Synthesis of Disialyl Lewis a (Le\(^{a}\)) Structure in Colon Cancer Cells by a Sialyltransferase, ST6GalNAc VI, Responsible for the Synthesis of \(\alpha\)-Series Gangliosides*

Akiko Tsuchida†, Tetsuya Okajima†, Keiko Furukawa‡, Takayuki Ando§, Hideharu Ishida§, Aruto Yoshida†, Yoko Nakamura†, Reiji Kannagi‡, Makoto Kiso§, and Koichi Furukawa***

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To whom correspondence should be addressed. Tel.: 81-52-744-2069; E-mail: koichi@med.nagoya-u.ac.jp.

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† To whom correspondence should be addressed. Tel.: 81-52-744-2070; Fax: 81-52-744-2089; E-mail: koichi@med.nagoya-u.ac.jp.

‡ The abbreviations used are: Le\(^{a}\), Lewis a (Gal[1,3]Fuc[1,4]GlcNAc[1,3]Galβ1,4Glcβ1-Cer); Le\(^{b}\), lactotetraosylceramide (Gal[1,3]Glc

\(\alpha\)NaCβ1,3Galβ1,4Glcβ1-Cer); nLc4, neolactotetraosylceramide (Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1-Cer); mAb, monoclonal antibody; PBS, phosphate-buffered saline; RT, reverse transcriptase; ST6GalNAc, sialyltransferase as defined in Ref. 25.

Biosynthesis of disialyl Lewis a (Le\(^{a}\)) was analyzed using previously cloned ST6GalNAc V and ST6GalNAc VI, which were responsible for the synthesis of \(\alpha\)-series gangliosides. Among lactotetraosylceramide (Lc4), neolactotetraosylceramide, and their sialyl forms, only sialyl Lc4 was sialylated with ST6GalNAc V and ST6GalNAc VI. The products were confirmed to be disialyl Le\(^{a}\) in TLC-immunostaining. Compared with the original substrate GM1b, the synthetic rates of disialyl Le\(^{a}\) were 22 and 38% with ST6GalNAc V and ST6GalNAc VI, respectively. Since sialyl Le\(^{a}\) could not be converted to disialyl Le\(^{a}\), disialyl Le\(^{a}\) was produced only from sialyl Lc4. Therefore, it appears that ST6GalNAc V/V1 and fucosyltransferase III (FUT-3) compete for sialyl Lc4, their common substrate. The results of either one transfection or co-transfection of two genes into COS1 cells revealed that both ST6GalNAc V/VI and FUT-3 contributed in the synthesis of disialyl Le\(^{a}\) but partly compete with each other. Many colon cancer cell lines expressed the ST6GalNAc VI gene more or less, and some of them actually expressed disialyl Le\(^{a}\). None of them expressed ST6GalNAc V. These results suggested the novel substrate specificity of ST6GalNAc VI, which is responsible for the synthesis of disialyl Le\(^{a}\) but not for \(\alpha\)-series gangliosides in human colon tissues.

Carbohydrate structures conjugated to proteins and ceramides on the cell surface are involved in the modification of cell-cell or cell-extracellular matrix interaction (1). Consequently, they play important roles in the regulation of cell proliferation, cell adhesion, cancer metastasis, tissue differentiation, and apoptosis (2, 3). In particular, sialylation of sugar chains has been suggested to be a very important process during development, cancer evolution, and progression (4), and sialic acid is often responsible for the synthesis of disialyl Le\(^{a}\) might result in masking the expression of oncofetal antigen by competing with each other in the biosynthesis of individual structures (9). The synthesis and expression of disialyl Le\(^{a}\) might affect the expression mode of sialyl Le\(^{a}\) antigen (i.e. an oncofetal antigen) by competing with each other in the biosynthesis of different antigens (9). The synthesis and expression of disialyl Le\(^{a}\) might result in masking the expression of sialyl Le\(^{a}\). However, little has been known about the biosynthetic pathway of disialyl Le\(^{a}\) or about the sialyltransferase responsible for the transfer of a sialic acid onto GlcNAc with \(\alpha\),\(\beta\) linkage.

To identify the \(\alpha\),\(\beta\)-sialyltransferase that catalyzes the synthesis of disialyl Le\(^{a}\) from monosialyl Le\(^{a}\) or the synthesis of disialyl lactotetraosylceramide (disialyl Lc4) from monosialyl Lc4 as a precursor for the synthesis of disialyl Le\(^{a}\), a sialyltransferase assay was performed using previously cloned \(\alpha\),\(\beta\)-sialyltransferases (10, 11). We demonstrated here that ST6GalNAc V/V1, which were cloned as the sialyltransferases responsible for the synthesis of \(\alpha\)-series gangliosides, could significantly synthesize disialyl Lc4, indicating the main synthetic pathway of disialyl Le\(^{a}\). Based on the analyses of expression levels of the transferase genes and resulting antigens in human colon cancer cell lines, ST6GalNAc VI appears most likely to be a key enzyme in the synthesis of disialyl Le\(^{a}\) structure.

MATERIALS AND METHODS

Nomenclature of Cloned Glycosyltransferases—Six members of the Galectin \(\alpha\)-6-sialyltransferase (ST6GalNAc) subfamily have been cloned so far: ST6GalNAc I (12), ST6GalNAc II (13), ST6GalNAc III (14), ST6GalNAc IV (14), ST6GalNAc V (10), and ST6GalNAc VI (11).
FUT-3 encodes the Lewis α (α3,3,1)-fucosyltransferase (15). ST3Gal IV (16) and β3Gal-T5 (17) are involved in the synthesis of sialyl Leα.

Cell Culture—Mouse fibroblast L cells, human colon cancer cell lines (Caco-2 cells, Colo320, DLD-1, HT-29, Lovo, SW1080, and SW1116), and COS1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal calf serum.

Construction of the Expression Vector—The expression vector pCDNA3.1-ST6GalNAc VI was prepared by inserting an Xhol and XhoI fragment from pMKneo-ST6GalNAc VI (11) into Xbal and Xhol sites of pCDNA3.1(+) vector.

Preparation of Membrane Fraction—L cells (3 x 10⁶) were plated in 10-cm dishes at least 48 h prior to transfection. Cells were transiently transfected with an expression plasmid (4 μg) by the DEAE-dextran method (18). After 48 h of culture in Dulbecco's modified Eagle's medium containing 7.5% fetal calf serum, the cells were harvested by trypsinization. Cells were pelleted, washed with phosphate-buffered saline (PBS), and lysed in ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride using a nitrogen cavitation apparatus (Farr Instrument Co., Moline, IL) at 400 psi for 30 min. Nuclei were removed by low speed centrifugation, and supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The pellet was resuspended in ice-cold 100 mM sodium cacodylate buffer, pH 7.0, and used as an enzyme source for the fusocyltransferase assay as described below.

Fusocyltransferase Assay—The fusocyltransferase assay was performed in a mixture containing 100 mM sodium cacodylate buffer, pH 7.0, 10 mM MgCl₂, 0.25% Triton X-100, 5 mM CDP-choline (Sigma), 0.1 mM GDP-fucose (Sigma), 25,000 dpm/nmol GDP-[^14C]fucose (Amersham Biosciences), 50 μg of a membrane fraction, and 10 μg of acceptors in a total volume of 50 μl. The reaction mixture was incubated at 37 °C for 1 h, and the enzyme products were isolated and analyzed by TLC as described above.

Preparation of Soluble Forms of ST6GalNAc V and VI—As described previously (10, 11), soluble forms of ST6GalNAc V and VI as fusion proteins with protein A were prepared for the sialyltransferase assay. Sialyltransferase Assay—The sialyltransferase assay was performed in a mixture containing 100 mM sodium cacodylate buffer, pH 6.0, 10 mM MgCl₂, 0.3% Triton CF-54, 0.66 mM CMP-NeuAc (Sigma), 6,000 dpm/μl CMP-[^14C]NeuAc (Amersham Biosciences), the enzyme solution, and 10 μg of acceptors in a total volume of 50 μl. The reaction mixture was incubated at 37 °C for 2 h. The products were isolated using a C₁₈ Sep-Pak cartridge (Waters, Milford, MA) and analyzed by TLC with a solvent system of chloroform/methanol/0.2% CaCl₂ (55:45:10). The radioactive material in each plate was visualized with an ABC Western system and a BAS 2000 image analyzer (Fuji Film, Tokyo, Japan). For kinetic analysis, incubation was performed using various concentrations of acceptor substrates, 0–0.2 μM GM1β or sialyl Leα.

TLC-Immunostaining—TLC-immunostaining was performed as described previously (19). Disialyl Leα was detected using anti-disialyl Leα mAb kindly provided by S. Hakomori (Pacific Northwest Research Institute, University of Washington, Seattle) (8) at a 1:3 dilution as a primary antibody. Briefly, after chromatography of the glycolipids, the TLC plate was heat-blotted onto a polyvinylidene difluoride membrane. The membrane was incubated with mAb for 1 h, washed, and incubated with biotinylated horse anti-mouse IgG at 1:200 dilution for 1 h. The antibody binding was visualized using an ABCPO kit (Vector, Burlingame, CA) and HRP-1000 (Konica, Tokyo, Japan).

Disialyl Leα and sialyl Leα was analyzed using the transfected cells after transient transfection of expression vectors. Two days after transfection, cells were trypsinized and washed twice with PBS and then used for flow cytometric analysis using anti-disialyl Leα mAb FII7, anti-sialyl Leα mAb H14 (Seikagaku Corp.), anti-sialyl Leα/sialyl Leα mAb 2B3 (Seikagaku Corp.), anti-GD1α mAb RA-17 (presented by Y. Hirabayashi (Brain Science Institute, Wako, Japan)), and anti-GM1 mAb GGR41 (presented by T. Tai (Tokyo Metropolitan Institute of Medical Science)) (21) on FACScalibur with Cell Quest™ version 3.1f software (Becton Dickinson). Fluorescein isothiocyanate-conjugated anti-mouse IgG (whole antibody) (ICN/Cappel) or anti-mouse IgM antibody (ZYMED) was used as second antibodies.

Immunofluorescence Assay—COS1 cells were cultured on cover glasses in 24-well plates and incubated at 37 °C for 24 h and transiently transfected with expression vectors as described above. The cells were fixed with cold acetone for 10 min or with 3% paraformaldehyde in PBS for 5 min. In the latter case, they were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Then they were processed for indirect immunofluorescence analysis as described previously (11). The staining was observed using the μ-Radiance™ confocal imaging system (Bio-Rad) and also with ORCA-ER-1394 imaging systems (Hamamatsu Photonics, Hamamatsu, Japan).

Analysis of ST6GalNAc V/VI Gene Expression—The expression levels of the ST6GalNAc V/VI gene in human colon cancer cell lines were determined by RT-PCR. RT-PCR was performed by Northern blot analysis with the ST6GalNAc V and VI gene-specific primers, 5′-GGTG-CCGACTGGTGTGGTGGC-3′ (nucleotides 22–41 in the coding sequence) and 5′-AATCTGGGACCCAGACTTCAAC-3′ (nucleotides 916–935) for ST6GalNAc V, 5′-CAGACCCCGAGAAGAATAAG-3′ (nucleotides 68–79 in the coding sequence) and 5′-GCCCCCGAAAGTGGACCC-3′ (nucleotides 529–547) for ST6GalNAc VI. For Northern blot analysis, total RNA was prepared using TRIZOL Reagent™ (Invitrogen) according to the manufacturer's instructions. Fifteen micrograms of total RNA was electrophoresed and blotted onto a nylon membrane (GeneScreen Plus) (PerkinElmer Life Sciences). They were hybridized with 32P-labeled ST6GalNAc V or ST6GalNAc VI cDNA probes as previously described (11).

Analysis of FUT-3, β3Ga-T5, and ST3Gal IV Gene Expression—The expression levels of the FUT-3, β3Ga-T5, and ST3Gal IV genes in human colon cancer cell lines were determined by RT-PCR. RT-PCR was performed with primers corresponding to the ST3Gal IV gene in which 5′-CGAGCGACCTCCATCCCTTCG-3′ (nucleotides 478–498 in the coding sequence) and 5′-GCTTCTCCATCCCTTCCTG-3′ (nucleotides 583–612 in the coding sequence) and 5′-AAAAGCTGGGGAGAGTGG-3′ (nucleotides 1234–1251) for FUT-3, 5′-CACACACCTCCCTCCTCG-3′ (nucleotides 948–965) for β3Ga-T5, and 5′-GCTCCTCCATCCTAAAGCG-3′ (nucleotides 329–348 in the coding sequence) and 5′-GACATATGGGCGAGGCG-3′ (nucleotides 948–929) for ST3Gal IV were used as forward and reverse primers, respectively.

The NIH Image program was developed at the National Institutes of Health and is available through the Internet by anonymous FTP from zippy.nlm.nih.gov or on a floppy disk from the National Technical Information Service (Springfield, VA) (part no. PB95-500195GED).
**Synthesis of Disialyl Leα by ST6GalNAc VI**

Various acceptor substrates were incubated in the standard assay mixture using ST6GalNAcV/VI-protA as the enzyme source. Each substrate was used at the concentration of 0.1 mM.

| Acceptor | Structure | Relative ratea | ST6GalNAcV | ST6GalNAcVI |
|----------|-----------|----------------|------------|-------------|
| GM1b     | NeuAc2,3Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1-Cer | 100 | 100 | 0 | 0 |
| Sialyl nLe4 | NeuAc2,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1-Cerb | 0 | 0 | 0 | 0 |
| nLe4 | Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1-Cer | 22 | 38 | 0 | 0 |
| Sialyl Le4 | NeuAc2,3Galβ1,3GlcNAcβ1,3Galβ1,4Glcβ1-Cer | 0 | 0 | 0 | 0 |
| Le4 | Galβ1,3GlcNAcβ1,3Galβ1,4Glcβ1-Cer | 0 | 0 | 0 | 0 |
| Sialyl Leα | NeuAc2,3Galβ1,3Fuc0,1,4GlcNAcβ1,3Galβ1,4Glcβ1-Cer | 0 | 0 | 0 | 0 |

a Relative rates are calculated as a percentage of the incorporated NeuAc to that onto GM1b.
b Ceramide mimic -CH2(C14H29)2 is chemically bonded to the oligosaccharides instead of ceramide.

**RESULTS**

**ST6GalNAc V and VI Synthesized Disialyl Le4**—To analyze the novel substrate specificity of ST6GalNAc V and VI, soluble fusion enzymes fused with protein A were prepared, and their sialyltransferase activities toward various lacto- and neolactoses glycolipids were examined. In previous studies, we reported that these enzymes synthesized α-series gangliosides such as GD1a, GT1a, and GQ1b. Among Le4, nLe4 and their sialylated forms, only sialyl Le4 was utilized with both enzymes (Fig. 1A). The relative incorporation rates were summarized in Table I. Incorporation of [14C]NeuAc toward sialyl Le4 was lower than toward GM1b in both ST6GalNAc V and VI.

To further confirm that the enzyme products are disialyl Le4, TLC-immunostaining of the products was performed using mAb FH9 to detect disialyl Le4. Lane 1, chemically synthesized disialyl Le4; lane 2, products of ST6GalNAc VI. The detection was carried out as described under “Materials and Methods.”

**Synthesis of Disialyl Leα in Cultured Cells**—To explore the function of the ST6GalNAc VI gene in vivo, we transiently transfected the expression vectors for ST6GalNAc VI and/or FUT-3 into COS1 cells, which have sialyl Le4 as a precursor of sialyl Leα and disialyl Le4, and have no inherent FUT-3 (15). Expression vectors pcDNA3.1-ST6GalNAc VI and pcXN2-FUT-3 were used. The flow cytometric analysis of the transfectant cells with ST6GalNAc VI alone exhibited no expression of sialyl Leα or disialyl Leα antigen as the mock transfectants (Fig. 5, A and B (a, b, c, and d)). When FUT-3 was transfected, not only sialyl Leα but disialyl Leα was expressed, suggesting the inherent ST6GalNAc VI gene expression (Fig. 5, A and B (c and g)). When ST6GalNAc VI was co-transfected with FUT-3, the expression level of disialyl Leα increased, whereas that of sialyl Leα decreased (Fig. 5, A and B (d and h)). The results of immunocytostaining revealed that disialyl Leα antigen mainly localized in the cytoplasm (Fig. 5B).

**Expression of ST6GalNAc VI in Human Colon Cancer Cell Lines**—To determine the expression pattern of ST6GalNAc V/VI and FUT-3 mRNA, RT-PCR analysis and Northern blot analysis were performed with seven human colon cancer cell lines (Caco-2, Colo320, DLD-1, HT-29, Lovo, SW1080, and SW1116). RT-PCR was also performed for β3Gal-T5 and ST3GalIV genes. To analyze the correlation between the expression levels of disialyl Leα and those of glycosyltransferase genes involved in the synthesis of disialyl Leα, RT-PCR analysis was conducted using the primers based on the human cDNA of ST6GalNAc V/VI, FUT-3, β3Gal-T5, and ST3Gal IV. As shown in Fig. 6A, a PCR product of ST6GalNAc VI with 446 base pairs was detected in all human colon cancer cell lines, although the intensity of the bands varied. Lovo cells expressed the ST6GalNAc VI gene at the highest level, whereas SW1080 cells showed a very weak band. In contrast, no bands were detected for the ST6GalNAc V gene in any of them, although the band as positive control was clearly detected with 912 base pairs. Northern blot analysis (Fig. 6B) also indicated the expression of ST6GalNAc VI mRNA with proportional levels to those in RT-PCR but not of V mRNA as shown in Fig. 6A. The
FUT-3 gene was expressed strongly in DLD-1 and SW1116, moderately in HT29, and weakly in Colo320 and Lovo. ST3Gal IV was broadly expressed, and ST3Gal-T5 was expressed highly in DLD-1 and Lovo and weakly in CACO-2 and HT-29 (Fig. 6C).

To investigate the expression of final enzyme products (i.e. cancer-associated antigens and \( \alpha \)-series ganglioside antigens in these cell lines), flow cytometric analysis and immunofluorescence assay were carried out. Table III summarizes the expression patterns of the ST6GalNAc VI mRNA, cancer-associated antigens, and \( \alpha \)-series ganglioside antigens in these cell lines.

It was found that \( \alpha \)-series ganglioside antigens (GD1\( \alpha \) and GQ1\( \beta \)) were minimal or zero in these cell lines, indicating that the main products of ST6GalNAc VI enzyme might be those of type I lacto-series glycolipids in human colon tissues. The expression levels of these antigens on the cell surface appeared to not necessarily correlate with the expression levels of inherent ST6GalNAc VI gene. Two lines with high levels of ST6GalNAc VI gene showed almost null expression of disialyl Le\( \alpha \). But the expression of sialyl Le\( \alpha \) was also low in these two lines. Sialyl Le\( \alpha \) is also at low levels, suggesting that these two lines lack precursors. On the other hand, cell lines expressing very low levels of ST6GalNAc VI did not express high levels of disialyl Le\( \alpha \) except for SW1116. Consequently, there were no critical controversial points in these results to take ST6GalNAc VI as a responsible enzyme for the synthesis of disialyl Le\( \alpha \) if we consider other factors such as low precursor levels or low activity of FUT-3.

To inquire about the correlation between the levels of glycosyltransferase genes and the levels of disialyl Le\( \alpha \) expression, we further evaluated the expression of responsible genes (\( \beta \)Gal-T5, ST3Gal IV, and FUT-3) and that of relevant antigens in these cell lines. Fig. 7A showed the expression pattern of these genes, and Fig. 7B is a summary of the antigen expression. The expression levels of FUT-3 correlated rather well with those of disialyl Le\( \alpha \). Otherwise, there was no clear correlation between the expression levels of glycosyltransferase genes and relevant sialyl compounds.

**Substrate Competition between ST6GalNAc VI and FUT-3**—As we did for COS1 (Fig. 5), we transiently transfected the ST6GalNAc VI and/or FUT-3 expression vectors into six colon cancer cell lines, which originally expressed both genes more or less. Flow cytometric analysis with mAbs FH7 and 1H4 revealed that the overexpression of ST6GalNAc VI cDNA resulted in the elevation of disialyl Le\( \alpha \) expression and suppres-

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**Table II**

| Acceptor            | ST6GalNAc V | ST6GalNAc VI |
|---------------------|-------------|--------------|
|                     | \( K_m \)   | \( V_{max} \) | \( K_m \)   | \( V_{max} \) |
| GM1b                | 0.56        | 4.28         | 0.33        | 10.0         |
| Sialyl-Lc4          | 0.90        | 0.25         |             | 2.3          |

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**Fig. 3.** Disialyl Le\( \alpha \) is synthesized from sialyl Lc4 via disialyl Lc4. A, fucosyltransferase assay was performed using membrane fraction of cells transfected with FUT-3 as described under "Materials and Methods," and the products were analyzed by TLC. Acceptors were Lc4, sialyl Lc4, disialyl Lc4, nLc4, and Lc4. B, to detect disialyl Le\( \alpha \), TLC-immunostaining was done with mAb FH7 (hybridoma supernatant) at a 1:3 dilution using the TLC plate in A. C, sialyltransferase activities of ST6GalNAc V/VI for fucosylated or nonfucosylated acceptors were analyzed, and the TLC pattern of the products is shown as an autofluorogram.

**Fig. 4.** Proposed pathway for the biosynthesis of disialyl Lc4 and disialyl Le\( \alpha \). Note that disialyl Le\( \alpha \) is synthesized from sialyl Lc4 via disialyl Lc4 but not from sialyl Le\( \alpha \).
The synthesis and expression of disialyl Lea, and they partly confirmed that both ST6GalNAc VI and FUT-3 contribute in competing to share sialyl Lc4 as a common acceptor substrate.

Discussion

In the present study, we elucidated that ST6GalNAc V/VI, which were isolated as synthases of α-series gangliosides (10, 11), could catalyze the synthesis of disialyl Lea, leading to the synthesis of disialyl Lea. ST6GalNAc V was defined as GM1b-specific α2,6-sialyltransferase to generate GD1α and was expressed specifically in the brain (10). ST6GalNAc VI was also a member of the α2,6-sialyltransferase family with the substrate specificity toward not only GM1b but also GD1a and GT1b, leading to the synthesis of GD1α, GT1ac, and GQ1ba, respectively (11). This gene is expressed in many tissues. Sialyl Lea appears quite similar to GM1b, and disialyl Lea also resembles GD1α in the three-dimensional structure, although sialylated GalNAc at C6 is not fucosylated in GD1α. Correspondingly, these enzymes discriminated the core structure of sialyl Lc4 and sialyl nLea (i.e. type I structure (Galβ1,3GlcNAc) and type II structure (Galβ1,4GlcNAc)). Namely, NeuAca2,3Galβ1,3HexNAc is important for the acceptor recognition. Compared with the synthesis of GD1α from GM1b, the efficiency of the synthesis of disialyl Lea is sufficiently high to be expected to actually play roles in cells. Consequently, these enzymes contain a multifunctional character as GM2/GD2/GA2 synthase (23) or GM1/GD1b/GA1 synthase (19) showed. However, this case is very rare since the directly substituted sugars by enzymes are variable (i.e. GalNAc and GlcNAc), although the whole steric connects are very similar between GM1b and sialyl Lea4.

In this study, we clearly demonstrated, for the first time, the biosynthetic pathway of disialyl Lea and the possibility of competition of ST6GalNAc VI with FUT-3 (i.e. synthesis of monosialyl Lea and that of disialyl Lea4 (and disialyl Lea)). The synthesis of disialyl Lea was achieved in COS1 cells by transfection of both ST6GalNAc VI and FUT-3 or FUT-3 alone. The expression rate of disialyl Lea was not so high as expected, partly because the efficiency of co-transfection might be not so high. Sialyl Lea was not converted to disialyl Lea with ST6GalNAc VI. Taken together, sialyl Lea4 is only one substrate examined so far leading to the synthesis of disialyl Lea. Therefore, synthesis of sialyl Lea and disialyl Lea4 (and disialyl Lea) should compete with each other sharing a common substrate, monosialyl Lea4. Transfection of the expression vector of ST6GalNAc VI into a sialyl Lea-expressing cell line could induce a mild reduction in the expression level of sialyl Lea in addition to new expression of disialyl Lea. These results really suggested the possibility that overexpression of disialyl Lea, based on the action of ST6GalNAc VI, might result in the suppression of the expression level of sialyl Lea. If this is the case, the expression level of ST6GalNAc VI gene might be low in fetal and colon cancers but high in normal colonic mucosa
corresponding with an onco-fetal nature of sialyl Leα. This issue is now under investigation in our laboratory.

Among ST6GalNAc families (I–VI), ST6GalNAc I and II preferentially act on nonsialylated substrates. ST6GalNAc III was poorly active for a type I structure (14), and a human homolog has not yet been reported. ST6GalNAc IV was a protein-dominant (O-glycan) enzyme (14). Consequently, we concentrated our efforts on the function of ST6GalNAc V and VI. The results obtained in this study elucidated that these sialyltransferases could act on multiple substrate structures including gangliosides and lacto-series. Thus, we have defined a novel substrate specificity of previously cloned sialyltransferases.

Whether these sialyltransferases actually exert a catalytic activity in the tissues is a critical point to be clarified. Analyses with seven human colon cancer cell lines showed that ST6GalNAc VI but not V was expressed at various levels as analyzed with RT-PCR. Northern blotting with these cell lines also demonstrated similar results. The intensity in flow cytometry or immunocytostaining of disialyl Leα did not correlate well with the expression levels of ST6GalNAc VI gene. However, two cell lines with poor expression of the ST6GalNAc VI gene also showed only low level expression of disialyl Leα. Two cell lines with high levels of the ST6GalNAc VI gene (Caco-2 and Colo320) scarcely showed disialyl Leα expression. However, they expressed the lowest levels of sialyl Leα among the cell lines examined, suggesting that FUT-3 level and/or sialyl Lc4 level are very low, resulting in inefficient synthesis of disialyl Leα despite high levels of ST6GalNAc VI expression. α-Series ganglosides such as GD1α and GQ1bα were very poor in all of these cell lines. Taken together, it appears quite likely that ST6GalNAc VI is really active and contributes to the synthesis of disialyl Lc4 and disialyl Leα in human colon tissues.

As for the correlation between sialyl Leα/disialyl Leα and FUT3/ST6GalNAc VI, it seemed difficult to find a simple competitive relation between these two enzymes by comparing the expression patterns of these genes and their products among cell lines as shown in Fig. 7. This is because the intra-Golgi localization of the ST6GalNAc VI and FUT3 might bias one product over another, resulting in the deviated efficiency of the utilization of the common substrate. Furthermore, the expression levels of other glycosyltransferase genes involved in the synthesis of sialyl Lc4 were various, depending on the cell lines, probably forming different situations in the individual lines. On the other hand, when either one or both of these transferase genes were transfected, we could find the reasonable effects of the expression of individual genes on the expression of sialyl Leα and disialyl Leα as shown in COS1 (Fig. 5) and colon cancer cell lines (Fig. 8). Namely, ST6GalNAc VI is essential for the expression of disialyl Leα, and it partly competes with FUT-3 for the acceptor substrate, sialyl Lc4. However, it simultaneously needs the help of FUT-3 to generate disialyl Leα. The importance of FUT-3 in the synthesis of disialyl Leα was indicated by the fact that FUT-3 expression levels fairly well correlated with disialyl Leα levels (Fig. 7A). Our results of tran-

**Fig. 6. Expression pattern of the ST6GalNAc V/VI gene in various human colon cancer cell lines.** A, RT-PCR was performed using single strand cDNAs reverse-transcribed from various human colon cancer cell lines as described under “Materials and Methods.” The ST6GalNAc V-gpmIKneo vector was used as a positive control for template. In parallel, β-actin cDNA was amplified to confirm the quality of the cDNA used. Lane 1, Caco-2; lane 2, Colo320; lane 3, DLD-1; lane 4, HT-29; lane 5, Lovo; lane 6, SW1080; lane 7, SW1116. B, Northern blot analysis was carried out using total RNAs from the same panel as similar pattern (data not shown). 28 and 18 S RNA bands as detected with ethidium bromide staining. Control band for ST6GalNAc V was detected using RNA from human brain (PC). 28 and 18 S RNA bands as detected with ethidium bromide are shown as controls (bottom). C, RT-PCR for FUT-3, ST3Gal IV, and βGal-T5 was also performed under the conditions described under “Materials and Methods.” Northern blotting of FUT-3 also showed a similar pattern (data not shown).

**Table III**

**Expression patterns of the ST6GalNAc VI mRNA, disialyl Leα, sialyl Leα, GD1α, and GQ1bα in various human colon cancer cell lines.**

| Cell lines | mRNA for Vα | mRNA for Vβ | Disialyl Leα | Sialyl Leα | GD1α | GQ1bα |
|------------|-------------|-------------|-------------|-----------|------|-------|
| Caco-2     | –           | –           | ++          | +         | –    | –     |
| Colo320    | –           | –           | ++          | +         | –    | –     |
| DLD-1      | –           | –           | +           | +         | –    | –     |
| HT-29      | –           | –           | ++          | +         | –    | –     |
| Lovo       | –           | –           | +           | +         | –    | –     |
| SW1080     | –           | –           | ±           | ±         | –    | –     |
| SW1116     | –           | –           | +           | +         | –    | –     |

*RT-PCR and Northern blot analyses were performed with various human colon cancer cell lines as described under “Materials and Methods.”* The level of gene expression is shown as very weak (±), weak (+), moderate (++), strong (+++), and very strong (++++) of disialyl Leα and disialyl Leβ as shown in COS1 (Fig. 5) and colon cancer cell lines (Fig. 8). Namely, ST6GalNAc VI is essential for the expression of disialyl Leα, and it partly competes with FUT-3 for the acceptor substrate, sialyl Lc4. However, it simultaneously needs the help of FUT-3 to generate disialyl Leα. The importance of FUT-3 in the synthesis of disialyl Leα was indicated by the fact that FUT-3 expression levels fairly well correlated with disialyl Leα levels (Fig. 7A). Our results of tran-

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The expression of disialyl Leα, sialyl Leα, GD1α, and GQ1bα were detected by flow cytometric analysis (FACS) or immunofluorescence assay (IF) as described in “Materials and Methods.” The expression level is presented as ++, 70–100%; +++, 40–70%; +, 5–40%; ±, 0–5%; –, 0%.

FACS, fluorescence-activated cell sorting.

IF, immunofluorescence.
sient expression as shown in Fig. 8 demonstrate well the dual aspects of FUT-3 in the synthesis of disialyl Lea.

Beyond the result obtained in this study, the regulatory mechanisms for the expression of disialyl Lea in normal/cancer tissues and the biological roles of the structure in the normal tissues and in colonic malignant cells remain to be investi-
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Synthesis of Disialyl Lewis a (Le$^a$) Structure in Colon Cancer Cell Lines by a Sialyltransferase, ST6GalNAc VI, Responsible for the Synthesis of $\alpha$-Series Gangliosides

Akiko Tsuchida, Tetsuya Okajima, Keiko Furukawa, Takayuki Ando, Hideharu Ishida, Aruto Yoshida, Yoko Nakamura, Reiji Kannagi, Makoto Kiso and Koichi Furukawa

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