues and that in nonmalignant epithelia was calculated in individual patient and analyzed statistically. *P* indicates the significance in the difference of the ratio between Dukes' A and B group and the Dukes' C and D group, as ascertained by Students' *t* test.

synthesizes 2→3, 2→6 disialyl Lewis^a determinant in nonmalignant epithelial cells occurred on malignant transformation, which led to the preferential expression of 2→3 sialyl Lewis^a determinant in cancer cells. It implied also that the down-regulation of ST6GalNAc6 gene expression in cancer cells may well be because of epigenetic changes such as histone deacetylation and DNA methylation.

Siglec-7-Mediated Adhesion of Lymphocytes to Nonmalignant Epithelial Cells *in Situ*. When frozen tissue sections prepared from 24 patients with colon cancers were immunohistochemically examined, the disialyl Lewis^a determinant was found to be expressed preferentially in nonmalignant epithelia ($P = 0.016$ in χ^2 test) and the monosialyl Lewis^a determinant to be expressed preferentially in cancer cells ($P = 0.011$ in χ^2 test), confirming our earlier results (13, 14). Typical results of the immunohistochemical study are shown in Fig. 6, *A* and *B*.

Because Siglec-7 is known to be expressed on lymphocytes and monocytes, the above results showing the preferential expression of disialyl Lewis^a in nonmalignant epithelial cells suggested that it could mediate adhesion of these leukocytes to normal epithelial cells. We chose six representative tissue sections (cases A–F) and performed confocal microscopic observation. The results indicated that a significant number of lymphocytes and monocytes expressing Siglec-7

were present in the connective tissue of lamina propria of colonic mucosal membranes (Fig. 6*C*), part of which exhibited a significant tendency to adhere to colonic epithelial cells (Fig. 6*D*, indicated by *arrows*). Color resolution of the contact surface indicated that lymphocytes and monocytes adhered to the epithelial cells not by their cell body but rather through pseudopodia-like structures (Fig. 6*E*). Adhesion of lymphoid cells to epithelial cells through a pseudopodia-like structure was noted previously by electron-microscopic observations (26, 27), and our results indicated that these lymphoid cells express Siglec-7, and the epithelial cells express its ligand, disialyl Lewis^a.

In cancer tissues, in contrast, the expression of 2→3, 2→6 disialyl Lewis^a determinant showed a significant decrease as described earlier (13–15, 28), which was accompanied by the disappearance of Siglec-7-expressing lymphocytes and monocytes (cases A–D; Fig. 6, *C* and *F–H*). The weak expression of 2→3, 2→6 disialyl Lewis^a determinant observed in cancer cells of some patients (cases E and F; Fig. 6, *I* and *J*) was accompanied occasionally by moderate infiltration of Siglec-7 expressing lymphocytes and/or monocytes (Fig. 6*J*).

DISCUSSION

The results obtained in the present study indicate collectively that the loss of expression of 2→3, 2→6 disialyl Lewis^a, the determinant expressed preferentially in nonmalignant colonic epithelial cells, is involved, at least partially, in the enhancement of expression of the 2→3 sialyl Lewis^a determinant, a cancer-associated carbohydrate determinant, in colon cancer cells. Impairment of 2→6 sialylation at the GlcNAc moiety is proposed to occur on malignant transformation of colonic epithelial cells, which leads to the loss of 2→3, 2→6 disialyl Lewis^a determinant and gain of the 2→3 sialyl Lewis^a determinant in cancer cells. The 2→3 sialyl Lewis^a determinant in cancer cells is involved in hematogenous metastasis, whereas the 2→3, 2→6 disialyl Lewis^a determinant in nonmalignant epithelial cells mediates normal interaction with intramucosal lymphoid cells. This can be regarded as a typical example of the classical concept of incomplete synthesis for the abnormal expression of carbohydrate determinants in cancers. We reported earlier a very similar relationship between the distribution of sialyl Lewis^x, another ligand for E-selectin, which was expressed preferentially on cancer cells, and the distribution of sialyl 6-sulfo Lewis^x, which was localized predominantly in nonmalignant colonic epithelial cells (29). In this case, some impairment of 6-sulfation was suggested to occur on malignant transformation of colonic epithelial cells, which led to the loss of sialyl 6-sulfo Lewis^x determinant and gain of sialyl Lewis^x in cancer cells (29).

A significant down-regulation of ST6GalNAc6, the sialyltransferase responsible for the synthesis of 2→3, 2→6 disialyl Lewis^a determinant, must be involved closely in the observed phenomenon. Many
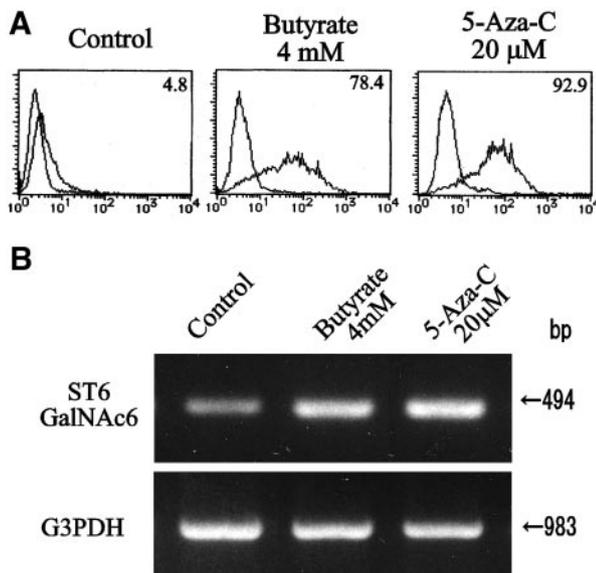

Fig. 5. Induction of the 2→3, 2→6 disialyl Lewis^a determinant and ST6GalNAc6 mRNA expression in cultured human colon cancer cells DLD-1 treated with histone deacetylase inhibitor butyrate or DNA methylation inhibitor 5-azacytidine. *A*, flow cytometric analysis of the 2→3, 2→6 disialyl Lewis^a determinant. *B*, reverse transcription-PCR analysis of ST6GalNAc6 mRNA expression. DLD-1 cells were treated either with 4 mM butyrate or 20 μM 5-azacytidine for 3 days. For experimental details, see "Materials and Methods."
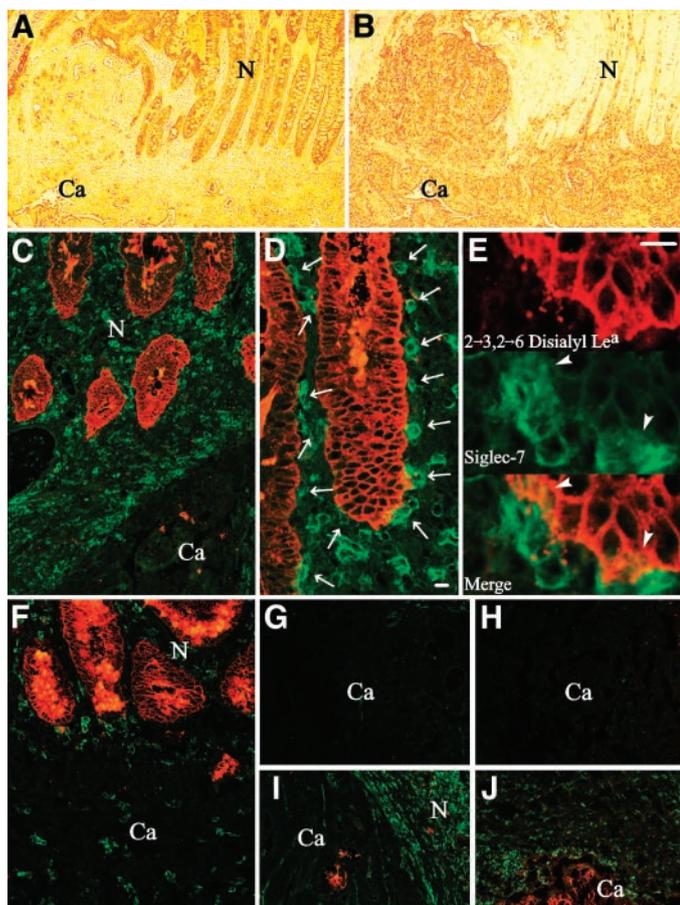
Fig. 6. Microscopic observation of epithelial cells expressing disialyl Lewis^a and leukocytes expressing Siglec-7 in human colonic mucosa. *A* and *B,* expression of 2→3, 2→6 disialyl Lewis^a and 2→3 monosialyl Lewis^a in a representative colon cancer tissue analyzed by routine avidin-biotin-complex immunohistochemical staining using FH7 or N19-9 antibody. In confocal microscopic analysis in *C–J,* distribution of Siglec-7, as detected by polyclonal anti-Siglec-7 antibody, is shown in *green,* and distribution of the 2→3, 2→6 disialyl Lewis^a determinant is shown in *red. C,* lower magnification of nonmalignant colonic mucosa adjacent to colon cancer tissue. *D,* adhesion of Siglec-7-expressing lymphoid cells to colonic epithelial cells (*arrows*). *E,* higher magnification showing adhesion of Siglec-7-expressing lymphoid cells (*green*) to nonmalignant colonic epithelial cells expressing the 2→3, 2→6 disialyl Lewis^a determinant (*red*) through pseudopodia-like extensions (*arrowheads*). *C* and *F–J,* comparison of distribution of both molecules in cancer tissues and nonmalignant mucosa in 6 typical tissue sections. Most cancer tissues were devoid of 2→3, 2→6 disialyl Lewis^a determinant and Siglec-7 expression as shown in portions labeled *Ca* in *C* and *G–H,* although some cancer cells expressed weakly 2→3, 2→6 disialyl Lewis^a determinant as in *I* and *J. C–E* are sections from the same patient (case A), whereas sections for *F–J* were prepared from five independent patients (cases B–F). *Ca,* cancer tissues; *N,* nonmalignant mucosa. *Bars,* 10 μm.

researchers have tried in vain to elucidate the mechanism for the increased expression of sialyl Lewis^a in cancers by looking for evidence of the up-regulation of its synthetic enzymes (4, 6–9, 30). However, the present results suggest clearly that the problem revolves not necessarily around the up-regulation of its synthetic enzymes, but rather the down-regulation of such an enzyme, which leads to synthesis of other determinants having a structure more complicated than sialyl Lewis^a.

The main cause for the down-regulation of the ST6GalNAc6 gene transcription is proposed to be epigenetic changes such as histone deacetylation and DNA methylation. It is well known that the expression of A- and B-determinants is decreased significantly in cancers, and DNA methylation of the genes for A- or B-enzyme is proposed to be responsible for their decrease in cancers (31, 32). We propose that these epigenetic changes would be one of the major mechanisms causing cancer-associated changes in carbohydrate determinants in

early stage cancers, especially for the mechanism referred to previously as incomplete synthesis.

The 2→3, 2→6 disialyl Lewis^a determinant, which is expressed preferentially on nonmalignant epithelial cells, is shown to have no apparent binding ability to E-selectin, yet a significant binding activity to another cell adhesion molecule, Siglec-7. Siglec-7 was described first as an inhibitory receptor p75/adhesion inhibitory receptor molecule-1 present on lymphocytes and monocytes, where the ligand binding is supposed to inhibit the cytotoxic and proliferative activity of the leukocytes (33–37). The physiological significance of the 2→3, 2→6 disialyl Lewis^a determinant on nonmalignant epithelial cells can be proposed to protect epithelial cells from unexpected cytotoxic attack by the autologous lymphocytes and monocytes. Siglec-7-positive natural killer cells are known to comprise usually <5% of normal peripheral blood mononuclear cells (33), but in the present study, we found that many lymphocytes in colonic mucosa strongly express Siglec-7.

Induction of sialyl Lewis^a expression in cancers of the digestive organs is accompanied by an increased ability of cancer cells to adhere to endothelial cells through the interaction with endothelial E-selectin (2, 4, 5). This interaction was demonstrated earlier to be involved also in tumor angiogenesis (38). The current study indicated that the gain of sialyl Lewis^a expression is accompanied by the loss of the 2→3, 2→6 disialyl Lewis^a determinant on epithelial cells, which binds to a leukocyte inhibitory receptor, Siglec-7, and may play a role in protecting normal epithelial cells. Confocal microscopic observation indicated that normal intramucosal trafficking of lymphocytes bearing an inhibitory receptor, Siglec-7/p75/adhesion inhibitory receptor molecule-1, in the colon is diminished accompanying the cancer-associated change in cell-adhesive carbohydrate determinants.

ACKNOWLEDGMENTS

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