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Murine monoclonal antibody recognizing human α(1,3/1,4)fucosyltransferase

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We prepared a mouse monoclonal antibody, FTA1-16, that specifically recognizes human α(1,3/1,4)fucosyltransferase without crossreactivity to any other members of the α(1,3)fucosyltransferase family. The specificity was confirmed by both immunofluorescense staining of native antigens in the Golgi apparatus and Western blotting analysis, using stable transformant cells transfected with each gene of the α(1,3)fucosyltransferase family. Western blotting analysis on a series of human tumour cell lines from various tissues revealed that some epithelial cancer cell lines from digestive organs expressed an amount of α(1,3/1,4)fucosyltransferase in good correlation with expression of sialyl Lewis a antigen. Immunohistochemical staining by FTA1-16 on colon cancer tissues revealed enhanced expression of the enzyme in cancer cells in comparison to normal cells. Finally, the antigenic epitope recognized by FTA1-16 was determined using truncated recombinant peptides which were expressed in *E. coli*. A minimal length determined was a fragment, amino acid positions 132–153, of the α(1,3/1,4)fucosyltransferase.

Keywords: monoclonal antibody, α(1,3/1,4)fucosyltransferase, Fuc-TIII, Lewis type enzyme, sialyl Lewis a, sLe^a

Abbreviations: Fuc-T, fucosyltransferase; β1,4GalT; β1,4galactosyltransferase; mAb, monoclonal antibody; RT-PCR, reverse transcriptase-polymerase chain reaction; ORF, open reading frame; PVDF, polyvinylidene difluoride; ELISA, enzyme linked immunosolvent assay; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

Introduction

Carbohydrate molecules containing fucose residues seem to play important roles in cell-cell recognition. Sialylated Lewis antigens such as sialyl Lewis x (sLe^x), SAα2,3Galβ1,4(Fucα1,3)GlcNAc-R, and sialyl Lewis a (sLe^a) SA-α2,3Galβ1,3(Fucα1,4)GlcNAc-R, for examples, are known to be the ligands for E-selectin [1–3]. The sLe^x and sLe^a antigens are regarded as tumour-associated antigens and their expression level on tumour cells seems to be related to their metastasizing capacity [4, 5]. The antigenic determinant on the stage-specific embryonic antigen-1 (SSEA-1) during mouse early embryogenesis is also defined as the Lewis x (Le^x) epitope [6], Gal-β1,4(Fucα1,3)GlcNAc-R. It has been suggested that the SSEA-1 antigen may participate in the cell-compaction phenomenon at the eight-cell stage of mouse embryogenesis [7]. To date, cDNAs for five members of the α(1,3)Fuc-T family have been cloned [8–16]. The cDNA encoding a human α(1,3/1,4)fucosyltransferase(Fuc-TIII) has been cloned by an expression cloning method [8]. So far, three α(1,3)fucosyltransferase (Fuc-T) genes (*Fuc-TIV, V, VI* genes), in addition to the *Fuc-TIII* gene, have been cloned by crosshybridization using the Fuc-TIII cDNA as a probe since all of the four genes had highly conserved homologous sequences. Another cDNA for Fuc-TVII that has a lower homologous sequence to the *Fuc-TIII* gene has been cloned by a novel method of expression cloning [16].

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Fuc-TIII catalyses the transfer of fucose residue to the GlcNAc residue of both type 1 and type 2 chains, Galβ1,3GlcNAc- and Galβ1,4GlcNAc-, with the α1,4 and α1,3 linkages resulting in the production of Le^a, Galβ1,3(Fucα1,4)GlcNAc-, and Le^x antigens, respectively. It can also transfer fucose to the sialylated-type 1 and -type 2 chains to produce the sLe^a and sLe^x antigens, respectively. It was recently proved that the *Fuc-TIII* gene is the Lewis (*Le*) gene that determines the expression of human Lewis histo-blood group antigens, Le^a and Le^b, on erythrocytes [17–19]. We recently demonstrated the molecular genetic mechanism of inactivation of Fuc-TIII enzyme in Lewis-negative (Le(−)) individuals who had no Lewis antigens on erythrocytes. In brief, the Le(−) individuals are the homozygotes for *le* genes (mutated *Le* genes) which are inactivated by missense mutations in the catalytic domain of the enzyme [19].

Recent studies in molecular analyses of the α(1,3)Fuc-T family revealed that the FucTs seemed to be characteristically expressed in a variety of tissues and cells [16, 20]. The specific sequences distinguishing each *Fuc-T* gene were used to design specific primer sequences for RT-PCR [20] or competitive PCR assays [16] for the detection of each mRNA of the homologous *Fuc-T* genes. Those analyses revealed that the cell lines derived from digestive organs express a relatively high amount of the mRNAs of *Fuc-TIII* and *VI* genes, but haematopoietic cell lines express low levels of these messages. In contrast, the *Fuc-TIV* gene is mainly expressed in haematopoietic cell lines and not in the cell lines from digestive organs. The *Fuc-TVII* gene is restrictively expressed in myeloid cells to function in the synthesis of the sLe^x determinant, the ligand for E-selectin [16]. Although RT-PCR and competitive PCR methods are sensitive assays for measuring the amount of mRNA, they cannot yield any information about a translated protein. For further analyses of correlation between the Fuc-Ts and the expression of Lewis antigens in cells and tissues, we need to obtain monoclonal antibodies (mAbs) which can distinguish each Fuc-T. They would be useful tools to determine tissue distribution and intracellular localization of the enzymes. We first tried to establish a mAb against Fuc-TIII utilizing a recombinant Fuc-TIII for immunization.

In this study, we describe the establishment and characterization of the mAb, FTA1-16, that has fine specificity against the human Fuc-TIII. We also determined the antigenic epitope recognized by FTA1-16 which might be useful information for establishing mAbs against the other Fuc-Ts.

Table 1. Primers and reaction conditions for PCR

PCR product	*Sense*	*Antisense*	*PCR conditions*						
			Denature		*Annealing*		*Extention*		*Cycles*
			Temp. (°C)	*Time (min)*	*Temp. (°C)*	*Time (min)*	*Temp. (°C)*	*Time (min)*	
Fuc-TIII (Immunogen)	1 TCGAATTCAA GGTGTACCC	2 CCGAATTC AGGTGAACC AAGCC	94	1	60	2	72	3	30
Full Length Fuc-TIII	3 CTCGAATTCA CCCATGGATCCCC TGGGTGCAGC	4 CTCAAGCT TCTCTCAGGT GAACCAAGC CGCTATG	94	1	55	2	72	2	30
Full Length Fuc-TV	3 CTCGAATTCA CCCATGGATCCC CTGGGTGCAGC	4 CTCAAGCT TCTCTCAGGT GAACCAAGCC GCTATG	94	1	55	2	72	2	30
Full Length Fuc-TVII	5 CTCGGATCCA ATCTCGGGTCT CTTGGCTG	6 CTCGAATTC GGTGGTTTGA TTTCGACACC	94	1	55	2	72	2	30
ΔM6	7 CTCGAATTCAG ACACGGTCATC GTGCACCACTG	8 CTCAAGCTT CTCTCAGGTG AACCAAGC CGCTATG	94	1	72	2	72	2	30
ΔM8	9 CTCGAATTCAG ACACGGTCATCG TGCACCACTG	10 CTCAAGCTT TGAAGTATCT GTCCAGGGCT TCCAG	94	1	72	2	72	2	30

Buffer for PCR contains 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 and 2.5 mM $MgCl_2$.

Materials and methods

Primers for PCR amplification

All the primers used in this study are listed in Table 1 together with the conditions for PCR amplification.

Cell lines and cell culture

Human cancer cell lines, GOTO, IMR32, NB-1, T98G, A431, Daudi, Raji and AT(L)-5KY, were obtained from the Japanese Cancer Research Resources Bank (JCRB, Japan).

Four kinds of culture media, (A), (B), (C) and (D), were used in this study. Their contents were as follows. (A): RPMI 1640 supplemented with 10% fetal bovine serum, 50 U ml^{-1} penicillin and 50 $\mu g\,ml^{-1}$ streptomycin. (B): Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin. (C): Eagle's Minimum Essential medium supplemented with 10% fetal bovine serum and 60 $\mu g\,ml^{-1}$ kanamycin. (D): 45% RPMI 1640, 45% Eagle's Minimum Essential medium, 10% fetal bovine serum, 30 $\mu g\,ml^{-1}$ kanamycin, 25 U ml^{-1} penicillin and 25 $\mu g\,ml^{-1}$ streptomycin. Cells were cultured with the appropriate medium at 37 °C under 5% CO_2. The list of human tumour cell lines and the medium for them were as follows: GOTO (neuroblastoma) (D); IMR32 (neuroblastoma) (C supplemented with nonessential amino acids); NB-1 (neuroblastoma) (D); T98G (glioblastoma) (C supplemented with nonessential amino acids and pyruvate); A-431 (epidermoid carcinoma) (B); Capan-2 (adenocarcinoma from pancreas) (B); ES-2 (squamous cell carcinoma from oesophagus) (B); ES-6 (squamous cell carcinoma from oesophagus) (B); KATO III (gastric carcinoma) (B); COLO 201 (adenocarcinoma from colon) (B); Hep G2 (hepatocellular carcinoma) (B); Namalwa cells (Burkitt lymphoma) (A); Daudi (Burkitt lymphoma) (A); Raji (Burkitt lymphoma) (A); AT(L)-5KY (B lymphoblastoid, ataxia telangiectasia) (A); U-937 (histiocytic lymphoma) (A); HL-60 (promyelocytic leukaemia) (A); PA-1 (ovarian teratocarcinoma) (B); NT2 (embryonic teratocarcinoma) (B supplemented with pyruvate); MCF7 (breast epithelial adenocarcinoma) (B); and HeLa (epitheloid carcinoma from cervix) (B).

Preparation of anti-Fuc-TIII mAb

A PCR amplification for the *Fuc-TIII* gene from human genomic DNA was done using primers 1 and 2 in Table 1. The amplified fragment was inserted into a bacterial expression vector, pWA51, which was modified from pGEMEX-1 (Promega, WI). The plasmid expressed a 30 kDa fusion protein in frame consisting of five amino acids encoded by the vector sequence and the peptide of the amino acid position 98 to 361 in the Fuc-TIII sequence. The recombinant protein expressed in *E. coli* accumulated in the insoluble fraction. After washing the insoluble inclusion body with 0.5% Triton X-100 followed by 2 M guanidine HCl, the protein was solubilized in 4 M guanidine HCl and dialysed against 10 M urea. Mice were immunized with the dialysate in complete Freund's adjuvant.

Screening of hybridomas reacting to the recombinant Fuc-TIII was done as follows. Microtitre plates were coated with the dialysed peptide and blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). After incubation for 2 h at 37 °C, the plates were washed with PBS containing 0.05% Tween-20 and then incubated with culture supernatants of hybridomas. The antibodies binding to the plates were detected by incubation with peroxidase conjugated anti-mouse whole Ig antibody (Dako, Japan). After 1 h, 1 mg ml^{-1} of 2,2′-Azino-di[3-ethylbenzthiazolinesulfonate(6)] (Wako Junyaku, Japan) in 40 mM phosphate-citrate buffer (pH 4.0) was added and absorbance was measured at 405 nm. mAb was purified with an Affigel protein A MAPS-™II kit (Bio-Rad Laboratories, CA). Finally, a monoclonal antibody reacting to the recombinant Fuc-TIII was established and named FTA1-16. The subclass of FTA1-16 was IgG2a.

Construction of expression plasmids containing Fuc-T *genes*

The DNA fragment encoding the full-length open reading frame (ORF) of the *Fuc-TIII* gene was obtained by PCR with primer 3 and 4 from genomic DNA of a Lewis antigen-positive (Le(+)) individual, and inserted into a pBluescript SK(−) vector with *Eco* RI and *Hind* III sites [17]. Sequencing of the inserted DNA revealed that no nucleotide substitution occurred by PCR. The *Fuc-TIII* gene in the pBluescript vector was excised with *Eco* RI and *Hind* III, blunted and ligated with two *Sfi* I adaptors, CTCTAAAG and CTTTAGAGCAC. After digestion with *Sfi* I, the gene was inserted into a *Sfi* I-digested pAMo vector [21] and the plasmid constructed was named pAMo-FTIII. The pAMo-plasmid containing the *Fuc-TV* gene was prepared by the same method and named pAMo-FTV. For construction of the pAMo-plasmid containing the *Fuc-TVII* gene (pAMo-FTVII), the product obtained by RT-PCR using mRNA of U937 cells as template was subcloned into pBluescript. After confirming the sequence, the gene was inserted into the pAMo vector.

The pAMo-FTIV and pAMo-FTVI which contain the *Fuc-TIV* and *Fuc-TVI* genes, respectively, in the pAMo vector were obtained from Kyowa Hakko Kogyo Co., Ltd (Japan).

Establishment of stable transformant cells

Namalwa cells were transfected with each of the expression plasmid DNAs, pAMo-FTIII, pAMo-FTIV, pAMo-FTV, pAMo-FucTVI and pAMo-FTVII by electroporation. Transfected cells were cultured in RPMI 1640 containing 10% fetal calf serum under 5% CO_2. After 24 h culture, G418 (Sigma) was added to the culture at a final concentration of 1.2 mg ml^{-1} to obtain stable transformants. The stable transformant cell lines with each of the *Fuc-T* genes were named Namalwa-FTIII, -FTIV, -FTV, -FTVI and -FTVII.

Fluorescence microscopy

Each of the stable transformant Namalwa cells was fixed on a glass slide with PBS containing 4% paraformaldehyde. Blocking and permeabilization of the cells were done simultaneously in PBS containing 0.05% Triton X-100 and 1% BSA (solution A). After incubation with 10 μg ml^{-1} of FTA1-16 in solution A, the cells were washed four times with 0.05% Triton X-100 in PBS (solution B) and incubated with FITC-conjugated anti mouse IgG in solution A. The cells were washed four times with solution B and observed with a fluorescence microscope.

Western blotting

Tumour cell lines and stable transformant cell lines were subjected to Western blotting analyses. The cells were disrupted in 0.1% Triton X-100, 20 mM HEPES (pH 7.4) with a well-type sonicator. After 10 min centrifugation at 10 000 rpm, the solubilized proteins were recovered and used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins separated in a 10% gel were transferred to an Immobilon PVDF membrane [22]. The membrane was blocked with PBS containing 3% BSA and incubated with 10 μg ml^{-1} of FTA1-16, 2D3 (anti-sLe^a) [23], KM-93 (anti-sLe^x) [24] or MAb8628 (monoclonal antibody against human β1,4galactosyltransferase) [25, 26]. FTA1-16 was pre-absorbed with cell lysates of wild-type Namalwa cells in PBS containing 1% BSA (solution C). After washing three times with PBS containing 0.01% Tween 20, the membrane was incubated with HRP-conjugated second antibody or biotinylated second antibody in solution C followed by a mixture of streptavidin and HRP-conjugated biotin from the Vectastain kit (Vector, CA). Detection of HRP was carried out with the Konica staining kit (Konica, Japan).

Immunoprecipitation

Composition of the TSA solution was as follows: 0.1 M of Tris-HCl (pH 8.0), 0.14 M of NaCl, 0.025% of NaN_3. Namalwa-FTIII cells (5×10^7 cells) were lysed in 1 ml of solution D (1 mM of PMSF, 0.5 mg ml^{-1} of Triton X-100, 1 mM of iodoacetic acid, 0.2 U ml^{-1} of aprotinin in TSA solution) and the lysate was stored at 4 °C for 60 min. After 10 min of centrifugation at 3000 × **g**, the supernatant was recovered and centrifuged at 100 000 × **g** for 60 min. The supernatant was pre-cleared using the following method. Two hundred ml of the supernatant was incubated with 1:1 slurry of protein G Sepharose/TSA for 2 h at room temperature. The mixture was microfuged for 5 s and the supernatant was recovered (pre-cleared supernatant). Five μl of FTA1-16 was added to the pre-cleared supernatant and shaken gently at 4 °C for 90 min. Then, 25 μl of 1:1 slurry of Protein G Sepharose/TSA was added to the mixture and shaken gently at 4 °C for another 90 min. The protein G Sepharose was washed twice with 0.1% Triton X-100 in TSA and once with TSA and 0.05 M Tris-HCl (pH 6.8). Proteins which bound to the protein G Sepharose were solubilized in SDS-solubilization buffer and used for SDS-PAGE. Proteins separated in 10% SDS-PAGE were detected by silver staining. A control experiment was carried out with IgG fraction prepared from normal mouse serum instead of FTA1-16.

Immunohistochemistry

Human colon tissue from a patient with colon carcinoma diagnosed as moderately differentiated tubular carcinoma was fixed in 4% paraformaldehyde in phosphate buffer immediately after resection by operation, and was subjected to paraffin embedding. Deparaffinized 4 μm sections of cancer and normal parts of the colon were washed in PBS three times, and were treated with H_2O_2-methanol followed by treatment with 40% horse serum in PBS after washing with PBS. Detection for Fuc-TIII was performed by application of FTA1-16 as the first antibody followed by biotinylated sheep anti-mouse IgG (Amersham Japan, Japan) and then by avidin-peroxidase complex (Wako Junyaku, Japan). Between each step, the glass slides were washed with PBS. For the peroxidase reaction, 0.1 mg ml^{-1} of DAB 4HCl (Dotite, Japan) in 0.1 M Tris-buffer (pH 7.6) was used.

Epitope mapping

Lane 1 in Fig. 6A schematically represents a full-length Fuc-TIII protein which contains 361 amino acid residues and lanes 2–8 represent truncated peptides (ΔP2–ΔP8). The genomic DNA of the full-length *Fuc-TIII* gene was inserted in pBluescript SK(−) vector between *Eco* RI and *Hind* III sites and named pBS-FTIII. The plasmids containing deletion mutants of the *Fuc-TIII* gene were prepared as follows. pΔM2 for production of the truncated peptide ΔP2 was prepared by self-ligation of *Eco* RV digest of pBS-FTIII. pΔM3 for ΔP3 was prepared by self-ligation of *Eco* 47III digest of pBS-FTIII. pΔM4 and pΔM5 were prepared by self-ligation of *Apa* I digest and *Kpn* I digest of pBS-FTIII, respectively. pΔM6 was prepared by insertion of a PCR product of primers 7 and 8 in Table 1 using pBS-FTIII as a template. pΔM7 was prepared by self-ligation of *Sty* I digest of pΔM6. pΔM8 was prepared by insertion of a PCR product of primers 9 and 10 using pBS-FTIII as a template. These plasmids expressed fusion proteins with

β-galactosidase in frame in *E. coli* strain XL1-Blue by adding isopropyl β-D-thiogalactopyranoside with a final concentration of 0.2 mM. Recovered cells were disrupted in 1% Triton X-100, 20 mM HEPES (pH 7.4) with a well type sonicator and microfuged for 10 min at 10 000 rpm. An equal volume of SDS-solubilization buffer was added to the Triton X-100-insoluble fractions. The mixtures were separated in 12.5% SDS-PAGE and transferred to an Immobilon PVDF membrane. The Western blotting procedure is described above. Some deletion mutants lost their original stop codon by deletion and their ORFs were terminated by stop codons in the vector sequence. Such mutants, therefore, contained additional amino acids at the carboxyl terminus of the truncated Fuc-TIII peptides. The length of additional amino acid residues from the vector sequence is illustrated with an open bar at the carboxyl terminus in Fig. 6A.

Results

Binding specificity of mAb FTA1-16

mAb FTA1-16 was established by immunization with the recombinant Fuc-TIII protein. Since the members of the α(1,3)Fuc-T family share highly homologous sequences, crossreactivity of FTA1-16 to the other Fuc-Ts is expected. To examine the binding specificity of FTA1-16, we first employed indirect immunofluorescence staining of stable transformant cells expressing each of the five *Fuc-T* genes. The expression of mRNA and enzyme activity corresponding to each *Fuc-T* gene in the stable transformant cells were confirmed by Northern blotting analysis and the measurement of activity prior to the experiment (data not shown). As seen in Fig. 1B, the Namalwa-FTIII cells, which are stable transformants with the *Fuc-TIII* gene, were clearly stained by FTA1-16 with a typical staining pattern of the Golgi apparatus in the cells. As a negative control, Namalwa cells stably transformed with the pAMo vector alone (Namalwa-pAMo) gave no staining (Fig. 1A). It was a surprise to observe no crossreactivity of FTA1-16 to any of the other stable transformants (Fig. 1C–F). To confirm the fine specificity of FTA1-16 against Fuc-TIII, the cell lysate from each of the transformant cells was prepared for Western blotting analysis. In the cell lysate of Namalwa-FTIII cells (Fig. 2, lane 2), FTA1-16 detected a band that was considered to be a Fuc-TIII protein with a molecular weight of 46 kDa. No specific band was detected by FTA1-16 in the lysates prepared from Namalwa-pAMo (lane 1), Namalwa-FTIV (lane 3) and Namalwa-FTVI (lane 4) cells.

Immunoprecipitation of Fuc-TIII protein by FTA1-16

The immunoprecipitation method for antigens by antibodies is very useful to purify antigens. We examined whether FTA1-16 could immunoprecipitate the Fuc-TIII protein or not. The lysate of Namalwa-FTIII cells was incubated with FTA1-16 followed by incubation with Protein G Sepharose as described in Materials and methods. Since the mobility of immunoglobulin heavy chain in SDS-PAGE under reducing conditions was similar to that of Fuc-TIII, SDS-PAGE analysis for the precipitated material was carried out under nonreducing conditions. Silver staining on the SDS-PAGE gel showed two bands with an approximate molecular weight of 45 kDa and 90 kDa in the lane of the sample immunoprecipitated by FTA1-16 in addition to the immunoglobulin-bands of FTA1-16 itself (Fig. 3, lane 1). The lower band (45 kDa) as indicated by an arrow in lane 1 was considered to be the Fuc-TIII protein since the size was equal to that of the band by Western blotting analysis. The upper band at around 90 kDa may be a dimer of Fuc-TIII. No specific protein band was detected in the sample immunoprecipitated by the unrelated mouse IgG as a negative control (lane 2).

Western blotting analysis on a variety of human tumour cell lines

Figure 4A–D shows the results of Western blotting analyses by FTA1-16 and MAb8628 on a variety of human tumour cell lines including four brain tumour cell lines in panel A, six epithelial cancer cell lines from digestive organs in panel B, six leukaemia cell lines in panel C and others in panel D. Capan 2 (pancreas adenocarcinoma) and MCF7 (breast epithelial adenocarcinoma) produced the largest amount of Fuc-TIII among the cell lines examined and they obviously gave two specific bands with approximate molecular weights of 45 kDa and 42 kDa. The specific bands of Fuc-TIII were weakly observed in A-431 (epidermoid carcinoma), COLO201 (colon adenocarcinoma) and Hep G2 (hepatocellular carcinoma) cells, but not in the other cell lines. Figure 4A–D shows the result of Western blotting analysis by MAb8628 (anti-human β1,4GalT). The β1,4GalT is known to be a glycosyltransferase ubiquitously expressed in many tissues and organs except for the trace amount expression in brain and testis. MAb8628 revealed strong bands of β1,4GalT in all of the epithelial cancer cell lines in panels B and D except for ES-6 cells. It gave intermediate-level signals in all of the leukaemia cell lines (except for AT(L)-5KY cells) in panel C and in two teratocarcinoma cell lines, PA-1 and NT2, in panel D. No specific bands could be observed in neuroblastoma cell lines as expected, but interestingly the glioblastoma T98G expressed a comparable amount of β1,4GalT to that in the leukaemia cell lines (panel A). The expression of sLe^a and sLe^x antigens was also determined by Western blotting analysis (data not shown) and is summarized in Fig. 4. Capan 2, A-431 and COLO201 cells expressed abundant sLe^a antigens, and ES-6 and KA-

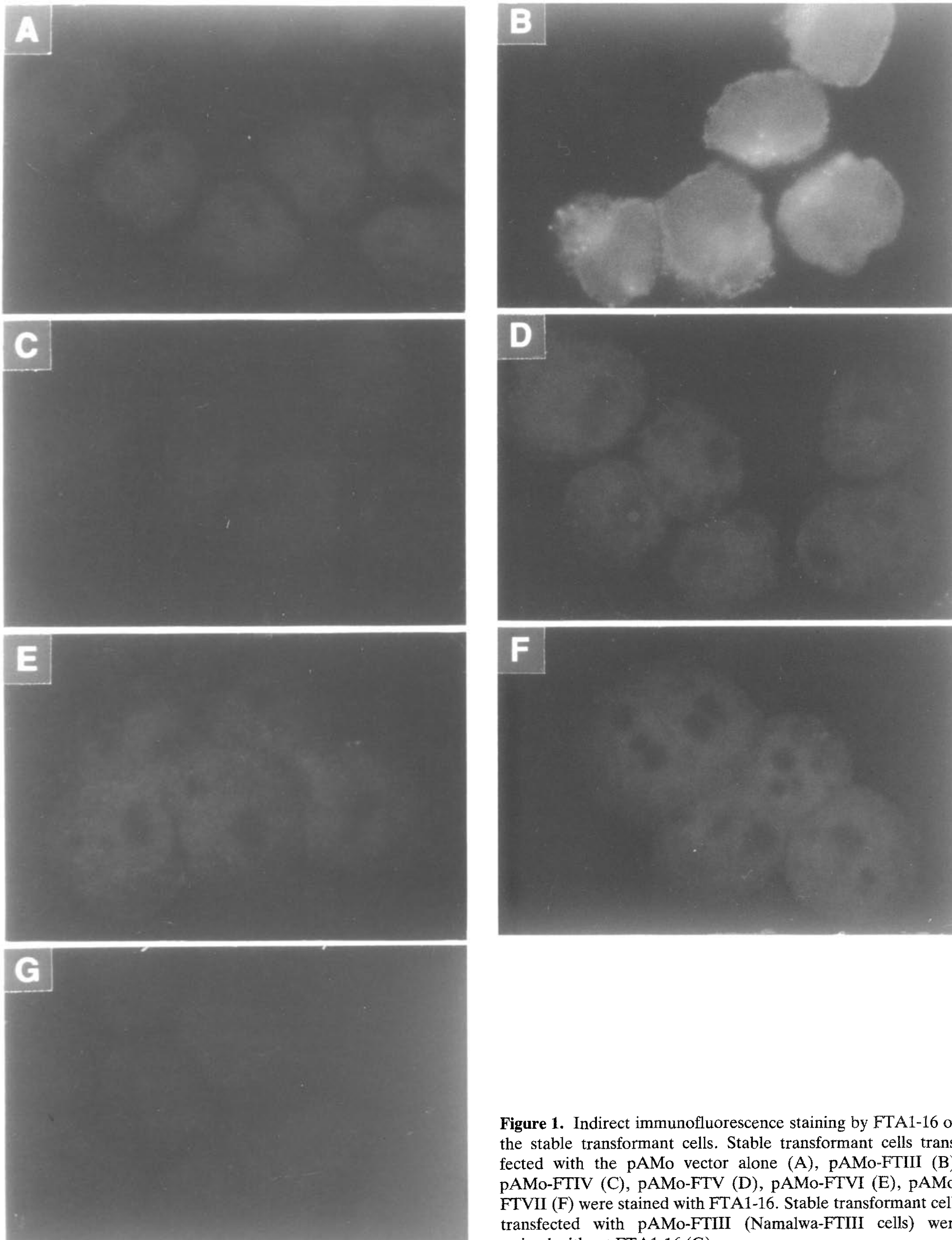

Figure 1. Indirect immunofluorescence staining by FTA1-16 on the stable transformant cells. Stable transformant cells transfected with the pAMo vector alone (A), pAMo-FTIII (B), pAMo-FTIV (C), pAMo-FTV (D), pAMo-FTVI (E), pAMo-FTVII (F) were stained with FTA1-16. Stable transformant cells transfected with pAMo-FTIII (Namalwa-FTIII cells) were stained without FTA1-16 (G).

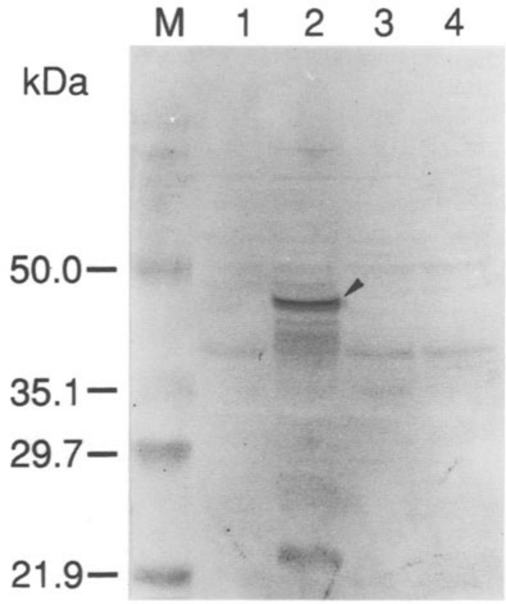

Figure 2. Western blotting analysis by FTA1-16 on the cell lysates of the stable transformant cells. Molecular weight markers (lane M), the cell lysates from Namalwa-pAMo cells (lane 1), Namalwa-FTIII cells (lane 2), Namalwa-FTIV cells (lane 3) and Namalwa-FTVI cells (lane 4) were electrophoresed on SDS-PAGE for Western blotting analysis.

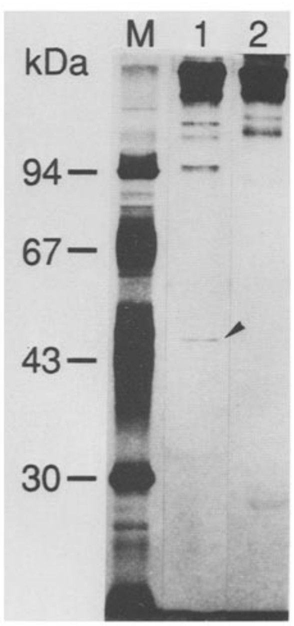

Figure 3. Immunoprecipitation of Fuc-TIII from Namalwa-FTIII cells by FTA1-16. Molecular weight markers (lane M), the samples immunoprecipitated by FTA1-16 (lane 1) and by control mouse IgG (lane 2) were electrophoresed in nondenatured SDS-PAGE and subjected to silver staining. An arrow indicates the Fuc-TIII protein.

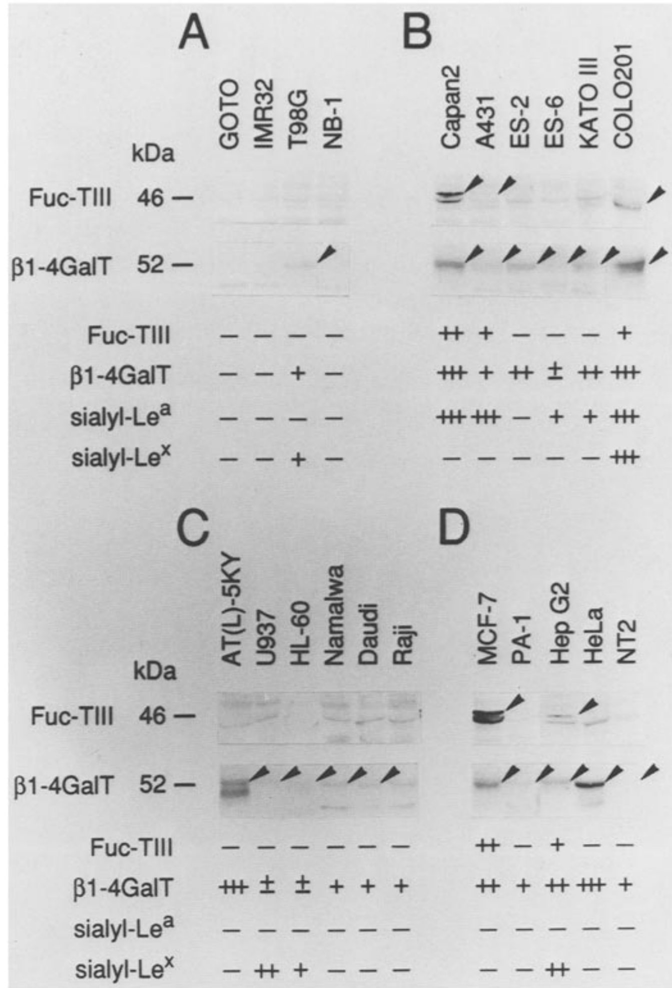

Figure 4. Western blotting analysis on a series of human tumour cell lines. Cell lysates from cancer cells were electrophoresed on SDS-PAGE for Western blotting analysis and transferred to an Immobilon PVDF membrane. Fuc-TIII and *β*1,4GalT were detected by FTA1-16 and MAb8628, respectively. The specific bands are indicated with arrows. A, brain tumour cell lines; B, epithelial cancer cell lines from digestive organs; C, leukaemia cell lines; D, other tumour cell lines. The results obtained were summarized with the indication of − to +++ depending upon the intensity of the detected bands. Summary of the bands detected by 2D3 (anti-sLe^a) and KM-93 (anti-sLe^x) were also indicated (data not shown).

TOIII cells expressed them at an intermediate level. The sLe^a expression was well correlated with the Fuc-TIII expression in the cells of Capan 2, A-431 and COLO201, but not correlated in MCF7 cells. The positive cell line of sLe^x expression detected by Western blotting analysis was COLO201 alone among the epithelial cancer cell lines in panel B and Hep G2 alone among the cell lines in panel D. From the restricted number of the sLe^x positive cell lines in this study, we could not make any conclusions about correlated expression between sLe^x antigens and Fuc-TIII. The sLe^x antigens detected positively in leukaemia cell lines, U937 and HL-60 in panel C, are known to be synthesized by Fuc-TVII, not by Fuc-TIII.

Immunohistochemistry on normal tissue and cancer tissue of colon

Fuc-TIII was detected in normal mucosal epithelium and carcinoma tissues of a colon cancer patient (Fig. 5). In the normal tissue, antigen positive cells distributed predominantly at the upper parts of the glands (Fig. 5a). The typical staining pattern of the Golgi region in surface epithelial cells of colon is circled on the right of Fig. 5a. In cancer cells, the antigen expression was more pronounced and diffuse (Fig. 5b) than in normal mucosa. As in the case of cancer cells, the staining pattern was very heterogenous, showing the positive staining not only in the Golgi area but also in cytoplasm and other unknown regions.

Epitope mapping of FTA1-16

To determine an antigenic epitope recognized by FTA1-16, we constructed seven deletion-mutant DNA plasmids of the *Fuc-TIII* gene in a bacterial expression vector and expressed them in *E. coli*. The full-length protein and the seven truncated peptides of Fuc-TIII expressed in *E. coli* were electrophoresed in denatured SDS-PAGE and analysed by Western blotting (Fig. 6). Panel A in Fig. 6 schematically represents the full-length protein (Fig. 6A 1) and the seven truncated peptides (Fig. 6A 2–8). The band with an approximate molecular weight of 43 kDa, estimated from the Fuc-TIII amino acid sequence, was detected in the full-length protein as indicated by an arrow in Fig. 6B, lane 1. Discrepancy of the molecular weight between the Fuc-TIII expressed in mammalian cells as in the previous section and that in *E.*

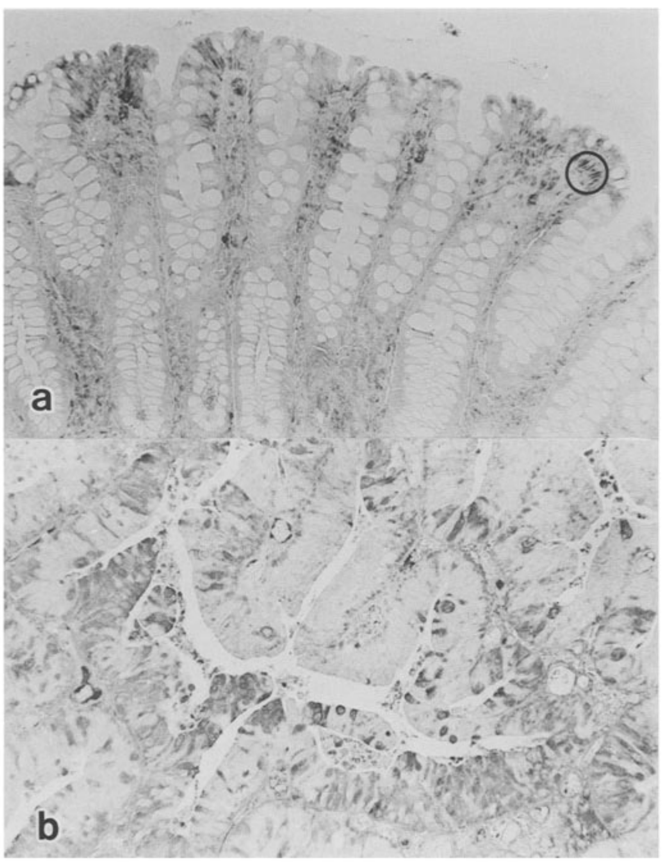

Figure 5. Immunohistochemical staining by FTA1-16 on normal tissue and cancer tissue of colon. Immunohistochemical staining on normal mucosa (a) and cancer tissue (b) of colon. The typical staining of the Golgi region in the normal tissue is circled in the upper panel. Note that most of the goblet cells are antigen free in the normal tissue whereas the cancer tissue exhibits more diffuse and random distribution of Fuc-TIII. Magnification: ×132 (a and b).

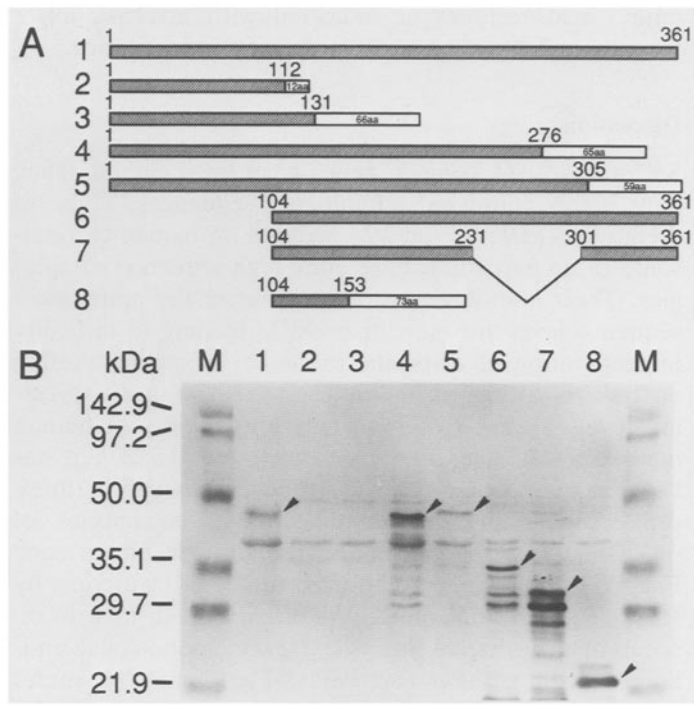

Figure 6. Determination of the antigenic epitope recognized by FTA1-16. The full-length protein and seven truncated peptides expressed in *E. coli* are schematically represented in panel A. The position of the amino acid residue in the Fuc-TIII sequence is numbered at both ends of truncated peptides (panel A). The length of the truncated peptide with the Fuc-TIII sequence is indicated with a shaded bar. As described in Materials and methods, the length of the peptide directed by the vector sequence is added at the carboxyl terminus as indicated by an open bar with the number of amino acid residues. Molecular weight markers (M) and each of the bacterial lysates containing the recombinant proteins (lanes 1–8) were electrophoresed in denatured SDS-PAGE and subjected to Western blotting analysis by FTA1-16 (panel B). The arrows in panel B indicate the specific bands of the recombinant proteins recognized by FTA1-16.

coli may be due to the absence or presence of glycosylated chains. Although many nonspecific bands were observed in all the lanes in Fig. 6B, they were considered to be degradative products obtained during manipulation and due to nonspecific binding of the second antibody to unrelated proteins of *E. coli*. Specific bands as indicated by arrows in Fig. 6B were detected in some truncated Fuc-TIII samples (lanes 4–8). Samples in lanes 2 and 3, however, did not reveal any specific bands. The antigenic epitope for FTA1-16 was located in the lane 8 sample which is a peptide from position 104 to 153 of the Fuc-TIII amino acid sequence and not in the lane 2 peptide (position 1–131). Taken together the above results indicate that the epitope should be localized within position 132–153 of the Fuc-TIII amino acid sequence. The amino acid sequence of Fuc-TIII in this region is presented in Fig. 7 with arrangement to the corresponding sequences of the other α(1,3)Fuc-Ts. Five amino acid residues as indicated with asterisks differ from the other peptides within this peptide sequence.

Discussion

So far, five *α(1,3)Fuc-T* genes have been cloned. They have highly conserved homologous sequences. Three of them (*Fuc-TIII*, *V* and *VI*) mapped on human chromosome 19, in particular, have quite high sequence homologies. Their homologies to each other at the amino acid sequence level are more than 90%, leading to difficulty in preparation of a specific probe for Northern blotting analysis to distinguish them. The transcriptional expression levels of *α(1,3)Fuc-T* genes in a variety of human malignant cell lines have been reported [16, 20]; it has been indicated that the α(1,3)Fuc-T activities in those cells are mixtures of multiple molecular species of α(1,3)Fuc-Ts. Oligonucleotide primers specific to each Fuc-T gene have been employed to detect transcripts by RT-PCR. The PCR method is often too sensitive to do accurate quantitative analysis. Hence, monoclonal antibodies distinguishing each α(1,3)Fuc-T may be useful tools for further analysis of α(1,3)Fuc-Ts at the translational level.

```
                        * * *  *      *
Fuc-TIII  132-153  WIWFNLEPPPNCQHLEALDRY-F
Fuc-TIV   167-189  WVWMNFESPSHSPGLRSLASNLF
Fuc-TV    145-166  WIWFSMESPSNCRHLEALDGY-F
Fuc-TVI   131-152  WIWFSMESPSHCWQLKAMDGY-F
Fuc-TVII  116-137  WVWASMESPSHTHGLSHLRGI-F
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Figure 7. Comparison of amino acid sequences in the corresponding region defined as the FTA1-16-epitope among five α(1,3)Fuc-Ts. The asterisks indicate the amino acid residues of the Fuc-TIII which are not shared by the other Fuc-Ts. The numerals indicate the positions of amino acid residues in each Fuc-T sequence corresponding to the position of the epitope-peptide of Fuc-TIII.

The monoclonal antibody, FTA1-16, specifically reacting to human Fuc-TIII, was established. It functioned well as a probe for Western blotting analysis, immunoprecipitation and immunohistochemical study. Indirect immunofluorescence analysis on the stable transformant cells showed specific staining of Fuc-TIII in the Golgi apparatus. This result demonstrated that FTA1-16 can recognize the native form of Fuc-TIII in the cells even though it was established by immunization of the denatured form. By Western blotting analysis on the stable transformants, we could confirm the restrictive specificity of FTA1-16 to Fuc-TIII and its capacity to recognize both the native and denatured forms. Furthermore, it was demonstrated that FTA1-16 would be a useful tool for purification of Fuc-TIII since it could immunoprecipitate Fuc-TIII very efficiently.

We have proved by molecular genetic analysis of the *Fuc-TIII* gene that the enzyme encoded by the *Fuc-TIII* gene determines the Lewis histo-blood group phenotype [17, 19]. More recently, the enzyme having α(1,4)Fuc-T activity secreted in saliva was proved to be the product of the *Fuc-TIII* gene [27]. Consequently, it is concluded that the *Fuc-TIII* gene is the Lewis gene and the Fuc-TIII enzyme is the Lewis-type enzyme. As α(1,4)Fuc-T activity has been found in a variety of epithelial tissues, particularly in those of digestive organs [28–30], and not found in myeloid cells, Lewis antigens on erythrocytes have been considered to be synthesized in epithelial cells of digestive organs and not in erythrocytes themselves. Western blotting analysis on a panel of tumour cell lines and immunohistochemical staining on colon tissue in this study clearly demonstrated that Fuc-TIII is mainly expressed in the epithelial cancer cell lines derived from colon and pancreas and not expressed in myeloid cell lines. Localization was at the surface epithelial cells of normal colon mucosa, in accord with the above observations on the tissues which express α(1,4)Fuc-T activity. Good correlation between the amount of Fuc-TIII and sLe^a was observed by Western blotting analysis on the cell lines. The more Fuc-TIII the cells expressed, the more sLe^a antigens they expressed, except for MCF7 cells that may lack type 1 chain precursors for sLe^a. As regards correlation between sLe^x- and Fuc-TIII-expression, we could not obtain conclusive results because of the restricted number of sLe^x-positive cell lines. The sLe^x antigens with type 2 chain precursors in intestinal cancer cells are thought to be synthesized not only by Fuc-TIII but also by Fuc-TVI as reported by others [3]. There is, therefore, no discrepancy with sLe^x expression in intestinal cancer cells which do not have Fuc-TIII. The sLe^x antigens in leukaemia cell lines, U937 and HL-60 cells, are synthesized by Fuc-TVII, not by Fuc-TIII. In fact, neither of the two cell lines have Fuc-TIII as seen in Fig. 4, panel C. The two bands of Fuc-TIII detected in the cell lines by Western blotting might represent the Golgi membrane-bound form and the soluble form. Western blotting analysis by MAb8628 (anti-human β1,4GalT) showed that: 1) β1,4GalT is a ubiquitous enzyme which is more abundantly produced than Fuc-TIII. This was also confirmed by detection of the transcripts using the competitive PCR method (data not shown). 2) Distribution of β1,4GalT among the cell lines seemed to be similar to that of Fuc-TIII. The cells producing β1,4GalT produced a comparable amount of Fuc-TIII. The cells, ES-2, ES-6, KATO III and all of the leukaemia cell lines, in which Fuc-TIII could not be detected by Western blotting expressed trace amounts of the Fuc-TIII transcripts. Such cells also produced small amounts or intermediate amounts of the β1,4GalT protein and transcripts. The mechanisms of transcriptional regulation of both genes might be similar. 3) β1,4GalT is more heterogenously glycosylated than Fuc-TIII since the β1,4GalT-bands diffused more widely than those of Fuc-TIII in all of the positive cells.

The immunohistochemical experiment in this study is the first report which has revealed the localization of Fuc-TIII. In normal colon tissue, Fuc-TIII apparently exists in the Golgi region of the epithelial cells and it was predominantly localized in the upper parts of the glands. The expression of Fuc-TIII in colon cancer cells was more enhanced and diffusely spread in a heterogenous staining pattern. Capability of FTA1-16 in staining paraffin-embedded specimens will allow us to detect more detailed tissue distribution.

Fuc-TIII catalyses the production of Lewis histo-blood group antigens, Le^a and Le^b. As described in our previous papers [17, 19], the *Fuc-TIII* genes (*Le* genes) from Le(−) individuals had missense mutations leading to amino acid substitutions in the catalytic domain of the enzyme. The single amino acid substitutions encoded in the *le* genes of Le(−) individuals have been proved to be the cause of enzyme inactivation resulting in no expression of Lewis antigens on erythrocytes [17–19]. As reported in the ABO blood-type system [31], the *O* genes inactivated by point mutations can produce transcripts which might be translated as nonfunctional enzyme. We do not know whether the mutated *Le* genes in Le(−) individuals (*le* genes) can produce nonfunctional Le enzymes or not. Detection of the nonfunctional transcripts and translated products is now under investigation on the Le(−) individuals (*le/le* homozygotes) using the RT-PCR method and FTA1-16, respectively.

The antigenic epitope recognized by FTA1-16 was determined. It may be useful for establishment of monoclonal antibodies against the other α(1,3)Fuc-Ts. It is reasonable that Fuc-Ts having highly homologous primary sequences with Fuc-TIII may have a tertiary structure similar to Fuc-TIII. The amino acid sequence in the other Fuc-Ts corresponding to position 132–153 of the Fuc-TIII sequence should form an antigenic epitope. Blocking experiment with FTA1-16 revealed that it could not block Fuc-TIII activity (data not shown). This indicates that the FTA1-16 epitope may not be involved in the active sites of the enzyme.

The level of CA19-9 tumour marker, defined as sLe^a [32], is influenced by the Lewis blood type. Although Fuc-TIII can synthesize sLe^a *in vitro*, it is not yet proved whether the CA19-9 antigen produced by cancer cells is the product of Fuc-TIII. We are now examining immunohistochemically the correlated expression between sLe^a antigen and Fuc-TIII in cancer tissues.

A recent study [33] regarding the patterns of fucosylation by Fuc-TIII and Fuc-TIV indicates that they might be localized in different compartments in the Golgi apparatus. It would be an interesting experiment to study the precise intracellular localization of the enzyme by electron microscopical study using comparative observations on two glycosyltransferases, Fuc-TIII and β1,4GalT.

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