Colonic Epithelial Cells Express Specific Ligands for Mucosal Macrophage Immunosuppressive Receptors Siglec-7 and -9

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Immune cells are known to express specific recognition molecules for cell surface glycans. However, mechanisms involved in glycans-mediated cell-cell interactions in mucosal immunity have largely been left unaccounted for. We found that several glycans preferentially expressed in nonmalignant colonic epithelial cells serve as ligands for siaic acid-binding Ig-like lectins (siglecs), the immunosuppressive carbohydrate-recognition receptors carried by immune cells. The siglec ligand glycans in normal colonic epithelial cells included disialyl Lewisα, which was found to have binding activity to both siglec-7 and -9, and sialyl 6-sulfo Lewisx, which exhibited significant binding to siglec-7. Expression of these siglec-7/-9 ligands was impaired upon carcinogenesis, and they were replaced by cancer-associated glycans sialyl Lewisα and sialyl Lewisx, which have no siglec ligand activity. When we characterized immune cells expressing siglec ligands in colonic lamina propriae by flow cytometry and confocal microscopy, the majority of colonic stromal immune cells expressing siglec-7/-9 turned out to be resident macrophages characterized by low expression of CD14/CD89 and high expression of CD68/CD163. A minor subpopulation of CD8+ T lymphocytes also expressed siglec-7/-9. Siglec-7/-9 ligation suppressed LPS-induced cyclooxygenase-2 expression and PGE2 production by macrophages. These results suggest that normal glycans of epithelial cells exert a suppressive effect on cyclooxygenase-2 expression by resident macrophages, thus maintaining immunological homeostasis in colonic mucosal membranes. Our results also imply that loss of immunosuppressive glycans by impaired glycosylation during colonic carcinogenesis enhances inflammatory mediator production. The Journal of Immunology, 2012, 188: 4690–4700.
For this purpose, we screened ligand activities of two sets of carbohydrate determinants, which show unique alterations upon malignant transformation. One pair was the set of disialyl Lewis^a^, preferentially expressed in nonmalignant epithelial cells, and sialyl Lewis^a^, a well-known cancer-associated determinant (7). The other pair was a combination of sialyl 6-sulfo Lewis^a^, preferentially expressed in nonmalignant epithelial cells, and a cancer-associated determinant, sialyl Lewis^x^ (12). We also tried to characterize immune cells expressing siglecs in colonic mucosal membranes and examined the effects of siglec ligation on their functions.

### Materials and Methods

#### Cells and Abs

Anti-disialyl Lewis^a^ (FH7, murine IgG3) and anti-disialyl Lewis^x^ (FH9, murine IgG2a) were prepared as described previously (13). The anti-sialyl Lewis^a^ Ab N19-9 (murine IgG1) was obtained from Alexis Biochemicals (Lausen, Switzerland). The Abs G152 (murine IgM), directed to sialyl 6-sulfo Lewis^a^, and G72 (murine IgM), reactive to both sialyl 6-sulfo Lac-NAC and sialyl 6-sulfo Lewis^x^, were prepared as described previously (14). An Ab directed to nonsulfated sialyl Lewis^a^, CSLEX-1 (murine IgM), was obtained from American Type Culture Collection (Manassas, VA). The stable ST6GalNAc6 transfectant cells were established by the transfection of cDNA for human ST6GalNAc6 to human colon cancer SW1083 cells, as described previously (7). The ECV304 cells stably transfected with FUT7 or GlcNAc6ST-1 cDNA, or cotransfected with both genes, were prepared as described previously (15).

#### ELISA for binding of recombinant siglecs to synthetic carbohydrate determinants

In some experiments, the binding specificity of recombinant siglec-7–Fc or siglec-9–Fc was ascertained by ELISA using pure carbohydrate determinants. The synthetic carbohydrate determinants were prepared as described previously (7) and kindly supplied by Professor Makoto Kiso (Gifu University, Gifu, Japan). Recombinant siglec-7–Fc, mutant siglec-7–Fc (R124K), and siglec-9–Fc were prepared as described previously (16). ELISA was performed using synthetic carbohydrate determinants immobilized at the bottom of 96-well culture plates by a standard method described previously (7, 14, 17). GD3 ganglioside (Avanti Polar Lipids) was used as a positive control for the binding of siglec-7–Fc in ELISA assays. Recombinant siglec-7 was preincubated with affinity-purified biotinylated rabbit anti-human IgG (Dako, Glostrup, Denmark), followed by incubation with peroxidase-streptavidin (Dako), before application to the assay plates. Recombinant mutant siglec-7–Fc lacking sialic acid-binding activity (7) served as a negative control.

#### Cell-binding assays for recombinant siglecs

Binding of recombinant siglecs to SW1083, ECV304, and their transfectant cells was ascertained by flow cytometric analyses, as described previously (18, 19). For this, recombinant siglecs were preincubated with affinity-purified biotinylated rabbit anti-human IgG, followed by incubation with PE-streptavidin, before application to the staining of cells. Cells were preincubated with blocking anti-carbohydrate Abs (20 μg/ml) for 30 min at 37°C when indicated. Percentage inhibition was calculated from the decrease in the mean fluorescence intensity of the cells after subtracting the background fluorescence obtained with negative-control cells. Binding of cells to immobilized siglec-7 or -9 on 96-well plates was evaluated in some experiments. Recombinant siglec-7 (2 μg/ml) in Tris-HCl buffer (pH 9.4) were immobilized at the bottom of 24-well plates overnight, and the wells were blocked with 2% BSA in PBS. 2’-bis-(carboxyethyl)-5-((and-6)-carboxyfluorescein acetoxyethyl ester-labeled ECV304 cells and their stable transfectant cells (1 × 10^6 cells/0.5 ml well) were added, and the plate was placed on a rotating platform for incubation under shear (90 rpm) for 20 min at room temperature. After three washings, the adherent cells were lysed with 0.5% Nonidet P-40 and counted with the Arvo 1420 multilabel counter (Wallac).

#### Immunohistochemical and flow cytometric analyses of siglec-expressing cells

Frozen sections, of 10-μm thickness for immunohistochemical examination, were prepared from surgical specimens obtained from 23 patients with colorectal cancer (11 originating in colon and 12 originating in rectum) at the Aichi Cancer Center Hospital and the Hospital of the International Medical Center of Japan after informed consent was obtained from all patients. Nine female and 14 male patients were included, with an average age of 58.3 y. The stage of the patients varied from Duks A to D; 22 cases were histologically diagnosed as moderately differentiated adenocarcinoma, and 1 case was diagnosed as poorly differentiated adenocarcinoma. No cases of mucinous adenocarcinoma were included. The avidin–biotin complex technique was used, as described in the instructions for the kits (VECTASTAIN; Vector Laboratories, Burlingame, CA), for the immunohistochemical study (12). For immunohistochemical analyses using recombinant siglec-7–Fc, the immune complex of recombinant proteins, or normal human IgG (R&D Systems, Minneapolis, MN) as negative control, with biotinylated anti-human IgG Ab and peroxidase-streptavidin was prepared before application to the staining of frozen sections. Polyclonal rabbit anti–siglec-7 or -9 Ab (rabbit IgG, raised against recombinant siglec-7 or -9 and affinity purified (7)) was used as a primary Ab for confocal microscopy.

For preparation of lamina propria mononuclear cells (LPMCs) for flow cytometry, fresh colonic tissue specimens were washed twice in calcium- and magnesium-free HBSS (CMF-HBSS) supplemented with penicillin (10 U/ml), streptomycin (10 μg/ml), gentamicin (100 μg/ml), and amphotericin B (1 μg/ml). The 2 × 2-cm pieces of tissue were incubated for 15 min at room temperature in CMF-HBSS supplemented with antibiotics and 1 mM EDTA. Cells were harvested by incubations for 20 min at 37°C in CMF-HBSS supplemented with anti-biotin, 0.75 mM EDTA, and 1 mM DTT, the tissues were minced and incubated for 15 min at 37°C in HBSS supplemented with antibiotics, 0.1% BSA, 0.5 mg/ml collagenase, 0.4 mg/ml Dispase, 0.01 mg/ml DNase, and 10 mM HEPES. Cells in supernatants were collected and filtered through a 200-μm steel mesh. The cells in suspension were washed twice with CMF-HBSS with antibiotics and centrifuged over a layer of Percoll (100/400/50%) at 400 × g for 25 min at 20°C. The harvested cells were washed twice and subjected to flow cytometric analyses. See supplemental materials for Abs used for cell surface marker analysis. The unpaired Student t test was used to compare results of flow cytometric analyses.

### Effect of siglecs on LPS-induced production of COX2 and PGE2

The U937 cells transfected with cDNA for siglec-7 (U937/siglec-7 cells) or for siglec-9 (U937/siglec-9 cells), prepared as described previously (7, 21), were cultured for 3 d with 10 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA); after being cultured for one additional day without TPA, they were challenged with 1 μg/ml LPS for 3 h (22–24). The F(ab’)_2 fragment (10 μg/ml) of anti-siglec-7 Ab or anti-siglec-9 Ab was added to the culture together with TPA or LPS. The F(ab’)_2 fragment was prepared by incubation in pH-neutralizing monoclonal anti–siglec-7 Ab (3A5) or anti–siglec-9 Ab (2G2), or anti–siglec-9 Ab (1A3–3A, rat IgG2b) with rabbit anti-rat IgG (Fc-specific) Ab-immobilized agarose beads, as described previously (21). Control rat F(ab’)_2 fragment was obtained from Jackson ImmunoResearch (West Grove, PA). Cells were collected, and total cellular RNA was isolated according to the acid guanidinium thiocyanate-cesium chloride extraction method using an isolation kit (Gentra, Tokyo, Japan). First-strand cDNA was prepared using 500 ng total cellular RNA. Synthesis of cDNA was carried out in a 20-μl reaction volume using the Superscript Preamplification System (Life Technologies BRL, Grand Island, NY), according to the manufacturer’s protocol, with oligo-d(T) as initiation primer. The primers used in RT-PCR analysis for human COX1 with 5’-TGGCCAGCTCTGGGCCGCCTGC-3’ and 5’-GTCATCTACAACAGGCTCTTCT-3’, which give a 331-bp product, and those for COX2 were 5’-TTCAAAAGGATAGTGGGGAGATAAG-3’ and 5’-AGTACATCTTGGTTGTAAAGAT-3’, which give a 305-bp product. The primers for G3PDH were 5’-AAAGGTCATCCATGACAA-3’ and 5’-
CACCCTTGTGCTGTAGCCCA-3′, which give a 489-bp product. Quantitative real-time RT-PCR analyses for COX2 were performed using ABI PRISM 7000 (Applied Biosystems), as described previously (7). The TaqMan probe ID for COX2 was Hs 00153133_m1 (Assays-on-Demand), and TaqMan Endogenous Control (Applied Biosystems) was used for GAPDH. The results were calculated using the comparative Ct method. Relative transcript levels were determined by the equation \(2^{-\Delta\Delta Ct}\). The \(\Delta Ct\) value was determined by subtracting the average GAPDH Ct value from the average target Ct value, and it was used to calculate percentage inhibition of COX2 induction in the LPS-stimulated U937 cells treated with F(ab′)2 fragments of anti-siglec-7/-9 Abs. PGE2 in culture supernatant of the cells was evaluated using a Parameter PGE2 assay kit (KGE004; R&D Systems). The culture supernatants were collected for PGE2 determination 2 d after the addition of LPS for the determination of PGE2 production.

Results

Disialyl Lewisx as a ligand for siglec-7/-9

Normal colonic epithelial cells expressed disialyl Lewisx, and the expression tended to be decreased in colonic cancer cells. Fig. 1A shows its carbohydrate structure and a representative pattern of its expression in colonic cancer tissues. The loss of disialyl Lewisx determinant is accompanied by an increase in sialyl Lewisx expression of the transfectant cells, which showed no binding. Unexpectedly, cells transfected with FUT7 cDNA only, which expressed sialyl Lewisx, showed strong binding comparable to that of the cells transfected with GlcNAc6ST-1 cDNA alone, which expressed Lewisx, showed strong binding comparable to that of the cells expressing the counterpart glycan, sialyl Lewisx.

We previously showed that the disialyl Lewisx determinant serves as a ligand for an immunosuppressive receptor, siglec-7/-9 (7). In this study, we show that the determinant also serves as a ligand for another immunosuppressive receptor, siglec-9. The disialyl Lewisx determinant was found to bind to the recombinant Fc chimera of siglec-9 or siglec-7 in ELISA-based assays using pure synthetic carbohydrate determinants (Fig. 1B). Both siglec-9 and siglec-7 also bound to the pure disialyl Lewisx, the carbohydrate determinant lacking the fucose residue, indicating that the fucose residue is not essential for the binding of siglec-7/-9. See Supplemental Fig. 2A for the structure of disialyl Lewisx.

We next tried to ascertain whether the binding of siglecs to disialyl Lewisx actually occurred at the cellular level. To see the binding at the cellular level, we generated cells expressing disialyl Lewisx by transfecting a cancer cell line SW1083 with the gene for α,2,6 sialyltransferase that synthesizes disialyl Lewisx and screened for the binding of siglecs to the transfectant cells, first by flow cytometry using several recombinant siglec molecules. The results indicated that among the four siglecs of the CD33 family, only siglec-7 and -9 showed binding to the transfectant cells (Fig. 1C). None of the siglecs bound to the parental cancer cells, which express sialyl Lewisx, the cancer-associated glycan, but no disialyl Lewisx (see Supplemental Fig. 2B for expression of disialyl Lewisx-related glycans in these cells). The binding of siglec-9 to the transfectant cells was inhibited significantly by anti-disialyl Lewisx (FH7) or anti-disialyl Lewisx (FH9) Ab; it was almost completely inhibited by adding both Abs but was not affected by the anti-sialyl Lewisx Ab (N19-9) (Fig. 1C).

Cell–cell interaction mediated by disialyl Lewisx and siglec-7/-9

Interactions of siglecs and their ligands were also found to mediate cell-to-cell adhesion (Fig. 1D). The U937 cells transfected with siglec-7 or -9 adhered to the cells expressing disialyl Lewisx; this binding was specifically inhibited by FH7 or more strongly by both FH7 and FH9 Abs, but it was not affected by the anti-sialyl Lewisx Ab (N19-9) (Fig. 1D). These results indicated that disialyl Lewisx, the carbohydrate determinant preferentially expressed in nonmalignant colonic epithelial cells, specifically serves as a ligand for siglec-7 and -9, whereas sialyl Lewisx, the closely related determinant on cancer cells, does not.

Sialyl 6-sulfo Lewisx as a ligand for siglec-7/-9

There is another set of glycans that shows a similar distribution pattern between benign and malignant colonic epithelial cells; sialyl 6-sulfo Lewisx and sialyl Lewisx. Fig. 2A shows the carbohydrate structure of sialyl 6-sulfo Lewisx, as well as a representative pattern of its expression in colonic cancer tissues. This determinant is preferentially expressed on normal colonic epithelial cells, and its expression is markedly reduced in colonic cancer cells. We previously reported a statistically significant decrease in sialyl 6-sulfo Lewisx in colonic cancers, which was accompanied by an increase in sialyl Lewisx expression (p = 0.007, n = 23) (12). Sialyl Lewisx is closely related structurally to sialyl 6-sulfo Lewisx, and it lacks a sulfate residue compared with sialyl 6-sulfo Lewisx. Impaired sulfation upon malignant transformation was proposed to be responsible for this shift in glycan expression (12).

The ELISA-based binding assays of siglecs to the pure sialyl 6-sulfo Lewisx determinant were performed by a consortium for functional glycomics; the results are available at http://www.functionalglycomics.org. Siglec-7 was found to be reactive to several glycans having diverse carbohydrate structures, including sialyl 6-sulfo Lewisx, which showed the seventh-best binding. Siglec-9 was shown to be the most reactive to sialyl 6-sulfo Lewisx among >200 glycans tested, according to the data posted on the consortium Web site on September 15, 2005. However, the reactivity in ELISA does not always warrant reactivity at the cellular level. Therefore, we tested whether the interaction of sialyl 6-sulfo Lewisx with siglecs is functional at the cellular level by generating cells expressing sialyl 6-sulfo Lewisx and control cells expressing the counterpart glycan, sialyl Lewisx.

Our initial screening in flow cytometric analyses of the cells expressing sialyl 6-sulfo Lewisx using recombinant siglecs indicated a strong binding of siglec-7, whereas the binding of siglec-9 was negligible (Fig. 2B). The latter finding was at variance with the consortium results, in which siglec-9 was shown to have a much better binding activity than siglec-7 to sialyl 6-sulfo Lewisx. In the cell-binding analysis using immobilized recombinant siglecs (Fig. 2C), again the strong binding to siglec-7 was detected with the cells expressing sialyl 6-sulfo Lewisx, which were obtained by cotransfection of FUT7 and GlcNAc6ST-1 cDNAs. Cells transfected with FUT7 cDNA only, which expressed sialyl Lewisx but not sialyl 6-sulfo Lewisx, showed no binding. Unexpectedly, cells transfected with GlcNAc6ST-1 cDNA alone, which expressed sialyl 6-sulfo N-acetyllactosamine (LacNac) but not sialyl 6-sulfo Lewisx, showed strong binding comparable to that of the cells cotransfected with FUT7 and GlcNAc6ST-1 cDNAs and expressed both determinants (Fig. 2C; see Supplemental Fig. 2C for glycan expression of the transfectant cells used in these experiments). Because sialyl 6-sulfo LacNac lacks the fucose residue, this suggested that the fucose residue in sialyl 6-sulfo Lewisx is not essential for binding of siglec-7.

Cell–cell interaction mediated by sialyl 6-sulfo Lewisx and siglec-7/-9

In cell–cell adhesion experiments, the U937/siglec-7 cells strongly adhered to cells expressing only sialyl 6-sulfo LacNac (GlcNAc6ST-
FIGURE 1. Siglec ligand activity of disialyl Lewis^a^ glycan, which is preferentially expressed on nonmalignant colonic epithelial cells. (A) Structure of disialyl Lewis^a^ (upper left panel) and its preferential expression in nonmalignant colonic epithelial cells (upper right panel). The structure of sialyl Lewis^a^ determinant and its close association with cancer cells in a consecutive section are also shown (lower panels). Disialyl Lewis^a^ determinant was detected by FH7 and sialyl Lewis^a^ was detected by N19-9 Abs in immunohistochemistry. Scale bar, 100 μm. (B) Specific binding of recombinant siglec-7/-9 to pure carbohydrate determinants. Binding of recombinant siglec-7/-9-Fc was evaluated by ELISA using immobilized pure synthetic glycolipids bearing disialyl Lewis^a^ and disialyl Lewis^a^ structure at the bottom of 96-well plates. GD3, the known glycolipid ligand for siglec-7, was used as a positive control for siglec-7. The recombinant siglec-7 mutant-Fc lacking binding activity served as control. (C) Flow cytometric analyses of binding of recombinant siglec to cells expressing disialyl Lewis^a^ (left panel) and its inhibition by specific Abs (right panel). Parental cancer cells SW1083 (parent) expressed sialyl Lewis^a^ but not the disialyl Lewis^a^ determinant. The cells transfected with ST6GalNAc6 cDNA (ST6-Transfectant) strongly expressed disialyl Lewis^a^ and had severely attenuated expression of sialyl Lewis^a^, the cancer-associated determinant (Supplemental Fig. 2B). The recombinant siglec-3–Fc and siglec-5–Fc served as negative controls for rIg-Fc molecules instead of siglec-7 mutant-Fc in flow cytometric analyses, because these siglec do not bind to these cells. Results of flow cytometric analyses of inhibition of recombinant siglec-7/-9 binding to cells expressing disialyl Lewis^a^ determinant by specific Abs (right panel). The percentage binding was calculated from mean fluorescence intensities obtained by flow cytometric analyses. The inhibitor Abs used included clone 1-3-A (anti–siglec-9), 13-three dimensional (anti–siglec-7), N19-9 (anti-sialyl Lewis^a^), FH7 (anti-disialyl Lewis^a^), and FH9 (anti-disialyl Lewis^a^). (D) Cell–cell adhesion mediated by siglec-7 or -9 and its specific glycan ligands. Adhesion of U937 cells transfected with siglec-7 (U937/siglec-7) or siglec-9 cDNA (U937/siglec-9) to the parental SW1083 cells (Parent) or ST6GalNAc6-transfectant cells was evaluated by nonstatic cell-adhesion assays. Significant adhesion, observed with the ST6GalNAc6-transfectant cells, was tested for possible inhibition with N19-9, FH7, or FH7 + FH9 Abs. Values are mean ± SD from triplicate experiments. The results in (B) and (D) are representative of at least three independent experiments, and those in (C) are representative of two independent experiments. Ca, Cancer cells; N, nonmalignant epithelial cells.
with FUT7 cDNA (FUT7 Transfectant) expressed sialyl Lewisx but not the sialyl 6-sulfo Lewisx determinant. Cells cotransfected with FUT7 and GlcNAc6ST-1 cDNAs (FUT7+6ST Transfectant) strongly expressed sialyl 6-sulfo Lewisx and had severely attenuated expression of sialyl Lewisx, the cancer-associated determinant (Supplemental Fig. 2C). The recombinant siglec-2–Fc served as a negative control instead of siglec-7 mutant–Fc in flow cytometric analyses, because this siglec does not bind to these cells.

**FIGURE 2.** Siglec ligand activity of sialyl 6-sulfo Lewisx glycan, which is preferentially expressed on nonmalignant colonic epithelial cells. (A) Structure of sialyl 6-sulfo Lewisx determinant (upper left panel) and its preferential expression in nonmalignant colonic epithelial cells (upper right panel). The structure of sialyl Lewisx determinant and its close association with cancer cells in a consecutive section are also shown (lower panels). Sialyl 6-sulfo Lewisx determinant was detected by G152 and sialyl Lewisx was detected by CSLEX-1 Abs in immunohistochemistry. Scale bar, 100 μm. (B) Flow cytometric analyses of binding of recombinant siglecs to cells expressing sialyl 6-sulfo Lewisx. ECV304 cells transfected with FUT7 cDNA (FUT7 Transfectant) expressed sialyl Lewisx but not the sialyl 6-sulfo Lewisx determinant. Cells cotransfected with FUT7 and GlcNAc6ST-1 cDNAs (FUT7+6ST Transfectant) strongly expressed sialyl 6-sulfo Lewisx and had severely attenuated expression of sialyl Lewisx, the cancer-associated determinant (Supplemental Fig. 2C). The recombinant siglec-2–Fc served as a negative control instead of siglec-7 mutant–Fc in flow cytometric analyses, because this siglec does not bind to these cells.
cally significant in cell–cell adhesion assays using U937/siglec-9 cells (Fig. 2D). Other cell lines strongly expressing sialyl 6-sulfo Lewis\(^\text{a}\), such as a subclone of Namalwa cells (18) and SW480 cells transfected with 6-sulfotransferase cDNA (26), also failed to show statistically significant binding to siglec-9 (data not shown).

These results indicated that the sialyl 6-sulfo Lewis\(^\text{a}\) determinant, which is specifically expressed in nonmalignant colonic epithelial cells, served as a ligand for siglec-7 at the cellular level. Another glycan, sialyl 6-sulfo LacNAc, which was previously shown to also be preferentially expressed in nonmalignant epithelial cells (12), was found to serve as a ligand for siglec-7. The possibility that they serve as ligands for siglec-9 cannot be completely excluded, but their ligand activity to siglec-9 at the cellular level seemed to be much weaker than expected from the results of the ELISA-based assays using pure glycans. Interestingly, sialyl Lewis\(^\text{a}\), which is another glycan that is structurally similar and is preferentially expressed in cancer cells, failed to interact with any siglecs.

**FIGURE 4.** Siglec-7/-9–expressing cells in colonic mucosal membranes. (A) Confocal microscopic observation of cells expressing siglec-7 (upper panel) or siglec-9 (lower panel) in colonic mucosal membranes. Frozen sections of normal colonic tissues were stained with anti–siglec-7 (upper panel) or with anti–siglec-9 (lower panel) in green. A cognate ligand for siglec-7/-9, disialyl Lewis\(^\text{a}\), is stained in red using FH7 Ab. Original magnification \(\times\)200. (B) Morphology and distribution of siglec-7/-9–expressing cells in colonic lamina propria. The frozen sections were stained with anti–siglec-7 Ab in green and with anti–siglec-9 Ab in red. Arrowheads indicate siglec-9\(^+\) cells. Areas of normal colonic epithelial cells are shown by dotted white line and labeled “E” in the merged panel. Original magnification \(\times\)100. (C) Flow cytometric analyses of siglec-7/-9–expressing cells in LPMCs. Frequency of siglec-7/-9–expressing cells in CD33\(^+\) and CD33\(^-\) fractions in LPMCs, as detected by flow cytometry, and average percentage obtained from six samples (upper panel). Example of flow cytometric analysis of CD33\(^+\) LPMCs prepared from normal human colon (lower panel). (D) Summary of cell lineage-specific marker analyses of siglec-7/-9–expressing cells in colonic lamina propriae. Average percentage obtained from five samples is shown. CD3 (UCHT1, IgG1), CD4 (RPA-T4, IgG1), CD8 (DK25, IgG1), CD20 (L26, IgG2a), CD56 (B159, IgG1), CD14 (TU\(\text{¨}\)K4, IgG2a), and CD68 (PG-M1, IgG3) were used to characterize the leukocyte subpopulation in immunohistochemistry. The markers used for evaluation of cell number in each leukocyte subpopulation were CD56 for NK cells, CD8 for killer T cells, and CD68 for monocytes/macrophages. Abs used for leukocytes in peripheral blood are shown in Supplemental Fig. 3. (E) Representative flow cytometric analyses of siglec-7 expression in CD8\(^{\alpha}\)\(\beta\)-integrin\(^+\) cells in PBLs. Two-dimensional distribution of CD8 and siglec-7 in gated CD3\(^+\) cells (left panel, three-color analysis, 2.4% of CD3\(^+\)CD8\(^+\) cells express siglec-7), two-dimensional distribution of CD8\(\alpha\) and siglec-7 in gated CD3\(^+\)CD8\(\alpha\) cells (middle panel, four-color analysis, 27.3% of CD3\(^+\)CD8\(\alpha\)CD8\(\beta\)\(^-\) cells express siglec-7), and two-dimensional distribution of \(\beta\)7-integrin and siglec-7 in gated CD3\(^+\)CD8\(\alpha\) cells (right panel, four-color analysis, 4.2% of CD3\(^+\)CD8\(\beta\)\(\beta\)-integrin\(^+\) cells express siglec-7). See Table I for statistical analyses.
When the binding of recombinant siglec-7 and -9 was tested by immunohistochemistry using frozen sections, the results indicated that both siglec-7 preferentially bind to nonmalignant colonic epithelial cells but only weakly to cancer cells (Fig. 3). This confirmed the preferential expression of the ligand glycans for siglec-7 and -9, such as disialyl Lewis X and sialyl 6-sulfo Lewis X, in nonmalignant epithelial cells, and its decrease or loss in cancer cells.

Identification of siglec-7/9-expressing cells in colonic mucosal membranes

To determine the physiological significance of siglec-7/9 ligand expression in colonic epithelial cells, we examined the characteristics of siglec-7/9-expressing cells in colonic tissues. Colonic mucosal membranes were found to contain a significant number of siglec-7/9-expressing cells, some of which were localized in lamina propria very close to colonic epithelial cells expressing their cognate ligands, suggesting the occurrence of possible epithelial–mesenchymal interactions (Fig. 4A). The majority of the cells expressing siglec-7 in colonic lamina propria had a dendritically shaped appearance with several spines. Relatively few siglec-9-expressing cells were observed, and they tended to be round cells expressing siglec-7 in colonic lamina propriae. The majority of the cells expressing siglec-9 in colonic lamina propriae had a dendritic appearance with several spines. Relatively few siglec-9-expressing cells were observed, and they tended to be round shaped with a less dendritic appearance (Fig. 4B). Flow cytometry of LPMC s indicated that most siglec-7/9-expressing cells were CD33+, and there were more siglec-7-expressing cells than siglec-9-expressing cells (Fig. 4C). Siglec-7+, siglec-9+, and siglec-7/9−expressing cells made up 11%, 5%, and 2%, respectively, of the CD33+ cells in the LPMC fraction. Further marker analyses by flow cytometry were hindered because of extensive application of proteases during the course of LPMC preparation; because we noticed that the recovery of siglec-7− cells was not as good as expected, we tried to identify siglec-7/9-expressing cells in colonic mucosal membranes by direct confocal microscopic observation of the frozen sections.

Results of immunohistochemical staining using cell lineage-specific markers indicated that most siglec-7-expressing cells belonged to the monocyte/macrophage lineage (76%), with a smaller number of CD8+ T lymphocytes (19%). NK cells made up <5% of the total siglec-7− cells (Fig. 4D). This was in clear contrast to the siglec-7 distribution in peripheral blood leukocytes of healthy individuals, where the majority (75%) of siglec-7+ cells were NK cells, followed by CD8+ T lymphocytes (23%). Most siglec-9-expressing cells in the colon also belonged to the monocyte/macrophage lineage (64%), with a smaller number of CD8+ T lymphocytes (8%) and other cells (25%, mostly granulocytes). NK cells accounted for <4% of total siglec-9+ cells (Fig. 4D). Again, this was in clear contrast to the distribution of siglec-9-expressing cells in peripheral blood leukocytes of healthy individuals, where granulocytes were predominant and accounted for 58% of siglec-9+ cells, followed by NK cells (27%).

Closer scrutiny of peripheral lymphocytes indicated that CD8+ cells contained significantly more siglec-7+ cells than did CD4+ cells (p < 0.01), and CD8α+ cells contained more siglec-7+ cells (p < 0.02) (Fig. 4E) and siglec-9+ cells (p < 0.05) than did CD8β+ cells (Table I). The Tcrγδ+ cell subset also contained a significantly higher number of siglec-7+ cells (p < 0.05) (Fig. 4E) and siglec-9+ cells (p < 0.05) than did the Tcrαβ+ cell subset.

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*Ratio of siglec-7/9− cells/each subset (%) was calculated from the results of flow cytometry for at least four healthy individuals.

1 Versus CD16* CD3* subset.
2 Versus CD56* CD3* subset.
3 Versus TcRγδ+ subset.
4 Versus CD4*CD3* subset.
5 Versus CD8αβ*CD3+ subset.
6 Versus β7-integrin*CD4* subset.
7 Versus CCR9* CD3* subset.
Characterization of siglec-7/-9–expressing macrophages in colonic mucosa

The number of siglec-7+ macrophages was ~4-fold greater than the number of siglec-9+ macrophages in colonic lamina propria (Fig. 5A). Most macrophages were of the siglec-7+siglec-9− phenotype (78.9 ± 9.7%), and only 11.9 ± 4.6% were siglec-7−siglec-9+. The population of siglec-7 siglec-9− cells was minor and accounted for only 9.3 ± 6.2%. This was in clear contrast to the siglec-7+/− expressing monocytes in the peripheral blood of healthy individuals, where the majority showed the siglec-7+siglec-9− phenotype (69.0 ± 11.8%), followed by the siglec-7−siglec-9− phenotype (31.1 ± 11.8%). Essentially no siglec-7−siglec-9− cells, which are predominant in colonic mucosa, were observed in peripheral blood monocytes (0.3 ± 0.5%). These results indicated that siglec-7+ expressing macrophages/monocytes in colonic mucosa have characteristics that are quite different from those in peripheral blood monocytes. The predominant macrophages in colonic mucosa were of the siglec-7+siglec-9− phenotype, and the difference in this subpopulation between the macrophages in colonic mucosa and the monocytes in peripheral blood was statistically significant at p < 0.001 (78.9 versus 0.3%), as ascertained by the unpaired Student t test. In contrast, the predominant phenotype in peripheral blood monocytes was siglec-7+siglec-9−, and the difference in this subpopulation between the peripheral blood monocytes and macrophages in colonic mucosa was also statistically significant at p < 0.001 (69.0 versus 11.9%). When normal human peripheral monocytes were induced to differentiate into macrophage-like cells by a standard technique using M-CSF, the resulting macrophage-like cells lost their expression of siglec-7 and -9 and became siglec-7−siglec-9− after long-term culture (Supplemental Fig. 4). This result again suggested that the siglec-expression pattern of the resident macrophages in colonic lamina propria is different from that in macrophages derived from peripheral monocytes.

Further marker analyses revealed that siglec-7+ macrophages/monocytes in colonic mucosa strongly express CD68 and CD163 and only weakly express CD14 and CD89 (Fig. 5B–D). In contrast, the siglec-9+ macrophages/monocytes in colonic mucosa tended to express CD14 and CD89 more frequently than did the siglec-7+ macrophages/monocytes (Fig. 5B–D). These findings suggested that the siglec-7+ cells in colonic mucosa have characteristics similar to so-called “resident macrophages” (27, 28), whereas siglec-9+ cells, at least in part, exhibit characteristics more like monocytes and activated macrophages. A significant number of siglec-7+ cells in the colonic mucosa were HLA-DR (TAL.1B5, IgG1), CD11c (KB90, IgG1), CD83 (HB15e, IgG1), and CD209 (DC-SIGN, DZX02, IgG2a). (Table I, see Supplemental Fig. 3 for additional details). In terms of gut-homing markers, β7-integrin CD8α cells contained a significantly higher number of siglec-7+ cells than did β7-integrin CD8β cells (p < 0.05) (Fig. 4E). CCR9+CD3+ cells also contained significantly more siglec-7+ cells than did CCR9+CD3α cells (p < 0.002) (Table I). These results showed that siglec-7+/− cells were more frequently expressed in gut-related lymphocyte subsets in peripheral blood. The combination of two sets of four-color analyses using a set of CD3, CD8α, and anti–siglec-7 and another set of CD3, CD8, β7-integrin, and anti–siglec-7 (Fig. 4E) indicated that the majority of siglec-7+ cells in the CD8α subset were CD8α+β7-integrin+ cells.

Characterization of siglec-7+/− expressing macrophages in colonic mucosa

FIGURE 5. Characterization of macrophages/monocytes in colonic lamina propria expressing siglec-7 and -9. (A) Distribution of siglec-7+/− macrophages/monocytes in colonic lamina propria and comparison with that in peripheral blood monocytes. The number of cells positive for either siglec-7 or -9 in CD33+ cells (WM53, IgG1) was taken as 100%. (B) Macrophage/monocyte marker analyses of colonic LPMCs expressing siglec-7 and/or -9. The Abs used were HLA-DR (TAL.1B5, IgG1), CD11c (KB90, IgG1), CD83 (HB15e, IgG1), and CD209 (DC-SIGN, DZX02, IgG2a). (C) Example of confocal microscopy after double staining for anti–siglec-7 (upper panel) or siglec-9 (lower panel) Ab and anti-CD14 Ab, showing that siglec-9− cells frequently express CD14. (D) Example of confocal microscopy after double staining with anti–siglec-7 (upper panel) or siglec-9 (lower panel) Ab and anti-CD163 Ab, showing that siglec-7+ cells frequently express CD163. (E) Example of CD209 (DC-SIGN) expression in siglec-7+ macrophages/monocytes in lamina propria of colonic mucosal membranes. Arrows indicate macrophages/monocytes expressing both siglec-7 and DC-SIGN. (C–E) Original magnification ×200.
number of siglec-7+ cells expressed CD209, known as a marker of dendritic cells (Fig. 5B, 5E).

The stroma of colon cancer tissues also contained a significant number of cells expressing siglec-7 and/or -9. Most of them were macrophages, followed by neutrophils, CD8+ T cells, and NK cells, and their relative numbers were not statistically significantly different from those in nonmalignant colonic mucosal membranes. The majority of macrophages were also of the siglec-7+/siglec-9+ phenotype in the stroma of the colon cancer tissues (data not shown).

Roles of siglec-7/-9 in macrophage function

Disialyl Lewisx and sialyl 6-sulfo Lewisx, the carbohydrate ligands for immunosuppressive siglec-7/-9, are preferentially expressed in normal colonic epithelial cells, and their expression is lost during colonic carcinogenesis. In this context, we scrutinized the effect of siglec-7/-9 on COX2 expression in macrophages, because the enhanced COX2 expression in stromal cells is implicated in colon carcinogenesis (10, 29). Human monocytoid cells U937 are known to differentiate into macrophage-like cells when cultured with TPA and to produce COX2 upon further LPS stimulation. When U937 cells transfected with siglec-7/-9 were treated with TPA and LPS, ligation of siglec-7/-9 with agonistic Abs significantly suppressed induction of COX2 mRNA (Fig. 6A). The results of quantitative real-time RT-PCR analyses performed in parallel with conventional RT-PCR indicated that ligation of siglec-7 on the U937/siglec-7 cells resulted in suppression of COX2 gene transcription to 48.3 ± 9.1% (mean ± SD, p < 0.05) of control culture with control F(ab)2, and ligation of siglec-9 on the U937/siglec-9 cells suppressed COX2 induction to 22.7 ± 11.5% (p < 0.01). Both siglecs conferred suppressive effects on COX2 transcription, and suppression of COX2 was also reflected in the reduction of its major product PGE2 (Fig. 6B).

Discussion

Among the glycans in colonic epithelial cells, some are specifically expressed in normal epithelial cells, and some other glycans are preferentially expressed in cancer cells. It is noteworthy that only the glycans that are expressed in normal epithelial cells serve as ligands for siglec-7 and -9, whereas cancer-associated glycans do not, as indicated by the results shown in Figs. 1 and 2. We demonstrated that disialyl Lewisx preferentially expressed in nonmalignant colonic epithelial cells serves as a ligand for siglec-7 and -9, whereas sialyl Lewisx on cancer cells has no binding activity. Further cell-binding experiments indicated that sialyl 6-sulfo Lewisx, another glycan preferentially expressed on nonmalignant colonic epithelial cells, also serves as a ligand for siglec-7, whereas sialyl Lewisx, the corresponding glycan in cancer cells, does not.

Cancer-associated carbohydrate determinants, such as sialyl Lewisx and sialyl Lewisx, are known to serve as ligands for selectins and mediate cancer cell adhesion to vascular beds in the course of hematogenous metastasis (4–6, 20). Expression of these determinants is markedly increased in cancer cells compared with normal epithelial cells. Nonmalignant epithelial cells express disialyl Lewisx and sialyl 6-sulfo Lewisx glycans having more complex structures than sialyl Lewisx (7, 12). Their expression is lost at the early stage of colon carcinogenesis as result of the epigenetic silencing of glycogenes involved in their synthesis (30, 31). Loss of these normal glycans leads to accumulation of less-complex glycans, such as sialyl Lewisx and sialyl Lewisx, in cancers. Taken together, our results indicate that colonic epithelial cells lose their siglec ligands upon malignant transformation, and this is associated with the acquisition of selectin ligand glycans.

Our results also indicated that the majority of immune cells expressing siglec-7 and -9 in normal colonic mucosa are macrophages/monocytes. A large number of tissue macrophages bearing siglec-7 was found in colonic lamina propria, and they showed a surface marker pattern similar to that of so-called “res-
ident macrophages” (27, 28), characterized by low expression of CD14 and CD89, and eventually expressed dendritic cell markers. Most of them predominantly expressed siglec-7, and the expression of siglec-9 was less prominent. This type of siglec-7–dominant macrophage/monocyte was not identified in peripheral blood.

Another notable finding was that a significant population of CD8 T cells was found to express siglec-7/9 in colonic lamina propria, indicating that the distribution pattern of siglec-7/9 among mucosal immune cells is quite different from that in normal peripheral blood. Human peripheral blood T cells had been known to contain a very small number, if any, of siglec-7/9 T cells, and the significance of such siglec-7/9 CD8 T cells has largely been overlooked (32). Our detailed analysis of siglec-7/9 T cells in normal peripheral blood indicated that siglec-7/9 are preferentially expressed in gut-associated minor populations, such as TcRγδ cells, CD8αα cells, and the T cells expressing gut-homing receptors β7-integrin and CCR9. These findings imply that siglec-7/9 is somehow preferentially expressed in gut-associated T cells (33).

Most siglecs, including siglec-7/9, have ITIMs in their cytoplasmic domains, which inhibit immune cell activation by recruiting tyrosine phosphatases SHP-1 and SHP-2. Siglec’s suppression of killing activity and immune mediator production of immune cells is well documented in the literature (2, 8, 34–37). The biological significance of the interaction of resident macrophages/monocytes expressing siglec-7/9 and their specific glycan ligands on colonic epithelial cells could be the protection of mucosal membrane from excess activation by immune cells. The endogenous glycan ligands on the resident macrophages/monocytes will also play important roles in physiological settings, because cis-ligands are known to inhibit binding of siglec with exogenous glycan ligands (2, 6).

Colonic epithelial cells are rendered increasingly susceptible to cancer progression both in humans and mice by chronic inflammatory stimuli in mucosal stroma (9). In this context, COX2, known to be expressed in stromal cells, such as fibroblasts (10, 11) and macrophages (29), has especially attracted researchers’ attention. COX2 expression in mucosal stromal cells is known to play a crucial role in facilitating colonic tumorigenesis in the APCΔMin model (10, 11). Administration of COX2 inhibitors is known to be effective in preventing colonic carcinogenesis (9). Several intrinsic protective mechanisms for suppressing excessive production of COX2 by resident stromal cells must be operating in normal colonic mucosal membranes. Because only the carbohydrate determinants on normal epithelial cells, but not cancer-associated determinants, served as ligands for siglec, we focused on the effects of siglec ligation on COX2 production of macrophages in the current study.

Experiments using human macrophage cell lines transfected with siglec-7/9 indicated that the ligation of siglec-7/9 suppresses LPS-induced COX2 and PGE2 production. These results suggest that normal glycans of colonic epithelial cells exert a suppressive effect on tissue macrophage COX2 expression in colonic mucosa, thus maintaining immunological homeostasis in normal mucosal membranes. These results also imply that the cancer-associated impaired glycosylation of siglec-7 and -9 ligands serves to enhance COX2 production by mucosal macrophages. Because colonic tissue continuously encounter bacterial stimuli from fecal flora, colonic mucosa is equipped with various modules that suppress excess activation of mucosal immune cells and prevent inflammatory damage to the host. The interaction of siglecs on mucosal immune cells and their specific glycan ligands on nonmalignant epithelial cells might be one of such homeostatic systems, and its abrogation through cancer-associated abnormal glycosylation provides a plausible mechanism for the progression of colonic cancers.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interests.

References


Supplemental Figure 1. Distribution of disialyl Lewis\textsuperscript{a} glycan in colon cancer cells and non-malignant epithelia. Surgical specimens obtained from 23 patients with colorectal cancer were immunohistochemically analyzed for expression of disialyl Lewis\textsuperscript{a} glycan using a specific monoclonal antibody FH7. The density of the carbohydrate determinant was graded on a scale of 5; +++ indicating the determinant is expressed in more than 50%; ++, 20-50%; +, 5-20%; ±, less than 5%; and –, none of the epithelial or cancer cells. Wilcoxon-Mann-Whitney test was employed for statistical analysis of staining densities. The results indicated preferential expression of disialyl Lewis\textsuperscript{a} in non-malignant colonic epithelial cells compared to cancer cells, which was statistically significant at p=0.004.
Supplemental Figure 2. Specificity of monoclonal antibodies and expression of carbohydrate determinants in transfectant cells used in this study. Panel A, specificity of monoclonal antibodies directed to disialyl Lewisα-related carbohydrate determinants. The reactivities of antibodies to pure synthetic glycans are shown. ELISA was performed using each synthetic carbohydrate determinant immobilized at the bottom of 96-well culture plates by a standard method described previously. Serial dilution of immobilized carbohydrate determinants started from 20 ng/well. The specificities of these antibodies were previously ascertained using glycolipids prepared from natural sources. Panel B, expression of disialyl Lewisα-related carbohydrate determinants in parental SW1083 cells and the cells transfected with ST6GalNAc6 cDNA. Note that the parental SW1083 cells do not express disialyl Lewisα/c but only sialyl Lewisα, whereas the transfectant cells express disialyl Lewisα, but virtually no sialyl Lewisα. Expression of disialyl Lewisα in transfectant cells was weak, suggesting that the enzymatic products of ST6GalNAc6 were mostly converted to disialyl Lewisα because of the strong endogenous fucosyltransferase activity. Panel C, expression of sialyl 6-sulfomethyl Lewisα-related carbohydrate determinants in ECV304 and transfectant cells. Structures of glycans defined by monoclonal antibodies directed to sialyl 6-sulfomethyl Lewisα-related carbohydrate determinants are shown in the left. G152 is specific to sialyl 6-sulfomethyl Lewisα, G72 is reactive to both sialyl 6-sulfomethyl LacNAc and sialyl 6-sulfomethyl Lewisα, and CSLEX-1 is specific to sialyl Lewisα. The reactivities of these antibodies to pure synthetic glycans were described in ref. (14). Expression of sialyl 6-sulfomethyl Lewisα-related carbohydrate determinants in mock ECV304 cells and the cells transfected with either FUT7 or GlcNAc6ST-1 cDNA, or with both cDNAs, are shown in the right panel. Note that the ECV304 cells transfected with FUT7 cDNA express only sialyl Lewisα defined by CSLEX-1, while those transfected with GlcNAc6ST-1 cDNA is reactive only to G72, which bind to sialyl 6-sulfomethyl LacNAc. The ECV304 cells co-transfected with both genes express sialyl 6-sulfomethyl Lewisα, and are reactive to both G152 and G72 antibodies. Expression of sialyl Lewisα is attenuated because of substrate deprivation in the cells co-transfected with both genes.
Supplemental Figure 3.
An example of expression patterns of siglec-7 (left) and siglec-9 (right) in normal peripheral blood lymphocytes as ascertained by flow cytometric analyses. Significant expression of siglec-7 was noted in NK cells (CD16+CD3- or CD56+CD3-), NKT cells (CD16+CD3+ or CD56+CD3+), killer T cells (CD8+CD3+ and especially CD8αα+CD3+), TcRγδ+ cells and gut-homing T cells (β7-integrin+CD8+ and CCR9+CD3+). Significant expression of siglec-9 was noted in NK cells (CD16+CD3- or CD56+CD3-), NKT cells (CD16+CD3+ or CD56+CD3+), CD8αα-positive killer T cells (CD8αα+CD3+), and TcRγδ+ cells. Monoclonal anti-siglec-7 (2-7-E) and anti-siglec-9 (5-8-C, both rat IgG2a) were used for flow-cytometric analyses. Antibodies for CD3 (SK7), CD8 (SK1) and β7-integrin (FIB504) were obtained from Becton Dickinson. PC5-labeled antibodies for CD8α (B9.11), TcRαβ (BMA031), CD4 (13B8.2), CD19 (J4.119), CD33 (D3HL60.251), CD56 (N901) and PE-labeled antibodies for CD8β (2ST8.5H7), TcRγδ (IMMU510) were obtained from Immunoteck (Marseille, France).
Supplemental Figure 4.
Expression of siglec-7 and -9 in macrophage-like cells differentiated from normal human peripheral monocytes. Normal human peripheral monocytes were obtained from Lonza Walkersville Inc. (Walkersville, MD), and were cultured in the presence of 10 ng/ml M-CSF in RPMI1640 with 10% FCS and 20 U/ml DNAse I (Sigma D4513) according to the manufacturer's protocol. Initially, normal human peripheral monocytes showed a typical siglec-7+siglec-9+ pattern. After 6 days of culture the cells formed a well-spread monolayer and showed a typical macrophage-like morphology. During the course of differentiation of peripheral monocytes into macrophage-like cells, expression of both siglec-7 and -9 tended to decrease, and became almost undetectable after two week culture as shown below. This indicated that the pattern of siglec expression in macrophages derived from peripheral monocytes by a standard technique is quite different from those found in the resident macrophages in colonic lamina propria.