Suppression of Sialyl Lewis X Expression and E-selectin-mediated Cell Adhesion in Cultured Human Lymphoid Cells by Transfection of Antisense cDNA of an α1→3 Fucosyltransferase (Fuc-T VII)*

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The antisense cDNA approach was used to identify the endogenous fucosyltransferase species responsible for synthesis of the sialyl Lewis X (NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAcβ1→R) determinant in human lymphoid cells. The cultured human adult T-cell leukemia cell line, ED40515-N, expressed the message of α1→3 fucosyltransferase (Fuc-T) IV and VII, with a low level of the Fuc-T III and VI message, and manifested the sialyl Lewis X as well as Lewis X (Galα1→3Galβ1→4 [Fucα1→3]GlcNAcβ1→R) determinant at the cell surface. Transfection of this cell line with the pRe/CMV vector containing an antisense human Fuc-T VII construct (pRe/CMV/5'FT7AS) resulted in a significant decrease of endogenous Fuc-T VII message and a marked reduction in the cell surface expression of sialyl Lewis X determinant as well as a reduction in the enzymatic activity of α1→3 fucosyltransferase against sialylated type 2 chain substrate. This was accompanied by diminution of cell adhesive activity toward E-selectin on interleukin-1β-treated endothelial cells. These results indicated that the synthesis of the sialyl Lewis X determinants that were functionally active as E-selectin ligands was mainly mediated by Fuc-T VII in these lymphoid cells. On the other hand, the message of Fuc-T IV showed no significant change in the transfected clones, and the surface expression of the Lewis X antigen as well as the enzymatic activity of α1→3 fucosyltransferase against non-sialylated type 2 chain substrate was well preserved. The clear contrast between the diminished expression of sialyl Lewis X and the conserved manifestation of Lewis X in the transfected clones suggested that the synthesis of sialyl Lewis X and that of Lewis X are independently regulated by different fucosyltransferases in human lymphoid cells. Fuc-T VII must be involved in the synthesis of sialyl Lewis X, while the synthesis of Lewis X is mediated by an enzyme other than Fuc-T VII, most probably Fuc-T IV.

The sialyl Lewis X1 determinant on leukocytes serves as a ligand for selectin family cell adhesion molecules (1–3), and the selectin-carbohydrate interaction is considered to play important roles in inflammatory reaction and leukemic infiltration (4–6). Recently cDNAs of several α1→3/4 fucosyltransferases (Fuc-T), which are capable of synthesizing sialyl Lewis X, have been cloned (1, 7–13). Leukocytes and related cell line cells are known to contain mainly Fuc-T IV, VI, and VII species messages (8, 12–15). It is also well known that the exogenous introduction of large amounts of these fucosyltransferases to cells by the transfection of sense cDNAs considerably enhances the synthesis of sialyl Lewis X in various cell lines and makes the cells highly adhesive to selectins (1, 8, 13). However, definitive evidence has not yet been provided regarding which endogenous enzymes are actually involved in the synthesis of sialyl Lewis X under in situ conditions. In this study we used an antisense approach to identify the endogenous fucosyltransferase species responsible for the synthesis of the sialyl Lewis X moiety in cultured human lymphocytic leukemia cells.

As the object of antisense transfection experiments, we have chosen a cultured human lymphocytic leukemia cell line, ED40515-N, which contains several species of candidate fucosyltransferases. Sialyl Lewis X is known to be preferentially expressed on helper memory T-cells as well as NK cells among normal lymphocytes (16, 17), and adult T-cell leukemia frequently originates from helper memory T-cells following infection with the retrovirus called HTLV-1. The ED40515 cells were derived from a patient with adult T-cell leukemia. We and other investigators recently showed that sialyl Lewis X is strongly expressed on adult T-cell leukemia cells, and mediates adhesion of leukemic cells toward vascular endothelium, and hence is involved in the organ infiltration of the leukemic cells (18–20).

MATERIALS AND METHODS

Cells and Antibodies—The human lymphoid leukemia cell line, ED40515, was originally established from peripheral blood lymphocytes of a Japanese male patient with adult T-cell leukemia (21, 22). ED40515-N is a subclone of ED40515 cells, which is IL-2-independent, and transplantable to nude mice. Another human adult T-cell leuke- mia-derived cell line, ATL-2 (23), and the human natural killer-like leukemic cell line, YT (24), were obtained from the First Division of

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Department of Internal Medicine, Kyoto University. These cell lines were maintained in RPMI 1640 medium with 10% fetal calf serum as described previously (19).

The anti-sialyl Lewis X antibody 2F3 was established by immunizing a BALB/c mouse with a synthetic sialyl Lewis X carbohydrate determinant and is reactive to the authentic natural as well as synthetic sialyl Lewis X as described previously (19). The antibody 2F3 detects the sialyl Lewis X antigen expressed on helper memory T-cells and adult T-cell leukemia cells much more efficiently than other classical anti-sialyl Lewis X antigens, as reported earlier (19). The anti-Lewis X antibody LeuM1 was obtained from Becton Dickinson Immunocytometry System, San Jose, CA. Anti-human E-selectin and anti-VCAM-1 antibodies (BBA2 and BBA6, both murine IgG1) were obtained from British Biotechnology Ltd., Abingdon, Oxon, United Kingdom.

Northern Blot Analysis—Total RNA was isolated by the guanidinium CsCl method as described by Maniatis et al. (25). Poly(A)+ RNA was isolated from total RNA with oligo(dT)-Latex (Takara Shuzo, Co. Ltd, Kyoto, Japan) as described in the manual provided by the company. Measured amounts of the poly(A)+ samples were electrophoresed through 1.2% agarose gels containing formaldehyde and were transferred to a nylon membrane (Hybond-N, Amersham Corp., Buckinghamshire, Great Britain). Northern blots were prehybridized overnight at 42°C in 50% formamide, 5×SSPE (1×SSPE = 0.18 M NaCl, 0.01 M sodium phosphate at pH 7.4, 1 mM EDTA), 2× Denhardt’s reagent, 0.1% SDS, and 150 μg/ml of sheared salmon sperm DNA (25).

Blots were then hybridized overnight at 42°C in the same hybridization solution containing 32P-labeled probe.

The probe used for detection of Fuc-T III, V, and VI was the 1.7-kb XhoI-XbaI fragment isolated from the insert in pCDM7-Fuc-T III (7). It detected Fuc-T V and VI transcripts as well as Fuc-T III transcripts, since it was highly homologous to the corresponding nucleic acid sequences in Fuc-T V and VI (about 85 and 90% in 1.7-kb XhoI-XbaI fragment, respectively), and hence termed Fuc-T III/V/VI probe (9, 11).

The probe used for detection of Fuc-T IV was the 591-bp PvuII-AvaII fragment isolated from the insert in pcDNA1-Fuc-T IV (26) (hence termed Fuc-T IV probe (14)). The probe for Fuc-T VII was the 459-bp KpnI-NorI 1 fragment isolated from the insert in pCDM8-Fuc-T VII (12, 13) and hence termed Fuc-T VII probe. Sense and antisense hybridization probes were synthesized with an RNA transcription kit (Promega, Madison, WI) and pRC/CMV/5′FT7AS template. Briefly, this plasmid was linearized by NotI to synthesize the sense strand Fuc-T VII probe by SP6 polymerase, and it was linearized by XhoI to synthesize the antisense strand Fuc-T VII probe by T7 polymerase as recommended by the supplier. A human β-actin cDNA was used as a control for quality and even loading of the mRNA.

Blots were washed twice in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M trisodium citrate), 0.1% SDS at room temperature, twice in 0.25× SSC, 0.1% SDS for 20 min at 42°C, and twice in 0.1× SSC, 0.1% SDS for 20 min at 68°C. Blots were air-dried and then subjected to autoradiography with an intensifying screen at −70°C.

The amount of Fuc-T VII mRNA (sense) relative to β-actin was qualified with a FUJIX Bioimaging analyzer (LAS2000, Fuji Photo Film Co. Ltd., Tokyo, Japan).

RT-PCR Analysis—RT-PCR analysis was performed using specific primers to discriminate between the messages of fucosyltransferase genes detected by the Fuc-T III/V/VI probe and to detect smaller quantities of transcripts that were below the detection level in Northern blot analysis.

After 1 μg of poly(A)+ RNA was pretreated with DNase I (Takara Shuzo Co., Ltd., Kyoto, Japan), the first strand cDNA was synthesized using oligodeoxynucleotidic acid-dT, primer and avian myeloblastosis virus reverse transcriptase (LifeSciences, St. Petersburg, FL), ethanol-solution containing 32P-labeled probe. Blotswere thenhybridizedovernightat42°C inthesamehybridization

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FIG. 1. The construct of human Fuc-T VII antisense expression vector, pRc/CMV/5′FT7AS, used for transfection experiments. Ori, Col E1 origin of replication; neo*, neomycin-resistant gene; Amp*, ampicillin-resistant gene; SV40 Ori, simian virus 40 origin of replication.

prepared without reverse transcriptase to exclude the possibility of amplification of contaminating genomic DNA. These also served as negative controls for each primer set and each sample. No amplification of contaminating genomic DNA was detected in any case described in this study.

Transfection Experiments—The expression vector contains a 0.47-kb fragment of the human Fuc-T VII cDNA (13), including a part of 5′-untranslated region (from −137 to +334), which was inserted in an antisense orientation into the mammalian vector pRcCMV.

In the human Fuc-T VII antisense expression vector, pRc/CMV/5′FT7AS, the transcript (Fig. 1), transcription of the insert was driven by the hCMV promoter and enhancer. The bovine growth hormone 3′-flanking sequence provided a termination signal for RNA processing. A bacterial neomycin phosphotransferase gene (neo) expression cassette allowed G418 selection.

DNA (10 μg) was linearized with EcoRI and was transfected to the ED40515-N cells (2×106), harvested at mid-log phase growth, by the electroporation method at 180 V, 500 microfarads in phosphate-buffered saline, using ECM600 (BTX Inc., San Diego, CA). The cells were allowed to grow for 2 days before being subjected to selection for the ability to grow in medium containing 600 μg/ml Geneticin (G418-sulfate, Life Technologies, Inc.).

Assay of Fucosyltransferase Activity—An acceptor oligosaccharide prepared from 3′IVNeuAcα1,6Galβ1,4GlcNAcβ1,3Galβ1,4Glc (for the synthesis of sialyl Lewis X) or nLc4 (for the synthesis of Lewis X) was fluorescence-labeled with 2-aminopyridine and used as the substrate. The reaction mixture contained 25 mM HEPES (pH 7.2), 20 mM MnCl2, 0.45% Triton CF54, 0.04 mM oligosaccharide-aminopyridine, 2 mM GDP-fucose, 5 mM CDP-choline, and 200 μg of protein in a volume of 0.05 ml (27).

The assay mixture was incubated at 37°C for 3 h, and the reaction was stopped by boiling for 2 min. After centrifugation, the reaction mixture was analyzed by high performance liquid chromatography using an Amide-Silica column with the gradient of 200 mM acetic acid (pH 7.3) with triethylamine/acetonitrile (25:75 to 50:50, v/v). The reaction product was quantified by the fluorescence intensity with excitation at 310 nm and emission at 380 nm.

Cell Adhesion Experiments—Cell adhesion experiments were performed as described previously (19, 28, 29). HUVECs were stimulated with 2 μg/ml of recombinant IL-1β for 4 h in 24-well plates. The ED40515-N clones (5×104 cells/0.5 ml/well) were added, and the plate was incubated with rotation at 90 rpm for 20 min at room temperature (28, 29). The incubation was carried out in the presence of anti-VCAM-1 antibody (25 μg/ml) to exclude a possible effect of the VCAM-1-VLA-4 adhesion system on the experimental results, since the ED40515-N cells strongly expressed VLA-4. After nonadherent cells were washed out three times with phosphate-buffered saline, the number of attached cells was counted directly under a microscope. In inhibition experiments, the monoclonal anti-E-selectin antibody was preincubated with HUVECs at 50 μg/ml for 30 min at 37°C prior to the adhesion experiments with ED40515-N clones.
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RESULTS

Expression of Fucosyltransferase mRNA in Cultured Lymphocytic Leukemia Cells—ED40515-N cells expressed a significant amount of Fuc-T VII and a smaller amount of Fuc-T IV mRNA as ascertained by Northern blotting analysis (Fig. 2, left panel). The Fuc-T III/V/VI probe gave negative results in Northern blotting, while a small amount of Fuc-T III and VI was detected by RT-PCR (Fig. 2, right panel) in ED40515-N cells. Another cell line, ATL-2, also expressed a significant amount of Fuc-T VII, but was hardly reactive to the Fuc-T IV and Fuc-T III/V/VI probes in Northern blotting. ATL-2 cells seemed to contain a small amount of Fuc-T IV, III, and VI, as detected by RT-PCR. YT cells were used as a control, since the cells contain only the message of Fuc-T VII, which was the reason why the YT cells were very useful for the cloning of Fuc-T VII cDNA (13). As shown in Fig. 2, the YT cells were again confirmed to contain the message of only Fuc-T VII and not to contain any other message of known fucosyltransferases, as ascertained by both Northern blotting and RT-PCR techniques.

Expression of Sialyl Lewis X Antigen on Cultured Lymphocytic Leukemia Cells—Flow cytometric analysis indicated that the ED40515-N cells expressed both sialyl Lewis X and Lewis X moderately, while the ATL-2 and YT cells strongly expressed sialyl Lewis X and were essentially negative for Lewis X (Fig. 3). Sialidase treatment of ED 40515-N cells resulted in the elimination of the sialyl Lewis X expression and gave rise to a significant enhancement of Lewis X expression. Similarly, the sialidase treatment of the ATL-2 and YT cells resulted in the complete disappearance of sialyl Lewis X and de novo appearance of Lewis X, confirming the antigenic determinant that had been detected on the untreated ATL-2 and YT cells with the antibody to be an authentic sialyl Lewis X determinant.

Suppression of Sialyl Lewis X Expression in the ED40515-N Clones Transfected with Fuc-T VII Antisense cDNA—Lack of both Fuc-T IV and Lewis X antigen expression on the YT cells, when compared with the ED40515-N cells, which contained both Fuc-T IV and VII and expressed both sialyl Lewis X and Lewis X, led us to speculate on the division of labor among the fucosyltransferase isozymes. In order to identify the enzyme responsible for sialyl Lewis X synthesis in these cells, we tried to suppress the expression of Fuc-T VII message by transfecting antisense cDNA into ED40515-N cells.

By transfection of the Fuc-T VII antisense cDNA, a total of 21 stable transfectant clones were obtained by the limiting dilution of the transfected ED40515-N cells in two 96-well cell culture plates. Expression of sialyl Lewis X was markedly suppressed in some clones, and some others expressed a decreased, but still considerable, amount of sialyl Lewis X as analyzed by flow cytometry. The results of flow cytometric analysis indicated that the expression of sialyl Lewis X in these clones ranged from 7.1 to 50.7% (mean ± S.D. was 25.9 ± 16.7%) of the mock-transfected clones in terms of fluorescence intensity. Fig. 4 illustrates a few examples of the flow cytometric pattern, including those of the best three clones, 2A11, 2G9, and 2A3, which had markedly suppressed sialyl Lewis X expression.

Northern Blot Analysis of Fucosyltransferase Message in the ED40515-N Clones Transfected with Fuc-T VII Antisense cDNA—The expression of antisense and sense RNA in these clones was ascertained by Northern blotting analysis. As shown in Fig. 5, the band of transfected antisense cDNA was strongly expressed at −1.5 kb in both clones, indicating that successful transfection was achieved (panel a). The size of the transcript is within the expected range when assuming −300 bases of the transcript were derived from the bovine growth hormone 3′-untranslated region and several hundred bases of the transcript were derived from the poly(A) portion of the construct. The sense mRNA of Fuc-T VII was detected by the antisense probe at −2.0 kb, with concomitant faint bands at −2.4 and −3.0 kb, as reported earlier (13). The Fuc-T VII mRNA (sense) levels in these clones were reduced to about one-fourth or one-fifth of the mock transfectant (the left lane in panel b) or parental ED40515-N cells (not shown). When these
levels were expressed as a percentage of the corresponding β-actin RNA level, the value in the antisense transfected clone 2A11 was only 9.7% and was 12.5% in 2G9, but was 42.9% in the mock transfected clones. On the other hand, the three transcripts of Fuc-T IV were observed at 2.3, 3.0, and 5.8 kb (panel c), and no significant difference was observed in the content of Fuc-T IV mRNA among these cells.

Preserved Expression of Lewis X Antigen on the ED40515-N Clones Transfected with Fuc-T VII Antisense cDNA—We further analyzed expression of the Lewis X determinant on the transfected clones. As shown in Fig. 6, virtually no decrease was detected in the expression of the Lewis X determinant in these clones. The expression of Lewis X in the clone 2A11 was unchanged, while that in the clone 2G9 showed rather a slight increase.

Alteration of Fucosyltransferase Activity in ED40515-N Clones Transfected with Fuc-T VII Antisense cDNA—When the fucosyltransferase activity synthesizing sialyl Lewis X structure was assayed using an authentic sialylated substrate 3IVNeuAcα2,6Galβ1,4GlcNAc, the transfectant was found to have less than 5%, if any, of the enzymatic activity compared with the mock-transfected cells as shown in Fig. 7. Interestingly, the fucosyltransferase activity synthesizing Lewis X structure, which was assayed using LeX as the substrate, was not decreased, but increased from 16.5 to 42.7 pmol/h/mg of protein in the antisense transfected clone, representing an about 2.6-fold increase.

Impaired Cell Adhesive Activity to E-selectin in the ED40515-N Clones Transfected with Fuc-T VII Antisense cDNA—ED40515-N cells undergo a clearly E-selectin-dependent adhesion to IL-1β-treated human umbilical vein endothelial cells, as reported previously (19). Fig. 8 illustrates the results of cell adhesion experiments using the transfected
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FIG. 7. Suppression of fucosyltransferase activity against pyridylaminated NeuAcα2-3 Galβ1-4GlcNAcβ in the Fuc-T VII antisense cDNA-transfected ED40515-N cells. Edneo, mock transfectant cells. 

FIG. 8. Suppression of E-selectin-mediated cell adhesion to HUVECs by the transfection of Fuc-T VII antisense cDNA to ED40515-N Cells. Edneo, mock transfectant cells. * Anti-E-selectin indicates experiments in the presence of 25 μg/well anti-E-selectin antibody.

Several mechanisms of gene expression inhibition by antisense cDNAs have been proposed, including a transcriptional control (34), translational arrest (35–38), and inhibition of splicing (39). The transfection of Fuc-T VII antisense cDNA resulted in a reduction of the endogenous message of Fuc-T VII, but the reduction was only partial in the transfected clones. On the other hand, the reduction in the enzymatic activity and that in the cell surface expression of the sialyl Lewis X antigen was nearly complete in these clones. This discrepancy suggests an involvement of some post-transcriptional inhibitory mechanisms in the action of antisense cDNA. Saijo et al. (40) reported a more impressive result with a human nucleolar antigen p120, the protein expression of which was reduced to 44% without any detectable reduction of p120 mRNA in their antisense transfected cells.

The finding that the transfected clones nearly completely lacked the binding activity toward E-selectin on the IL-1β-treated endothelial cells indicates that Fuc-T VII is the major endogenous enzyme that synthesizes the functionally active E-selectin ligand carbohydrates on the lymphoid cells. Together with the recent findings that the message of this enzyme is co-expressed with putative L-selectin ligand in lymph nodes (41), Fuc-T VII seems to be heavily involved in the synthesis of carbohydrate ligands for selectins in various cells and tissues, including lymphoid organs.

The sialyl Lewis X determinant on leukemia cells is suggested to mediate the adhesion of leukemic cells to endothelial cells and to enhance the organ infiltration of the malignant cells. We and other investigators (18–20) recently showed that the sialyl Lewis X determinant, which is strongly expressed on the adult T-cell leukemic cells, is involved in the tissue infiltration of the leukemic cells. The results of the current experiments indicate that transfection of the antisense cDNA considerably suppresses the adhesive activity of the malignant cells to the vascular endothelial cells and suggest that this therapeutic maneuver would be useful also for the prevention of multiple organ infiltration of leukemic cells.

The preserved expression of Lewis X on the antisense-transfected clones is in clear contrast to the diminished expression of sialyl Lewis X on these clones and indicates that the synthesis of Lewis X determinant on ED40515-N cells is regulated by an enzyme other than Fuc-T VII. Although the Lewis X and sialyl Lewis X determinants had been given the resembling names of CD15 and CD15s, respectively, in the Cluster of Differentiation nomenclature (42), the synthesis of the two carbohydrate determinants are regulated independently by different endogenous fucosyltransferases. Recently CD15 has been shown to serve as a ligand for CD2, the structure of which is quite different from selectins (43). It would be quite natural that the synthesis of the ligands for biologically different molecules are regulated independently by a distinct set of glycosyltransferases. It has been reported that the transfection of sense Fuc-T IV cDNA results in the appearance of Lewis X, while the transfection of Fuc-T VII cDNA produces sialyl Lewis X expression on COS-7 cells (13, 15, 26). The presence of a considerable amount of Fuc-T IV in the Lewis X-positive ED40515-N cells, and also the lack of Lewis X expression accompanied with the coincidentally minute level of Fuc-T IV message in the YT and ATL-2 cells, suggest that the Lewis X determinant is most probably synthesized through the action of Fuc-T IV in these cells.

DISCUSSION

The human lymphocytic leukemia ED40515-N cells contained the message for multiple fucosyltransferases. Which enzyme contributed to the synthesis of sialyl Lewis X determinant in these cells was hard to determine, since Fuc-T III, VI, and VII, which were more or less expressed in the ED40515-N cells, have all been reported to be capable of synthesizing sialyl Lewis X (1, 11–13). Even Fuc-T IV is reportedly capable of synthesizing sialyl Lewis X eventually (8, 30). Use of the antisense approach enabled us to identify the endogenous fucosyltransferase species mainly responsible for the synthesis of sialyl Lewis X to be Fuc-T VII. The results of the current experiments clearly indicated that the fucosyltransferase activity in ED40515-N cells is strongly suppressed by the transfection of antisense cDNA of Fuc-T VII, and this resulted in the nearly complete disappearance of the selectin ligand sialyl Lewis X from the cell surface in the typical transfected clones. The antisense approach would be useful for the functional study and modulation of endogenous glycosyltransferases (31), where several species of isoenzymes are frequently present for the transfer of a particular carbohydrate residue, as typically seen in the cases of fucosyltransferases (32) and sialyltransferases (33).

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