Expression of glycoconjugates bearing the Lewis X epitope during neural differentiation of P19 EC cells

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Abstract The Lewis X (Le^x) bearing glycolipids were noticeably increased in amounts during the course of neural differentiation of P19 EC cells induced by retinoic acid (RA, all-*trans* form). Applying neoglycolipid technology and in situ TLC-LSIMS, the oligosaccharide chains of these scarce Le^x bearing glycolipids were partially characterized after released by endoglycoceramidase and subsequent conversion into neoglycolipids. In order to understand the enzymatic basis for the expression of Le^x bearing glycolipids, we measured glycolipid, glycoprotein and oligosaccharide fucosyltransferase (Fuc-T) activities using appropriate substrates in P19 EC cells with or without RA treatment. All three Fuc-Ts were increased after RA treatment and the highest activity was in the differentiated neural cells. We then investigated the two possible Fuc-T genes that might be responsible for these changes using RT-PCR analysis. Mouse Fuc-TIX (mFuc-TIX) transcript was detected in all cell types but it was only strongly expressed in RA-induced aggregates and neural cells. In the case of mouse Fuc-TIV (mFuc-TIV) gene, its transcript was only detectable in RA-induced aggregates and not found in either undifferentiated or RA-induced neural cells. These results strongly support that RA induces only a transient expression of the mFuc-TIV gene in cell aggregates but a more persistent expression of the mFuc-TIX gene at the transcription level throughout neural cell differentiation. The mFuc-TIX gene is probably the main cause for the increased expression of Le^x glycoconjugates during neural differentiation of P19 EC cells.

Key words: Retinoic acid; Le^x; Fucosyltransferase; Fuc-TIX gene; Neural cell differentiation; Embryonic carcinoma cell

1. Introduction

The expression of Lewis X (Le^x) carbohydrate epitope, 3-fucosyl *N*-acetyllactosamine, can be recognized by a number of monoclonal antibodies (MAbs), namely SSEA-1 [1–3], FORSE-1 [4], CD-15 [5,6] and L5 [7]. It is widely distributed in many tissues but there are distinctive differences in expression between species, such as their expression on granulocytes of human but not on those of mouse, rat, rabbit or pig [8]. It is also involved in many cell–cell interaction systems, such as in developing mouse embryo and compaction of mouse embryo of embryonal carcinoma F9 embryonic carcinoma (EC) cells [2,9], differentiation of TERA-2-derived human embryonal carcinoma cells [10,11], and in early neural development of chick embryo [6]. Two cloned mouse α1,3-fucosyltransferases (mFuc-Ts): mFuc-TIV [12,13] and mFuc-TIX [14] have been shown to be responsible for the synthesis of Le^x epitope in vitro. The mFuc-TIX transcript was detected in neural cells but not in glial cells including astrocytes [14] while the mFuc-TIV transcript was not expressed in brain but abundantly expressed in other tissues [12,14]. These observations suggest that mFuc-TIX and not mFuc-TIV participates in Le^x synthesis in neurons and may also be developmentally regulated [14]. P19 EC cells have been shown to be useful in studying early stages of neural differentiation and they differentiate into postmitotic neurons capable of synaptic formation upon stimulation with retinoic acid (RA) while muscle cells are formed when stimulated with dimethyl sulfoxide [15–17]. In our previous study of neural differentiation using P19 EC cells, we showed that RA also induced the expression of synthases of

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[2] Glycosphingolipids are named according to Svennerholm [29].

Abbreviations: BSA, bovine serum albumin; DHPE, L-1,2-dihexadecyl-*sn*-glycero-phosphoethanolamine; EC, embryonic carcinoma; Fuc, fucose; Fuc-T(s), fucosyltransferase(s); mFuc-T(s), mouse fucosyltransferase(s); Hex, hexose; dHex, deoxyhexose; HexNAc, *N*-acetylhexosamine; HPLC, high-performance liquid chromatography; HPRT, hypoxanthine phosphoribosyltransferase; Le^x, Lewis X (3-fucosyl *N*-acetyllactosamine sequence); LSIMS, liquid secondary ion mass spectrometry; MAb, monoclonal antibody; PA, pyridylaminated; PBS, phosphate-buffered saline; RA, retinoic acid (all-*trans* form); RT, reverse transcriptase; RT-PCR, reverse transcription-polymerase chain reaction; TLC, thin-layer chromatography; UV, ultraviolet light; Cer, ceramide; CMH, Galβ1-1Cer; CDH, Galβ1-4Glcβ1-1Cer; CTH, Galα1-4Galβ1-4Glcβ1-1Cer; Forssman, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; GA1, asialo-GM1, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; globoside, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer; paragloboside, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; Oligosaccharides used were: LNFP-III, lacto-*N*-fucopentaose-III, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc; LNnT, lacto-*N*-neotetraose, Galβ1-4GlcNAcβ1-3Galβ1-4Glc[2]

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acidic glycolipids in the ganglioside biosynthetic pathway [18]. In the present study, structures of the neutral glycolipids bearing the Le^x epitope derived from three P19 EC cell types (untreated, RA-induced aggregates and differentiated neural cells) were analyzed and their immunoactivities measured. Due to limited amounts of glycolipids available and the complexity in both sugar chains and ceramide (Cer) moieties, glycolipids were converted into neoglycolipids before analysis. This eliminates distribution of the same sugar chain among different fractions bearing different Cers, and hence increases the sensitivity of both structural and immunostaining detection. We also measured Fuc-T activities which were responsible for the synthesis of Le^x epitope on different glycoconjugates using appropriate substrates in these cells. In addition, we investigated the transcripts of two α1,3-Fuc-T genes: mFuc-TIV and mFuc-TIX by a reverse transcription-polymerase chain reaction (RT-PCR) method to show if one or both Fuc-T genes were involved during RA-induced neural differentiation.

2. Materials and methods

2.1. Materials and cells

GDP-[^{14}C]Fuc (10.1 GBq/mol) was from NEN Life Science (NY, USA). RA, cytosine arabinoside, GDP-Fuc, CDP-choline, fucose (Fuc), and asialofetuin were from Sigma (MO, USA). LNnT (lacto-*N*-neotetraose, Galβ1-4GlcNAcβ1-3Galβ1-4Glc)-PA was synthesized by pyridylamination of LNnT (Dextra, Reading, UK) using a reagent kit (Takara, Otu, Japan). Paragloboside (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer) (LNnT-Cer) was from Dia-Iatron (Tokyo, Japan). Bovine brain CMH (Galβ1-1Cer), CDH (Galβ1-4Glcβ1-1Cer) and GA1 (asialo-GM1, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer), human erythrocytes CTH (Galα1-4Galβ1-4Glcβ1-1Cer) and globoside (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer) and sheep erythrocytes Forssman (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer) glycolipids were from Sigma. Neoglycolipids, LNnT-L-1,2-dihexadecyl-*sn*-glycero-phosphoethanolamine (DHPE) and LNFP-III (lacto-*N*-fucopentaose-III, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc)-DHPE were prepared by conjugation of oligosaccharide standards (Dextra, Reading, UK) to DHPE (Fluka, Gillingham, UK) as described by Feizi et al. [19]. Specific anti-Le^x MAb, clone 73-30 [20], was from Seikagaku (Tokyo, Japan). Horseradish peroxidase-conjugated anti-mouse immunoglobulins antibody was from Amersham Pharmacia Biotech (Buckinghamshire, UK). P19 EC cells (kindly provided by Dr. H. Hamada of Osaka University, Osaka, Japan) were cultured as previously described [17,18]. Three types of cells were used in this study: untreated, RA-induced aggregates and differentiated neural cells. All differentiated neural cells were shown to express neural marker proteins by immunohistochemical analysis using anti-GAP-43, and -synaptophysin MAbs in addition to morphological changes observed under the microscope [21].

2.2. Extraction of neutral glycolipids and thin-layer chromatography (TLC)-immunostaining

Total lipids were extracted from 10^9 cells and were separated into neutral and acidic fractions by DEAE Sephadex A-25 (acetate form) according to Ledeen and Yu [22]. The total neutral glycolipids obtained from untreated cells, RA-induced aggregates and differentiated neural cells were 1.8 mg, 2.3 mg, and 3.1 mg (dry weight), respectively, and they were resolved on silica gel 60 high-performance TLC plates (HPTLC, Merck, Darmstadt, Germany) using the following solvent systems: solvent (A), $CHCl_3$/MeOH (1/1, v/v) and solvent (B), $CHCl_3$/MeOH/0.2% $CaCl_2$ (60/40/10, v/v/v). The resolved neutral glycolipids were stained with orcinol and primuline [19,23] and identified by comparing with commercial standards. TLC-immunostaining with anti-Le^x MAb was carried out similar to previously described [18]. Briefly, neutral glycolipids (2 μg) were resolved on HPTLC plate, stained with primuline, visualized and photographed under ultraviolet light (UV) at 365 nm [23]. Since preliminary experiments showed that all immunoreactive bands were found below the spot of GA1, sample plates from subsequent studies were therefore cut and only the immunoreactive portion was investigated further. The cut plate was first plasticized by dipping into 0.05% polyisobutyl methacrylate (Plexigum P28; Merck, Darmstadt, Germany) in cyclohexane for 2 min and air-dried. After wetting with phosphate-buffered saline (PBS), the plate was then submerged in PBS containing 3% bovine serum albumin (BSA) at room temperature for 1.5 h, overlaid with anti-Le^x MAb (diluted 1:100 with PBS containing 3% BSA) and incubated at room temperature for 1.5 h. After washing three times with PBS, immunoreactive bands were visualized using a Vectastain ABC kit (Vector Lab., CA, USA) and DAB substrate kit (Funakoshi, Tokyo, Japan) according to suppliers' instructions. Color development was stopped by dipping into PBS. A study on the acidic glycolipid fraction had already been reported elsewhere [18].

2.3. Analysis of neutral oligosaccharides and neoglycolipids

For the identification of Le^x bearing oligosaccharide structures, neutral glycolipids extracted from 5×10^9 RA-induced aggregates were treated with endoglycoceramidase (Seikagaku) according to the supplier's instructions. After passing through a Sep-Pak C18 cartridge (500 mg, Waters, Watford, UK) and desalted on a mixed bed column containing AG50-X12 (H form) and AG3-X4 (OH form) resins (Bio-Rad, CA, USA), the released neutral oligosaccharides were estimated for hexose (Hex) contents [19] and then fractionated on a TSK Amide-80 column (4.6×250 mm, Anachem, Luton, UK) using acetonitrile/water (elution gradient was from 35% to 50% aqueous in 40 min at a flow rate of 1 ml/min; detection was by UV at 205 nm). Sixteen fractions were collected (Fig. 2A), lyophilized and conjugated to DHPE [19]. Le^x immunoreactive components in these neoglycolipid fractions were identified by TLC-immunostaining as described above after their separation on HPTLC plates using $CHCl_3$/EtOH/H_2O (45/60/15, v/v/v) as solvent (Fig. 2B,C). The immunoreactive fractions: 7, 10 and 12 were further subjected to in situ TLC-liquid secondary ion mass spectrometry (LSIMS) analysis. LSIMS was performed on a VG ZAB 2E mass spectrometer in negative-ion mode. Spectra were acquired directly from TLC plate surface as previously described [24,25].

2.4. Assay of Fuc-Ts

Glycolipid, glycoprotein and PA-oligosaccharide Fuc-Ts were assayed using appropriate acceptor and donor substrates. Cells (10^8) were suspended in 100 μl of 0.5 M sucrose in PBS containing 0.3% Triton X-100 detergent, sonicated (0.5 cycle, 90% amplitude; UP 50H, Kubota, Japan), and used as enzyme source. The determinations of these three Fuc-T enzyme activities were optimized and linear up to 4 h under the present conditions. Briefly, glycolipid Fuc-T activity was assayed in 20 μl of 0.1 M HEPES, pH 7.0 containing 6 μg paragloboside (LNnT-Cer, acceptor substrate), 100 μM GDP-[^{14}C]Fuc, 5 mM CDP-choline (phosphodiesterase inhibitor), 10 mM Fuc (fucosidase inhibitor), 20 mM $MnCl_2$ and 10 μl enzyme source [26]. After incubation at 37°C for 2 h, the radio-labeled product was measured as previously reported [18]. Glycoprotein Fuc-T activity was measured in 20 μl of 0.1 M HEPES, pH 8.0 containing 100 μg asialofetuin (acceptor substrate), 100 μM GDP-[^{14}C]Fuc, 5 mM CDP-choline, 10 mM Fuc, 20 mM $CaCl_2$, and 10 μl enzyme source. After incubation at 37°C for 2 h, the reaction mixture was spotted onto Whatman No. 1 paper (2×2 cm), fixed by dipping into 10% (w/v) trichloroacetic acid, rinsed twice with fresh 10% (w/v) trichloroacetic acid and then rinsed once with EtOH/diethyl ether (2/1, v/v). Radioactivity on the paper was measured using a liquid scintillation counter. PA-oligosaccharide Fuc-T activity was measured in 20 μl of 0.1 M HEPES, pH 8.0 containing 0.1 mM LNnT-PA (acceptor substrate), 1 mM GDP-Fuc, 5 mM CDP-choline, 10 mM Fuc, 20 mM $CaCl_2$ and 10 μl enzyme source. After incubation at 37°C for 2 h, the reaction was stopped by boiling for 5 min and centrifuged at 10 000 rpm for 5 min. 10 μl of supernatant was then subjected to high-performance liquid chromatography (HPLC) analysis on a PALPAK Type N column (Takara, Otu, Japan), by isocratic elution with acetonitrile/200 mM acetic acid-triethylamine (69/31, v/v), pH 7.3, at a flow rate of 1.0 ml/min at 40°C. Elution profile was monitored by fluorescence (excitation and emission wavelengths at 320 and 400 nm, respectively) and the amounts of the products were determined by comparing their fluorescence intensities to the standard, LNnT-PA (Takara, Otu, Japan). Protein concentration was determined using Micro BCA Protein Assay Reagent kit (Pierce, IL, USA). Relative enzyme activities were expressed as CPM[^{14}C]Fuc/mg protein/h for the glycolipid and glycoprotein Fuc-Ts. The PA-oligosaccharide Fuc-T activity was expressed as LNnT-Fuc-PA pmol/mg protein/h.

2.5. Analysis of Fuc-T transcripts using RT-PCR methods

Total RNA was extracted from 10^9 untreated cells, RA-induced aggregates, and differentiated neural cells using ISOGEN reagent (Nippon gene, Tokyo, Japan) and 5 μg of it was treated with RNase-free DNase I (Boehringer Mannheim, Germany) at 37°C for 10 min to remove residual genomic DNA. It was followed by incubation at 95°C for 15 min to inactivate DNase I. Complementary DNA (first strand cDNA) was synthesized with an oligo (dT) primer from 1 μg of the DNase I-treated total RNA in a 20 μl total volume reaction mixture using a SUPERSCRIPT II kit (Life Technologies, MD, USA). 1 μl aliquot of the first strand cDNA was amplified by PCR in 50 μl reaction mixture containing 0.2 μM of each primer, 0.2 mM dNTP and 1.25 U of AmpliTaqGold (Perkin-Elmer, NJ, USA) with a pre-PCR heating step at 95°C for 9 min, followed by optimal numbers of PCR cycles at 95°C for 45 s, 63°C for 45 s and 72°C for 1 min (40 cycles for mFuc-TIV and mFuc-TIX, and 30 cycles for hypoxanthine phosphoribosyltransferase (HPRT), the transcript expression control). For analysis, 10 μl aliquot of PCR products and appropriate bp molecular marker standards (Gibco BRL, NY, USA) were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Primer sets used were as described previously [14]: mFuc-TIV forward, 5′-TTGCAGCCTGCGCTTCAACATC-AG-3′; mFuc-TIV reverse, 5′-ACTCAGCTGGTGGTAGTAACG-GAC-3′; mFuc-TIX forward, 5′-CAGCTGGGATCTGACTAACT-TACC-3′; mFuc-TIX reverse, 5′-CCACATGAATGAATGAATCA-GCTGG-3′; HPRT forward, 5′-TGACCTGCTGGATTACATTAA-AGCACT-3′, and HPRT reverse, 5′-ATAGTCAAGGGCATATC-CAACAACAAA-3′.

3. Results

3.1. Structures of extracted neutral glycolipids

Globoside and Forssman were shown to be the major neutral glycolipid components in the three P19 EC cell types: untreated, RA-induced aggregates and RA-induced neural cells. Le^x immunoreactive components were present in all but only in minute quantities. However, glycolipids bearing Le^x epitope were noticeably increased in both the aggregates and neural cells (Fig. 1B, lanes 2 and 3), when compared to untreated cells (Fig. 1B, lane 1). These Le^x epitope bearing glycolipids also had a slower TLC mobility than GA1, indicating that they contained oligosaccharide moieties larger than tetrasaccharide (Fig. 1A).

In order to increase the chance of determining oligosaccharide structures from limited glycolipid materials, oligosaccharides were first released by endoglycoceramidase from total neutral glycolipids extracted from RA-induced aggregates. The oligosaccharides released (40 μg Hex, as determined by orcinol staining [19]) were then fractionated by normal phase HPLC. Sixteen fractions were collected and oligosaccharides recovered in each fraction were converted into neoglycolipids (Fig. 2A). A portion of neoglycolipids (10%) were used for TLC-immunostaining with anti-Le^x MAb and Le^x immunoreactivity was found in fractions 7 (pentasaccharide region), 10 (heptasaccharide region) and 12 (octasaccharide region) (Fig. 2B,C). These Le^x immunoreactive neoglycolipids were analyzed by in situ TLC-LSIMS and their carbohydrate compositions, sequences and probable structures are summarized in Table 1. In fraction 7, two closely resolved neoglycolipid bands of equal intensity were found and gave LSI spectra having $[M-H]^-$ ions at *m/z* 1499 (upper band) and *m/z* 1515 (lower band). Fragmentation ions in the spectrum (Fig. 3) of the upper band at *m/z* 826, 988, 1337, 1353, together with a singlet ion at *m/z* 1157, diagnostic of a Fuc branch [25], clearly identified a pentasaccharide sequence with a non-reducing terminal Fuc branch. Since this band was Le^x immunoreactive, the structure was deduced to be Galβ1-4(Fucα1-3)GlcNAc-Hex-Hex-DHPE. The non-immunoreactive lower band contained no Fuc as indicated by $[M-H]^-$ at *m/z* 1515 and sequence ions at *m/z* 826, 988, 1191, and 1353, corresponding to Hex-Hex-HexNAc-Hex-Hex-DHPE. Similarly, fraction 10 gave two incompletely resolved neoglycolipid bands. The upper minor band, which was Le^x immunoreactive, gave an $[M-H]^-$ at *m/z* 1864 and partial sequence ions, from which the Galβ1-4(Fucα1-3)GlcNAc-Hex-HexNAc-Hex-Hex-DHPE sequence was deduced. The lower non-immunoreactive band contained no Fuc as indicated by a composition

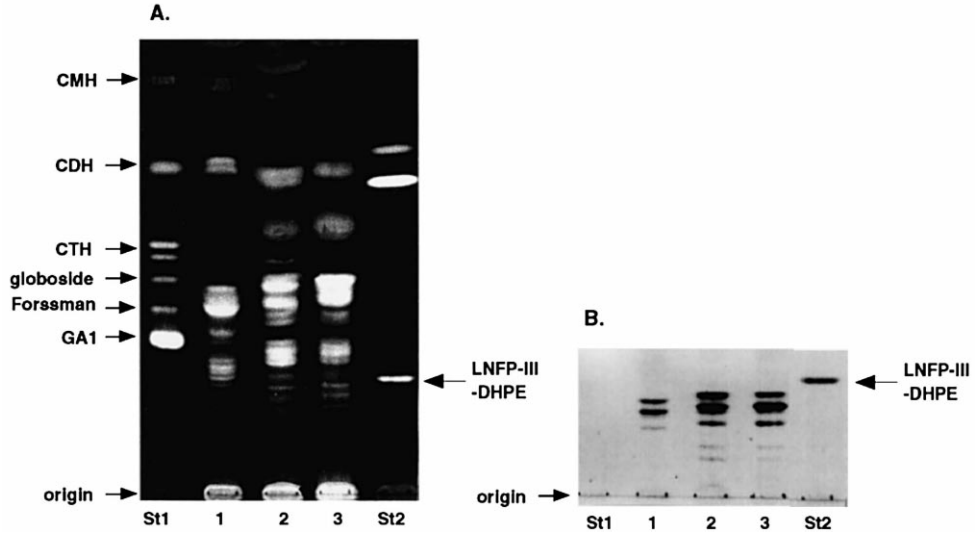

Fig. 1. TLC and immunostaining analyses of neutral glycolipids from P19 EC cells. TLC of neutral glycolipids extracted from undifferentiated and RA-treated differentiated P19 EC cells. The samples, 2 μg lipids, were developed on a HPTLC plate with solvent systems: (A) $CHCl_3$/MeOH (1/1, v/v), followed by (B) $CHCl_3$/MeOH/0.2% $CaCl_2 2H_2O$ (60/40/10, v/v/v). A: Primuline staining. B: Immunostaining analysis of the same TLC plate (A) with anti-Le^x MAb. Lanes: 1, undifferentiated cells; 2, RA-induced aggregates; 3, RA-induced neural cells; St1, glycolipid standards (arrow heads point to the relative positions of CMH, CDH, CTH, globoside, Forssman and GA1, respectively) and St2, neoglycolipid standard, LNFP-III-DHPE (the strong fluorescence band near the CDH position is DHPE reagent). 'Origin' points to the position of sample application.

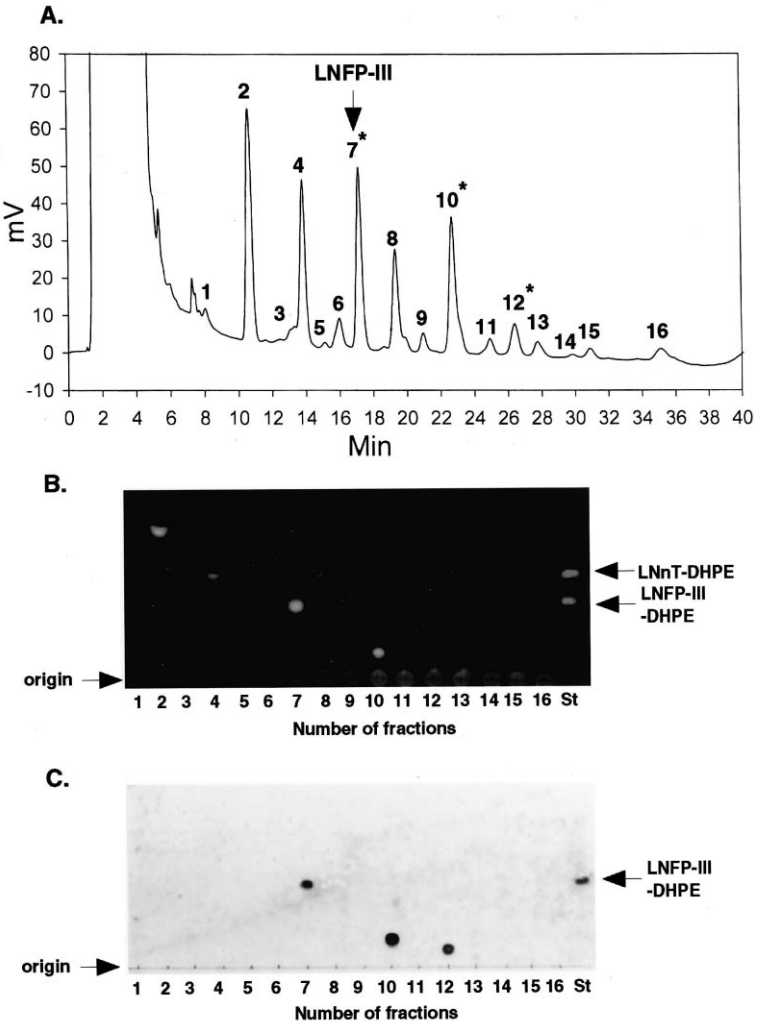

Fig. 2. HPLC, TLC and immunostaining analyses of oligosaccharides released from neutral glycolipids of RA-induced aggregates. A: Normal phase HPLC fractionation of oligosaccharides released by endoglycoceraminidase from total neutral glycolipids. Oligosaccharide fractions (numbers 1–16) were collected and converted to DHPE neoglycolipids (see B). Numbers with a star are fractions where positive Le^x immunoreactivity was found (see C). Arrow indicates elution position of the oligosaccharide standard, LNFP-III. B: Primuline staining; C: immunostaining analysis of the same TLC plate (B) with anti-Le^x MAb. Neoglycolipids (spots 1–16) prepared from the corresponding oligosaccharides fractions in A were first resolved by HPTLC with solvent $CHCl_3$/EtOH/H_2O (45/60/15, v/v/v) and then immunostained with anti-Le^x MAb; St, neoglycolipid standards (arrow heads point to the LNnT-DHPE and LNFP-III-DHPE). Arrow with 'origin' indicates the position of sample application.

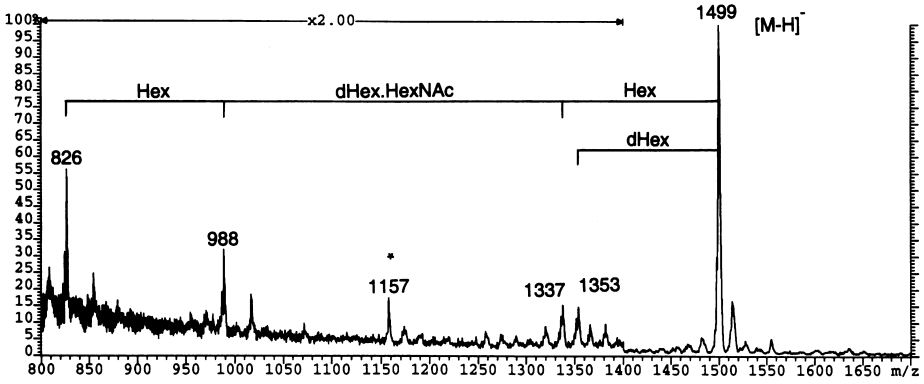

Fig. 3. LSI mass spectrum of the upper neoglycolipid band from neutral oligosaccharide HPLC fraction 7. The assignment of the oligosaccharide-DHPE structure was made from the deprotonated molecule *m/z* 1499 and the sequence ions *m/z* 1353, 1337, 988 and 826. The singlet ion *m/z* 1157 is diagnostic of a Fuc branch [25]. Full interpretation is in Section 3.1.

Table 1
Partial sequence of the major Le^x epitope bearing oligosaccharides released from P19 EC cell neutral glycolipids obtained by TLC-LSIMS of the derived neoglycolipids

HPLC fractions		$[M-H]^-$	Deduced structures
7	upper	1499	Galβ1-4(Fucα1-3)GlcNAc-Hex-Hex
10	upper	1864	Galβ1-4(Fucα1-3)GlcNAc-Hex-HexNAc-Hex-Hex
12	upper	2010	Galβ1-4(Fucα1-3)GlcNAc-(dHex.Hex_3.HexNAc)
	lower	2026	Galβ1-4(Fucα1-3)GlcNAc-(Hex_4.HexNAc)

Monosaccharide sequences established by fragment ions are indicated by a dash (-) and otherwise compositions are given as indicated by a period (.). $[M-H]^-$, deprotonated molecular ion; Galβ1-4(Fucα1-3)GlcNAc-, Le^x epitope; Hex, hexose; dHex, deoxyhexose; HexNAc, *N*-acetylhexosamine.

of (Hex_2.HexNAc)-Hex-HexNAc-Hex-Hex-DHPE deduced from a $[M-H]^-$ at *m/z* 1880 and partial sequence ions. In fraction 12, two unresolved neoglycolipid bands were present but at too low intensity to obtain sequence ions. However, the $[M-H]^-$ at *m/z* 2010 (upper part) and $[M-H]^-$ at 2026 (lower part) were consistent with the respective compositions of (dHex.Hex_4.$HexNAc_2$)-DHPE and (dHex.Hex_5.$HexNAc_2$)-DHPE. Since both bands were immunoreactive to anti-Le^x MAb, the oligosaccharide structures were likely to be Galβ1-4(Fucα1-3)GlcNAc-(dHex.Hex_3.HexNAc)-DPHE and Galβ1-4(Fucα1-3)GlcNAc-(Hex_4.HexNAc)-DHPE, respectively.

The lipid chains of the Le^x epitope bearing glycolipids were also analyzed by TLC-LSIMS. The majority of the Cer moiety (with a mass of 564) was deduced to contain a total of 34 carbon atoms, one hydroxyl group and one double bond. Because of limited materials available the detailed structures of the Cer chains could not be identified.

3.2. Fuc-T activities

Glycolipid, glycoprotein and oligosaccharide Fuc-T activities were all increased in both of the two RA-induced cell types with the highest activity found in the differentiated neural cells (Fig. 4A–C, lane 3). The oligosaccharide Fuc-T activity in RA-induced neural cells showed a 30-fold higher activity than the undifferentiated cells (Fig. 4C, lanes 1 and 3, respectively).

3.3. Expressions of mouse Fuc-TIV and Fuc-TIX genes by RT-PCR analysis

The mFuc-TIV transcript, 511 bp (Fig. 5A, was only expressed in RA-induced aggregates and was not detected in undifferentiated cells nor in RA-induced neural cells. On the other hand, the mFuc-TIX transcript, 530 bp (Fig. 5B), was detectable in all three cell types. It was weakly expressed in undifferentiated cells but visibly much stronger in RA-induced aggregates and stronger still in RA-induced neural cells. The control HRPT transcript (357 bp) was expressed at similar levels in all cell types (Fig. 5C). From these results, we concluded that RA induces only a transient expression of the mFuc-TIV gene in cell aggregates but a more persistent expression of the mFuc-TIX gene at the transcription level throughout neural cell differentiation.

4. Discussion

The biological relevance for the presence of these Le^x bearing glycans is interesting. Boubelik et al. [27] indicated that surface carbohydrates were involved in cadherin-mediated cell sorting in the absence of mixed aggregates formed between parental and Le^x- or embryoglycan-negative mutant P19 cells. In our present study, Le^x epitope bearing glycolipids were markedly increased with concomitant elevation of Fuc-T activities during RA-induced neural differentiation of P19 EC cells. Our findings on Fuc-T gene transcripts also support that

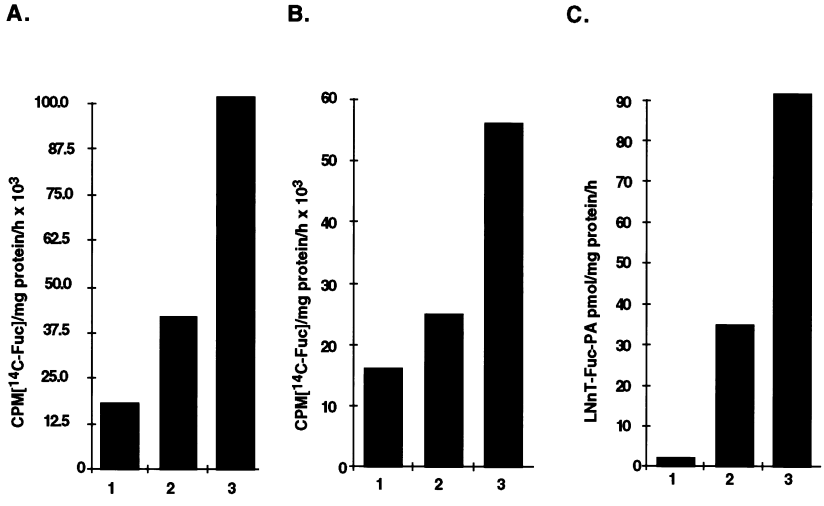

Fig. 4. Fuc-T activities in P19 EC cells before and after treatment with RA. A: Glycolipid Fuc-T activities; B: glycoprotein Fuc-T activities and C: PA-oligosaccharide Fuc-T activities. Lanes 1, undifferentiated cells; lanes 2, RA-induced aggregates and lanes 3, RA-induced neural cells. Fuc-T activities were measured as described in Section 2 and were averages of two experiments.

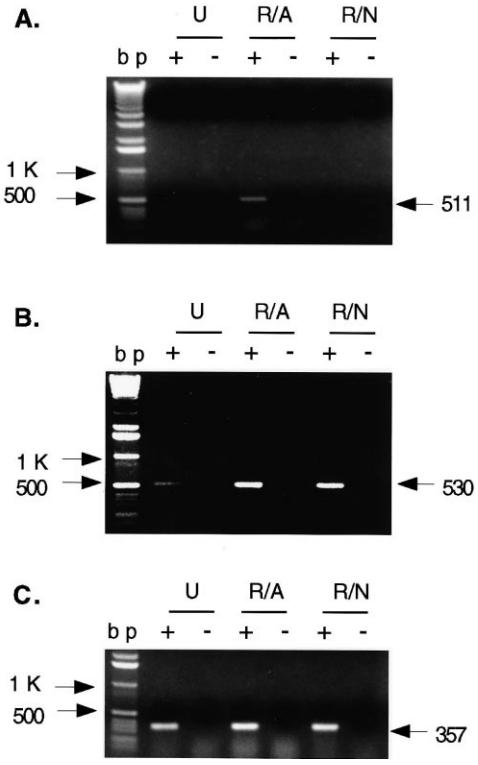

Fig. 5. Expression of mFuc-TIV, mFuc-TIX, and HRPT in P19 EC cells by the RT-PCR analysis. Total RNAs were isolated from undifferentiated cells (U), RA-induced aggregates (R/A), and RA-induced neural cells (R/N). Reverse transcription and amplification were performed in the presence of RT (reverse transcriptase) as described in Section 2. A: mFuc-TIV; B: mFuc-TIX and C: HRPT which was used to normalize for variations in the amount and quality of RNA loaded in each lane. Aliquots of the reactions were electrophoresed in 1% agarose gel and stained with ethidium bromide. bp, base pair molecular weight markers; + or − indicates with or without RT, respectively.

the mFuc-TIX gene and not mFuc-TIV was involved during this type of neural differentiation. Using the neoglycolipid technology [19] and mass spectrometry, we were able to isolate and identify at least four Le^x epitope bearing oligosaccharide chains from the very limited amounts of neutral glycolipids extracted from these cells. The shortest structure was a pentasaccharide with a partial sequence of Galβ1-4(Fucα1-3)GlcNAc-Hex-Hex-. A heptasaccharide, Galβ1-4(Fucα1-3)-GlcNAc-Hex-HexNAc-Hex-Hex-, and two octasaccharides, Galβ1-4(Fucα1-3)GlcNAc-dHex.Hex_3.HexNAc- and Galβ1-4(Fucα1-3)GlcNAc-(Hex_4.HexNAc)-, each containing either one or two Fuc residues, had also been deduced and partially sequenced. Recently, Nishihara et al. [28] reported that human Fuc-TIII, -TIV, -TV, and -TVI had very similar substrate specificity and preferred the transfer of Fuc to the internal GlcNAc residue of *N*-acetyl polylactosamine chain but Fuc-TIX preferred the peripheral GlcNAc residue. More experiments are therefore required to study particularly the acceptor specificity for these Fuc-Ts using hexa-, octa-, or even longer *N*-acetyl polylactosamine chains to confer the transfer of Fuc to internal GlcNAc residue of glycolipids and glycoproteins.

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