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Expression of Sialyl 6-Sulfo Lewis X Is Inversely Correlated with Conventional Sialyl Lewis X Expression in Human Colorectal Cancer[1]

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ABSTRACT

Sialyl 6-sulfo Lewis X determinant has been described recently as a major ligand for L-selectin on high endothelial venules of human peripheral lymph nodes. From our investigation of its distribution in human colorectal cancer tissues and cultured colon cancer cells, the sialyl 6-sulfo Lewis X determinant was preferentially expressed in the nonmalignant colonic epithelia rather than cancer cells ($P < 0.001$; $n = 23$). This was in contrast to the distribution of conventional sialyl Lewis X, which was preferentially expressed in cancer tissues rather than nonmalignant epithelia ($P = 0.007$; $n = 23$), indicating that 6-sulfation predominantly occurs in nonmalignant tissues and is suppressed upon malignant transformation. In confirmation of this, a nonsialylated determinant 6-sulfo Lewis X was also found to be preferentially localized in the nonmalignant epithelia. Significant expression of sialyl 6-sulfo Lewis X was observed in only 2 lines, whereas 8 were positive for conventional sialyl Lewis X, among 13 cultured colon cancer cell lines. Transfection of cells with fucosyltransferase (Fuc-T) VI induced expression of sialyl 6-sulfo Lewis X, whereas transfection of Fuc-T III did not, suggesting that the determinant was synthesized mainly by Fuc-T VI in colonic epithelia. Members of the sialic acid cyclase pathway, the de-*N*-acetyl sialyl 6-sulfo Lewis X and cyclic sialyl 6-sulfo Lewis X determinants, were also preferentially expressed in the nonmalignant epithelia rather than colonic cancer cells ($P < 0.001$; $n = 23$). Stimulation of the sialyl 6-sulfo Lewis X-positive colon cancer cell line with a calcium ionophore ionomycin markedly reduced sialyl 6-sulfo Lewis X and induced cyclic sialyl 6-sulfo Lewis X expression. These results suggested that the metabolic conversion of sialyl 6-sulfo Lewis X into cyclic sialyl 6-sulfo Lewis X by a calcium-dependent enzyme, sialic acid cyclase, as we hypothesized for human leukocytes previously (C. Mitsuoka *et al.*, Proc. Natl. Acad. Sci. USA, *96*: 1597–1602, 1999), also occurs in nonmalignant colonic epithelia.

INTRODUCTION

Malignant transformation is frequently accompanied with a drastic alteration of surface oligosaccharide expression (1–3). Carbohydrate determinants containing sialylated and/or fucosylated structures, such as sialyl Le^x[3] and sialyl Le^a, recently attracted special attention because they serve as ligands for the adhesion molecule E-selectin and are thought to play significant roles in blood-born metastasis of cancer (1, 4–12). Considerable structural heterogeneity of sialyl Le^x/sialyl Le^a-related carbohydrate determinants is known to occur in malignant and nonmalignant cells (1, 8, 9, 13). The most recently described modification of these determinants is 6-sulfation. Sialyl 6-sulfo Le^x was identified recently as a major L-selectin ligand on high endothelial venules of human peripheral lymph nodes (14–18). The G152 antibody specific to sialyl 6-sulfo Le^x strongly labeled high endothelial venules in human peripheral lymph nodes and inhibited the binding of L-selectin to high endothelial venules (16). The determinant was also found to serve as a ligand for selectins other than L-selectin (16, 17). The functional selectin ligand activity was successfully reconstituted by the transfection of cells with Fuc-T and 6-*O*-sulfotransferase, which produced sialyl 6-sulfo Le^x on the transfected cells (17). We detected this determinant on human leukocytes and some epithelial cells with the aid of the G152 antibody in preliminary experiments. It was of particular interest to study expression of this determinant in colon cancer, because an increase of sialylation and a decrease of sulfation of carbohydrate determinants have long been known to associate with malignant transformation of colonic epithelial cells. In this work, we studied expression of sialyl 6-sulfo Le^x and related 6-sulfated determinants in malignant and benign colonic tissues. We also sought evidence that would indicate that the conversion of sialyl 6-sulfo Le^x to cyclic sialyl 6-sulfo Le^x, which we proposed recently as a hypothetical metabolic pathway for inactivation of selectin ligand activity in leukocytes (19), takes place in colonic tissues.

MATERIALS AND METHODS

Antibodies, Clinical Samples, and Immunohistochemical Staining. 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 in rectum) operated at the Aichi Cancer Center Hospital. Nine female and 14 male patients were included, with an average age of 58.3 years. The stage of the patients varied from Dukes A to D, and 22 cases were histologically diagnosed as moderately differentiated and 1 case as poorly differentiated adenocarcinoma. No cases of mucinous adenocarcinoma were included. The avidin-biotin complex technique for the immunohistochemical study was performed as described in the instructions for the kits (Vectastain) provided by Vector, Inc. (Burlingame, CA). The density of each carbohydrate determinant was graded on a scale of 5; +++ indicating the determinant is expressed in >50%; ++, 20–50%; +, 5–20%; ±, <5%; and −, none of the epithelial or cancer cells. The Wilcoxon Mann-Whitney test was used for statistical analysis of staining densities. The antibodies G152 directed to sialyl 6-sulfo Le^x, G72 directed to sialyl 6-sulfo LacNAc, AG107 and AG223 both recognizing nonsialylated 6-sulfo Le^x were prepared as described previously (16, 20). A classical anti-sialyl Le^x antibody, CSLEX-1, was obtained from American Type Culture Collection (Manassas, VA; Ref. 21), and SNH-3 was a gift from Dr. Sen-itiroh Hakomori (Pacific Northwest Research Foundation, Seattle, WA; Refs. 10 and 12). The anti-Le^x antibody FH-2 was also a gift from Dr. Sen-itiroh Hakomori, and LeuM1 was obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA). The antibody SU59 directed to 3′-sulfo Le^x was a gift from Dr. Akihiro Hino (Nisshin Shokuhin Co. Ltd., Otsu, Japan; Ref. 16). This antibody considerably cross-reacts to 3′-sulfo Le^a; therefore, we refer to the determinant defined by this antibody as 3′-sulfo Le^x/Le^a. The anti-cyclic sialyl 6-sulfo Le^x antibody G159 (murine IgG1), established against a synthetic glycolipid antigen, was

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[3] The abbreviations used are: Le^x, Lewis X, Galβ1→4[Fucα1→3]GlcNAcβ1→R; Le^a, Lewis A, Galβ1→3[Fucα1→4]GlcNAcβ1→R; LacNAc, *N*-acetyl lactosamine; Fuc-T, fucosyltransferase; GlcNAc, *N*-acetyl glucosamine; sialyl Le^x, NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAcβ1→R; VIM-2, NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4[Fucα1→3]GlcNAcβ1→R.

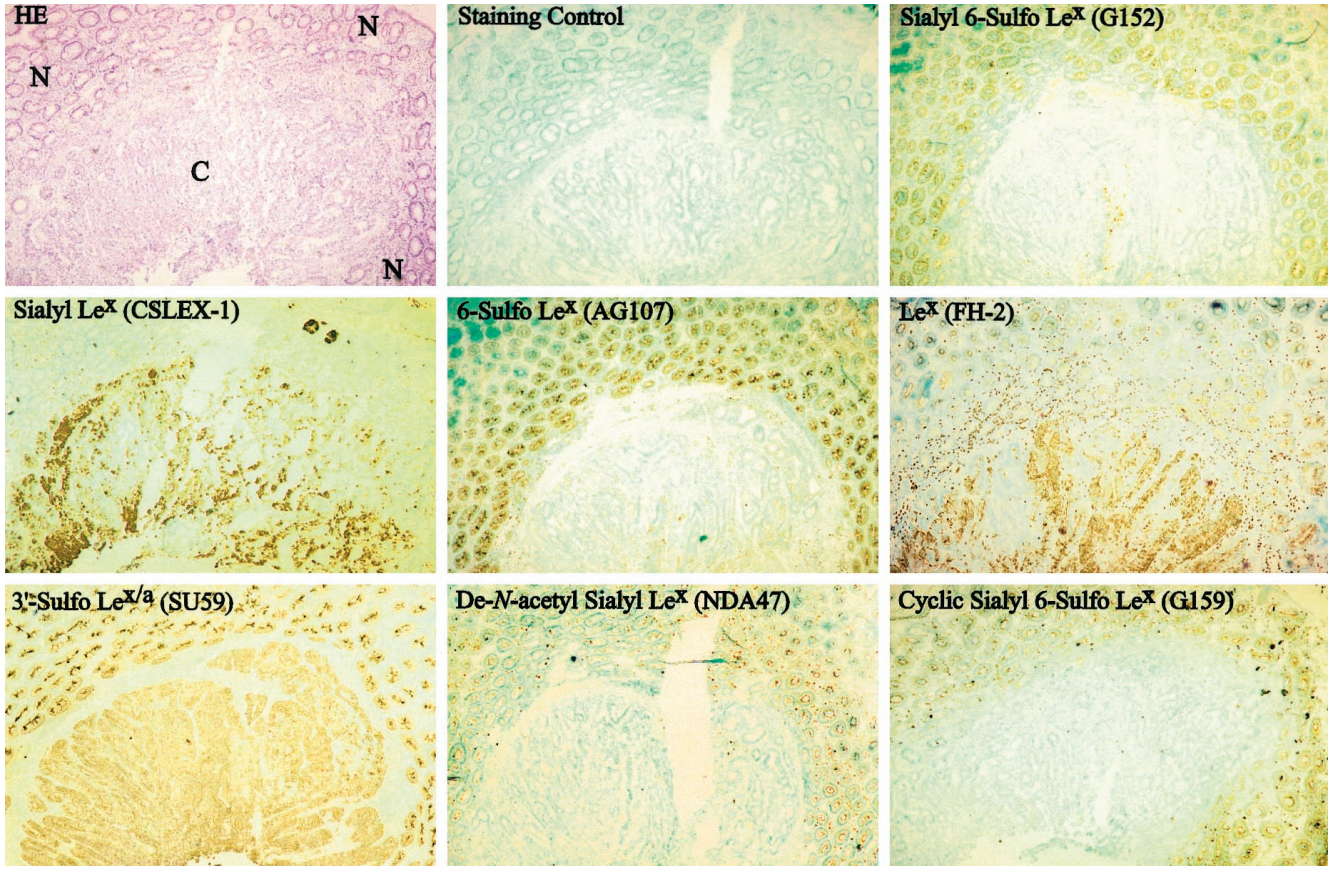

Fig. 1. Typical examples of immunohistochemical localization of sialyl 6-sulfo Le^x and related carbohydrate determinants in colon cancer cells and surrounding nonmalignant colonic epithelia. ×100; *HE*, H&E staining. Antibodies used for staining are indicated in *parentheses*. *C*, cancer cells; *N*, nonmalignant colonic epithelial cells.

prepared as described previously (19). The anti-de-*N*-acetyl sialyl 6-sulfo Le^x antibody NDA47 (murine IgM) was newly established against a synthetic glycolipid antigen, and will be described elsewhere.[4] Reactivity of these antibodies against pure carbohydrate determinants was determined by ELISA as reported previously (16).

Transfection of Namalwa-KJM-1 with Fuc-Ts. The cell line Namalwa KJM-1, a subline of the human Burkitt lymphoma cell line Namalwa, was chosen as a host for transfection of Fuc-Ts because it did not express sialyl Le^x-related carbohydrate determinants but expressed sialyl 6-sulfo lactosamine, as detected by the G72 antibody, and was thought to have substrates necessary for the synthesis of sialyl 6-sulfo Le^x and/or 6-sulfo Le^x determinants. Vectors containing the replication origin of EBV, such as pAMo, were shown to be replicated stably in an episomal state in this cell line (22). Namalwa KJM-1 cells were transfected with pAMo-Fuc-T III, pAMo-Fuc-T VI, pAMo-Fuc-T VII, pAMo-Fuc-T IV, or parental vector pAMo, as described previously (22). After transfection with these pAMo vectors, cells grown in selection medium containing G418 (0.5 mg/ml) were used in the experiments without further cloning procedures.

Flow Cytometric Analysis of Sialyl 6-Sulfo Le^x and Related Carbohydrate Determinants and Ionomycin Stimulation. Cultured human colon cancer cell lines, including C-1, WiDr, Colo201, HT-29, LS174T, SW1083, SW480, LoVo, Colo320, HCT-15, HCT116, CoR-1, and Caco-2, were maintained with DMEM supplemented with 10% FCS. These cancer cells and Namalwa KJM-1 transfectants were harvested at a semiconfluent stage and stained with the monoclonal antibody using purified antibody at 1 μg/ml or culture supernatant at a dilution of 1:5. Cells were then stained with 1:200 dilution of FITC-conjugated goat antimouse IgG (heavy and light chain specific; Silenus Laboratories, Hawthorn, Victoria, Australia) and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Stimulation of LS174T cells was performed with the final 10–80 μM of ionomycin (Calbiochem, San Diego, CA). The reaction was stopped with a final concentration of 0.5% paraformaldehyde at the indicated time, and the cells were subjected to staining and cytofluorometric analyses.

RESULTS

Distribution of Sialyl 6-Sulfo Le^x and Conventional Sialyl Le^x in Human Colon Cancer Tissues. By immunohistochemical studies on 23 cases of colon cancer tissues using a specific antibody G152, the sialyl 6-sulfo Le^x determinant was shown to be expressed in nonmalignant epithelial cells more strongly than in cancer cells. It was in clear contrast to the distribution of conventional sialyl Le^x, which was preferentially expressed in cancer cells. A typical example is shown in Fig. 1. Weak expression of sialyl 6-sulfo Le^x in cancer cells was noted in some cases, but such cases were all accompanied by the much stronger expression of conventional sialyl Le^x in the same specimen. The difference in the expression of sialyl 6-sulfo Le^x in nonmalignant colonic tissues compared with that in cancer cells was statistically significant at $P < 0.001$ ($n = 23$), as shown in Fig. 2. The sialyl 6-sulfo LacNAc determinant, which is defined by the G72 antibody, also predominated in nonmalignant epithelial cells, and the difference was statistically significant (Fig. 2). Specificity of these antibodies against pure carbohydrate determinants is shown in Fig. 3. G72 reacts to both sialyl 6-sulfo Le^x and sialyl 6-sulfo LacNAc, whereas G152 is specific to sialyl 6-sulfo Le^x.

On the other hand, the preferential expression of conventional sialyl Le^x in cancer tissue was statistically significant at $P = 0.007$ when the CSLEX-1 antibody was used and at $P = 0.02$ with the SNH-3

[4] D. Mitsuoka, S. Komba, H. Ishida, M. Kiso, and R. Kannagi. Identification of intermediate metabolites in sialic acid cyclase pathway, manuscript in preparation.

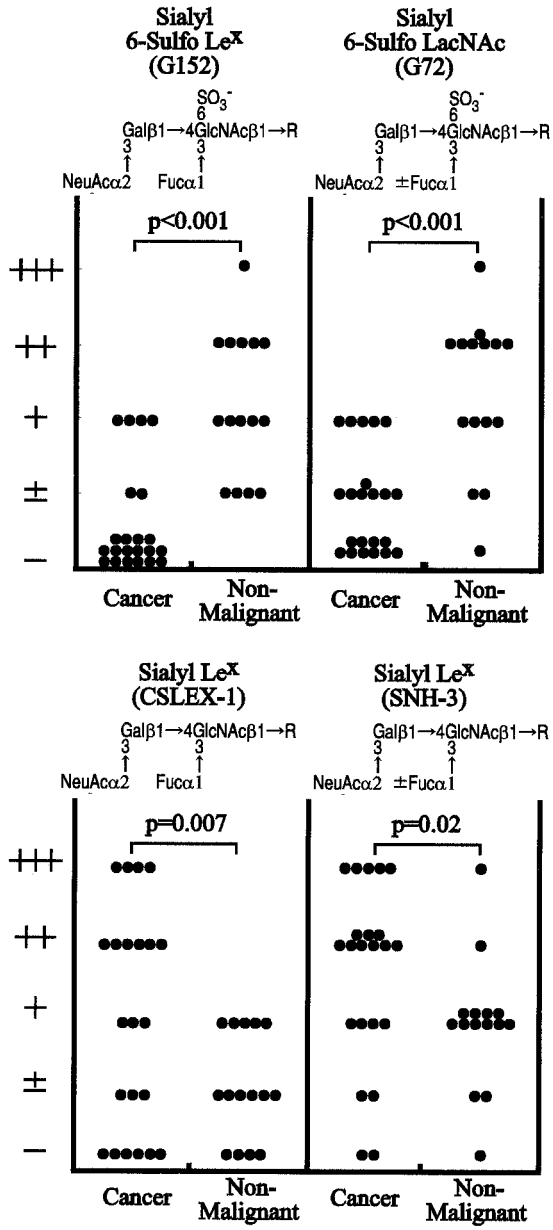

Fig. 2. Distribution of the sialyl 6-sulfo Le^x, sialyl 6-sulfo LacNAc, and conventional sialyl Le^x determinants in colon cancer cells and nonmalignant epithelia. Antibodies used for staining are indicated in *parentheses*, and the carbohydrate structures defined by the antibodies are shown in the "Results" section.

antibody (Fig. 2). SNH-3 recognizes both sialyl Le^x and the related VIM-2 determinant, whereas CSLEX-1 is specific to sialyl Le^x, and neither antibody is reactive to 6-sulfated determinants (Fig. 3). The predominant expression of conventional sialyl Le^x in cancer cells is compatible with the previous notion that this determinant is a cancer-associated antigen (1–3, 21, 23), whereas the preferential expression of sialyl 6-sulfo Le^x in nonmalignant tissues was unexpected, because the carbohydrate structure of the determinant is very similar to that of conventional sialyl Le^x, except for one sulfate residue attached at the C-6 position of the GlcNAc moiety.

Distribution of Nonsialylated 6-Sulfo Le^x and Conventional Le^x in Human Colon Cancer Tissues. To assess more exactly the significance of 6-sulfation in the determinants, we studied the distribution of nonsialylated 6-sulfo Le^x and conventional Le^x in human colon cancer tissues. The nonsialylated 6-sulfo Le^x determinant, as defined by the AG107 antibody, was also preferentially expressed in nonmalignant colonic epithelia compared with cancer cells, as typically seen in Fig. 1, and the difference was statistically significant (Fig. 4). Expression of the 6-sulfated determinants, including sialylated and nonsialylated 6-sulfo Le^x, tended to be stronger in nonmalignant epithelia adjacent to cancer cell nests than in the epithelia distant from the cancer cell nests, suggesting that some stimuli enhanced their expression. The conventional Le^x determinant defined by the FH-2 antibody was strongly expressed in cancer cells, as shown in Fig. 1, but this determinant was expressed in the cells at the base of the colonic crypt, which is a proliferation zone in this tissue, and the difference between its expression in nonmalignant epithelia and in cancer cells was statistically not significant (Fig. 4). The FH-2 antibody is reactive to conventional sialyl Le^x but not to sialyl 6-sulfo Le^x (Fig. 3). The 3′-sulfo Le^x/Le^a determinant defined by the SU59 antibody was strongly expressed in cancer cells as well as in sur-

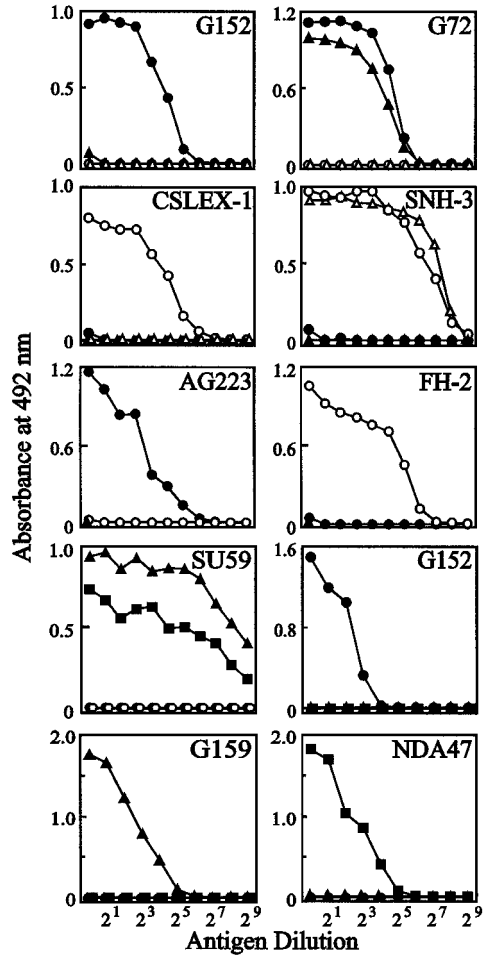

Fig. 3. Reactivity of the antibodies used in this study against pure carbohydrate determinants as ascertained by ELISA. In the assays for anti-sialyl 6-sulfo Le^x antibodies (*G152* and *G72*) and anti-sialyl Le^x antibodies (*CSLEX-1* and *SNH-3*), ● indicates reactivity against pure sialyl 6-sulfo Le^x; ○, against sialyl Le^x; ▲, sialyl Le^x; and △, the VIM-2 determinant. The antibody G152 is specific to sialyl 6-sulfo Le^x, and G72 is reactive to both sialyl 6-sulfo Le^x and sialyl 6-sulfo LacNAc, which lacks 3-fucosylation at penultimate GlcNAc (16). CSLEX-1 is specific to conventional sialyl Le^x, and SNH-3 is reactive to sialyl Le^x as well as some determinants such as the VIM-2 determinant, which lacks 3-fucosylation at penultimate GlcNAc (46). For nonsialylated series of antibodies including anti-6-sulfo Le^x (*AG223*), anti-Le^x (*FH-2*), and anti-3′-sulfo Le^x/Le^a (*SU59*), ● indicates reactivity against pure 6-sulfo Le^x; ○, against pure Le^x; ▲, 3′-sulfo Le^x; and ■, 3′-sulfo Le^a. In the above assays, serial dilution of antigen started from 40 ng/well. For a set of antibodies directed to the metabolites in sialic acid cyclase pathway (*G152*, *G159*, and *NDA47*), ● indicates reactivity against pure sialyl 6-sulfo Le^x; ▲, against cyclic sialyl 6-sulfo Le^x; and ■, against de-*N*-acetyl sialyl 6-sulfo Le^x. In these assays, serial dilution of antigen started from 20 ng/well.

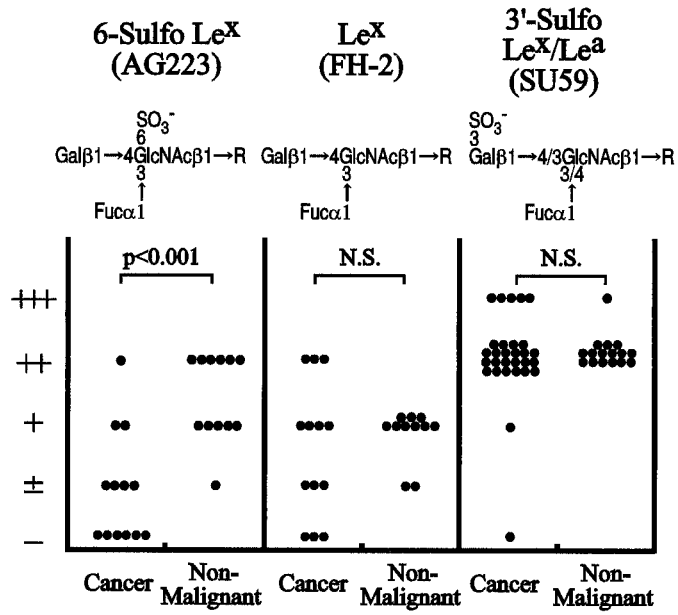

Fig. 4. Distribution of the nonsialylated determinants, 6-sulfo Le^x, conventional Le^x, and 3′-sulfo Le^x/Le^a, in colon cancer cells and nonmalignant epithelia. Antibodies used for staining are indicated in *parentheses*, and the carbohydrate structures defined by the antibodies are shown immediately *below*.

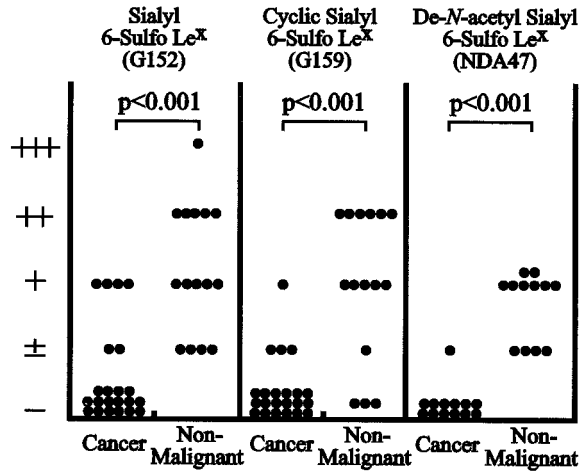

Fig. 5. Distribution of the metabolites in the sialic acid cyclase pathway, sialyl 6-sulfo Le^x, de-*N*-acetyl sialyl 6-sulfo Le^x, and cyclic sialyl 6-sulfo Le^x determinants in colon cancer cells and nonmalignant epithelia. Antibodies used for staining are indicated in *parentheses*. For specificity of the antibodies, see Fig. 3 and "Materials and Methods."

rounding nonmalignant epithelia (Fig. 1), and the difference was statistically not significant (Fig. 4).

Expression of Sialyl 6-Sulfo Le^x-related Determinants in Sialic Acid Cyclase Pathway in Human Colon Cancer Tissues. The de-*N*-acetyl sialyl 6-sulfo Le^x and cyclic sialyl 6-sulfo Le^x determinants, the two intermediate metabolites of the sialic acid pathway that we proposed recently for sialyl 6-sulfo Le^x (19), were detected in colonic tissues. These metabolites were detected by the antibodies specific to the respective determinants (Fig. 3). The de-*N*-acetyl sialyl 6-sulfo Le^x determinant defined by the NDA47 antibody was weakly expressed, whereas the cyclic sialyl 6-sulfo Le^x determinant defined by the G159 antibody was moderately expressed in human colonic tissues, as typically shown in Fig. 1. Both determinants were preferentially localized in the nonmalignant epithelia rather than cancer cells, and the difference was statistically significant (Fig. 5). All cases positive for de-*N*-acetyl sialyl 6-sulfo Le^x or cyclic sialyl 6-sulfo Le^x expressed sialyl 6-sulfo Le^x. The weaker expression of de-*N*-acetyl sialyl 6-sulfo Le^x determinant is most probably related to its rapid turnover as an intermediate product of the metabolic pathway.

Fuc-Ts Involved in Synthesis of 6-Sulfated Determinants. To know whether the hitherto reported Fuc-Ts are capable of synthesizing 6-sulfated determinants as described above, we analyzed the expression of these determinants in the cultured Namalwa KJM-1 cells transfected with Fuc-T cDNA. To date, isozymes Fuc-T III, VI, and IV are known to be expressed in colonic tissues and colon cancer cells (24, 25). The cells transfected with Fuc-T VI expressed both sialyl 6-sulfo Le^x and conventional sialyl Le^x, as well as the nonsialylated determinants such as 6-sulfo Le^x and conventional Le^x (Fig. 6). On the other hand, the cells transfected with Fuc-T III expressed conventional sialyl Le^x strongly, whereas sialyl 6-sulfo Le^x was apparently not expressed (Fig. 6). Marked expression of conventional Le^x was observed in the cells transfected with Fuc-T III, but 6-sulfo Le^x was virtually not expressed (Fig. 6). These findings indicated that Fuc-T VI was very active on the 6-sulfated carbohydrate substrates but suggested that 6-sulfated type 2 chain carbohydrates were not preferred substrates for Fuc-T III.

Fuc-T IV, which is known to be expressed frequently in cultured colon cancer cells (24) as well as in colonic cancer tissues (25), and the transcript of which increases upon malignant transformation (25), induced expression of both conventional Le^x and 6-sulfo Le^x, whereas the induction of sialylated determinants was not detectable (Fig. 6). This is compatible with the notion proposed previously that this enzyme prefers nonsialylated substrates to sialylated ones (26). Fuc-T VII, the isozyme preferentially localized in leukocytes and endothelial cells but not in colonic epithelia, is known to prefer sialylated substrates over nonsialylated ones (27, 28), and this is confirmed by the strong expression of conventional sialyl Le^x and 6-sulfo sialyl Le^x in Fuc-T VII transfectant cells.

Expression of Sialyl 6-Sulfo Le^x in Cultured Colon Cancer Cells and Effect of Ionomycin Treatment. Among the tested 13 human colon cancer cell lines, only 2 cell lines expressed the sialyl 6-sulfo Le^x determinant significantly. The LS174T cells expressed the deter-

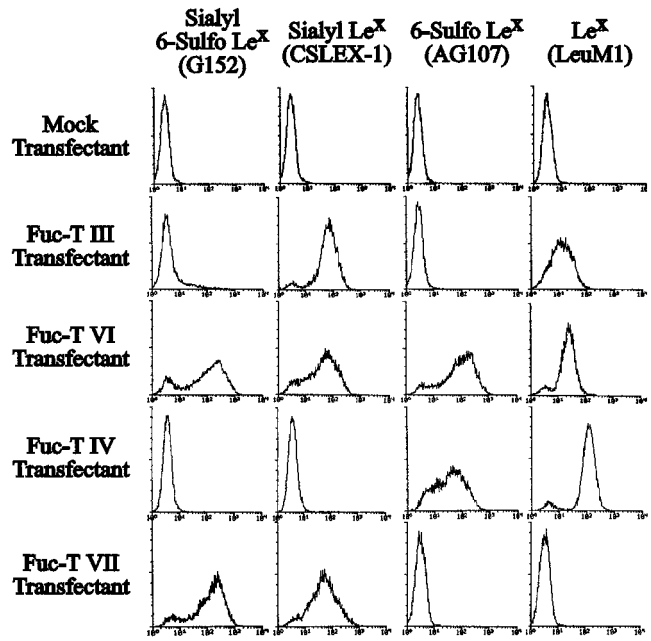

Fig. 6. Expression of sialyl 6-sulfo Le^x and related determinants on cultured human lymphoid cells transfected with four human α1→3/4 Fuc-Ts, Fuc-T III, VI, IV, and VII. *Abscissa*, relative fluorescence intensity; *ordinate*, cell number. For specificity of antibodies, see "Materials and Methods."

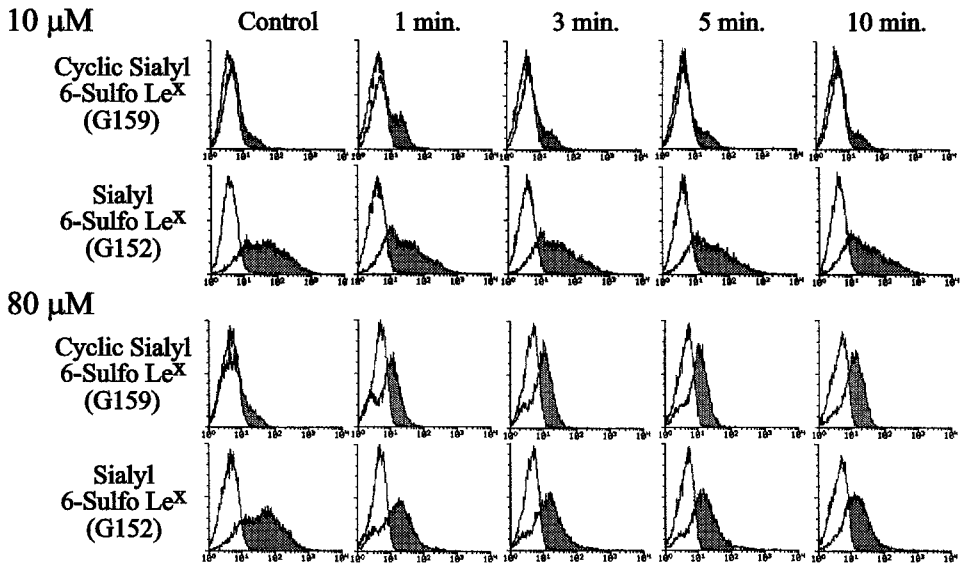

Fig. 7. Expression of the sialyl 6-sulfo Le^x and cyclic sialyl 6-sulfo Le^x determinants on the cultured human colon cancer cell line LS174T stimulated by a calcium ionophore, ionomycin. *Abscissa*, relative fluorescence intensity; *ordinate*, cell number. Final concentration of ionomycin was 10 μM in the experiments shown in the *upper panel* and 80 μM in the experiments shown in the *lower panel*.

minant moderately, but the Colo201 cells expressed the determinant only weakly. This was in contrast to the expression of conventional sialyl Le^x, which was clearly expressed in 8 of 13 lines. This again confirmed that the 6-sulfo determinant was less frequently expressed in cancer cells. The 2 cell lines positive for sialyl 6-sulfo Le^x were both associated with much stronger expression of conventional sialyl Le^x. The finding was also in contrast to the expression of 3′-sulfo Le^x/Le^a, which was obviously expressed on 5 of 13 lines, again confirming that the 6-sulfated determinant is less frequently expressed in cancer cells compared with the 3′-sulfated determinant. There was no correlation between the expression of 3′-sulfated and 6-sulfated determinants.

When the LS174T cells were stimulated with 10 μM of the calcium ionophore ionomycin, a transient expression of G159-defined cyclic sialyl 6-sulfo Le^x was induced 1 min after the addition of the ionomycin, which was accompanied by a small but significant decrease in the fluorescence intensity of G152-defined sialyl 6-sulfo Le^x (Fig. 7, *upper panel*). Induction of cyclic sialyl 6-sulfo Le^x was more prominent at 1 min and sustained thereafter at 80 μM of ionomycin, and this was associated with a marked reduction of sialyl 6-sulfo Le^x expression (Fig. 7, *lower panel*). These findings are compatible with the notion that sialyl 6-sulfo Le^x is converted to cyclic sialyl 6-sulfo Le^x by a calcium-dependent enzyme, sialic acid cyclase, as proposed to occur previously in leukocytes (19). The time course of the reaction at the increasing concentration of ionomycin is illustrated in Fig. 8. The reaction was rapid and saturable within 5–10 min after the addition of ionomycin, and around 30–70% of sialyl 6-sulfo Le^x at the cell surface was susceptible to the reaction in terms of fluorescence intensity. The magnitude of reaction was dependent on the concentration of ionomycin, and the reaction was irreversible above an ionomycin concentration of 40 μM.

DISCUSSION

The present study indicated that the sialyl 6-sulfo Le^x determinant, shown previously to be an L-selectin ligand on high endothelial venules in lymph nodes, is expressed in human colonic tissues. The determinant was preferentially expressed in nonmalignant colonic epithelia surrounding cancer nests, contrary to conventional sialyl Le^x, which was predominantly expressed in cancer cells. Our transfection experiments indicated that Fuc-Ts Fuc-T VI and VII are capable of synthesizing sialylated 6-sulfo determinants, and Fuc-T IV and VI can synthesize nonsialylated 6-sulfo determinants. Because Fuc-T VI and IV are reported to be significantly expressed in colonic tissues (24, 25, 29), it could be suggested that the sialyl 6-sulfo determinants were mainly synthesized by Fuc-T VI and nonsialylated 6-sulfo Le^x determinants by Fuc-T VI and IV in colonic tissues. This indicates that 6-sulfated determinants are synthesized by a common set of the enzymes involved in the synthesis of conventional sialyl Le^x and Le^x. It is well known that conventional sialyl Le^x has significance as a cancer-associated antigen in colonic tissues as well as in other tissues (1, 2), because its expression is significantly increased in cancer tissues compared with nonmalignant tissues, as was also confirmed in this study. To date, we and other researchers have focused on the increase of synthetic enzymes such as Fuc-Ts and/or sialyltransferases for a possible mechanism that would explain the accumulation of sialyl Le^x in cancer tissues (24, 25, 30, 31). However, transcripts for Fuc-Ts as well as their enzymatic activity are not

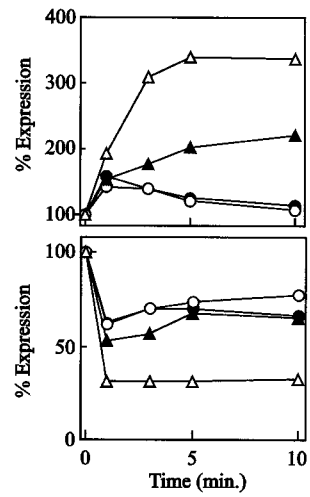

Fig. 8. Time course and effect of ionomycin concentration on the reciprocal expression of the sialyl 6-sulfo Le^x and cyclic sialyl 6-sulfo Le^x determinants on the ionophore-stimulated cultured human colon cancer cell line LS174T. *Upper panel*, percentage of relative expression of cyclic sialyl 6-sulfo Le^x as defined by the G159 antibody; *lower panel*, expression of sialyl 6-sulfo Le^x as defined by the G152 antibody. Ionomycin concentrations: ●, 10 μM; ○, 20 μM; ▲, 40 μM; and △, 80 μM.

significantly increased in colon cancer tissues except for Fuc-T IV (24, 25, 31, 32). Transcripts for the sialyltransferase ST-4, which was proposed to be involved in the synthesis of sialyl Le^x (22), was shown to be even significantly decreased in cancer tissues compared with nonmalignant colonic mucosa (25). The results of the present study suggest that a set of enzymes required for the sialyl Le^x synthesis is already present in nonmalignant colonic tissue, and that the suppression of synthesis of sialyl 6-sulfo Le^x could partly explain the accumulation of conventional sialyl Le^x in colonic cancer tissues. This is an indication of the so-called "incomplete synthesis of carbohydrate determinants" (1) as a possible cause for the abnormally increased expression of conventional sialyl Le^x determinant in colon cancer. Another possibility is the preferential acceleration of degradation of 6-sulfate residue in colon cancer cells.

The nonsialylated determinant 6-sulfo Le^x defined by the AG107 or AG223 antibody was also preferentially expressed in the nonmalignant colonic epithelia. Together with the findings on the sialylated determinants, this suggests that the sulfation at the C-6 position of GlcNAc is enhanced in nonmalignant tissues and is somehow suppressed in colon cancer cells. It has long been postulated from the results of histochemical studies using cationic dyes that sulfomucin tends to decrease upon malignant transformation of colonic epithelia. A part of the sulfate residue preferentially expressed in nonmalignant colonic epithelia must be 3′-sulfation, the modification at the C-3 position of terminal galactose, as proposed previously (33–35), but results of our current study indicated that 6-sulfation, which is the addition of sulfate at the C-6 position of the GlcNAc moiety, is another candidate for the preferential sulfation in nonmalignant colonic mucosa. Actually, our results indicated that 6-sulfated determinants tend to be more preferentially localized in nonmalignant epithelia than 3′-sulfated determinants. *N*-Linked oligosaccharides having 6-sulfated GlcNAc were reported to occur also preferentially in the normal counterpart of the carcinoembryonic antigen (36).

The physiological significance of the sialyl 6-sulfo Le^x in colonic tissues is not clear at this moment. It is proposed that the conventional sialyl Le^x determinant expressed in colonic cancer cells plays an important role in hematogenous metastasis through the binding to E-selectin on blood vessels (1, 4–12). We have shown previously that sialyl 6-sulfo Le^x is capable of binding to E-selectin as well as L-selectin (16, 17). The sialyl 6-sulfo Le^x determinant expressed in cancer cells could also play a role in the adhesion. However, the expression of sialyl 6-sulfo Le^x in cancer has been generally weak and always associated with the much stronger expression of conventional sialyl Le^x in both cancer tissue sections and cultured colon cancer cell lines. In fact, it may be of only secondary or minor significance in the adhesion to selectins. A possible function of sulfated carbohydrate determinants in normal epithelia is proposed to be the absorption of pathogenic microorganisms such as virus or bacteria (37–41), and this could be a physiological function of sialyl 6-sulfo Le^x expressed in nonmalignant colonic epithelia, where the determinant was preferentially localized at the lumenal surface of the cells.

Recently, we proposed that the sialyl 6-sulfo Le^x determinant is metabolized through a distinct pathway involving cyclization of sialic acid (19). This pathway involves deacetylation of the *N*-acetyl residue of the sialic acid moiety in sialyl 6-sulfo Le^x, producing the de-*N*-acetyl sialyl 6-sulfo Le^x determinant, followed by the cyclization of sialic acid by dehydration, forming the cyclic sialyl 6-sulfo Le^x determinant (19). Results of the present study indicated that both metabolites of the pathway, de-*N*-acetyl sialyl 6-sulfo Le^x and cyclic sialyl 6-sulfo Le^x, are present in colonic tissues and preferentially localized in the nonmalignant colonic epithelia as well as parental sialyl 6-sulfo Le^x. This indicates that the sialic acid cyclase pathway, which we postulated as a regulatory pathway for inhibition of selectin-ligand activity, occurs in nonmalignant epithelia but less frequently in cancer cells. The presence of a related metabolite, de-*N*-acetyl G_{D3}, was reported recently to occur in colonic tissue (42). Colonic epithelia have long been known to have a unique intramolecular modification of sialic acid moiety, such as *O*-acetylation (43, 44). It is reported that *O*-acetylation of sialic acid moiety also preferentially occurs in nonmalignant colonic epithelia and is suppressed in colon cancer tissues. This was proposed to be one of the possible mechanisms for the abnormal accumulation of nonacetylated conventional sialyl Le^x in colon cancer cells (43–45). Nonmalignant colonic epithelia are likely to be equipped with several means of intramolecular modification of sialic acid moiety expressed on their surface, thereby regulating cell adhesive and other cellular activities, and these regulatory systems seemingly become dysfunctional upon malignant transformation.

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