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Expression and localization of Lewis^x glycolipids and GD1a ganglioside in human glioma cells

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We analysed the glycolipid composition of glioma cells (N-370 FG cells), which are derived from a culture of transformed human fetal glial cells. The neutral and acidic glycolipid fractions were isolated by column chromatography on DEAE-Sephadex and analysed by high-performance thin-layer chromatography (HPTLC). The neutral glycolipid fraction contained 1.6 μg of lipid-bound glucose/galactose per mg protein and consisted of GlcCer (11.4% of total neutral glycolipids), GalCer (21.5%), LacCer (21.4%), Gb4 (21.1%), and three unknown neutral glycolipids (23%). These unknown glycolipids were characterized as Lewis^x (fucosylneolactonorpentaosyl ceramide; Le^x), difucosylneolactonorhexaosyl ceramide (dimeric Le^x), and neolactonorhexaosyl ceramide (nLc6) by an HPTLC-overlay method for glycolipids using specific mouse anti-glycolipid antibodies against glycolipid and/or liquid-secondary ion (LSI) mass spectrometry. The ganglioside fraction contained 0.6 μg of lipid-bound sialic acid per mg protein with GD1a as the predominant ganglioside species (83% of the total gangliosides) and GM3, GM2, and GM1 as minor components. Trace amounts of sialyl-Le^x and the complex type of sialyl-Le^x derivatives were also present. Immunocytochemical studies revealed that GD1a and GalCer were primarily localized on the surface of cell bodies. Interestingly, Le^x glycolipids and sialyl-Le^x were localized not only on the cell bodies but also on short cell processes. Especially, sialyl-Le^x glycolipid was located on the tip of fine cellular processes. The unique localization of the Le^x glycolipids suggests that they may be involved in cellular differentiation and initiation of cellular growth in this cell line.

Keywords: human glioma cells, N-370 FG cells, gangliosides, neutral glycolipids, and Lewis^x glycolipids.

Introduction

Glycosphingolipids are important constituents of the plasma membrane and constitute part of the glycocalyx network on the cell surface [1]. Gangliosides, sialic acid containing glycolipids are particularly abundant in the vertebrate nervous tissues [2]. They have been implicated in a variety of phenomena including cell-cell recognition, neurite outgrowth, synaptogenesis, transmembrane signalling, cellular growth and differentiation, and oncogenic transformation [1–4]. During the past decade considerable evidence has been accumulated showing altered glycolipid content and pattern in tumour tissues and transformed cells in culture [1, 5]. These alterations have been correlated with changes in cellular properties such as decreased contact inhibition and accelerated growth due to expression of tumour-specific glycolipid antigens [1, 6]. These changes, which may play an important role in tumour metastasis and/or cellular differentiation, are frequently accompanied by alterations in glycosyltransferase activities that are involved in the syntheses of these tumour-specific antigens [3, 7]. The N-370 FG cells are hybrid tumour cell lines derived from human fetal glioma cells. They exhibit many properties that are characteristic

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of oligodendroglial cells. To define the relationship between glycolipid expression and cellular properties, we undertook a detailed investigation to analyse the neutral glycolipid and ganglioside compositions of N-370 FG cells. We demonstrated that N-370 FG cells expressed a unique glycolipid composition, including the presence of galactosylceramide, a myelin and oligodendroglial specific glycolipid. Among the various glycolipids, the most unusual feature was the presence of a large amount of GD1a ganglioside as well as glycolipids bearing Lewis^x determinant such as III^{3}FucnLc$_4$ (Le^x), V^{3}FucIII^{3}FucnLc$_6$ (dimeric Le^x), and IV^{3}NeuAc,III^{3}FucnLc$_4$ (sialyl-Le^x), which are known to be tumour-associated antigens and are also expressed in earlier stages of embryogenesis [8–12]. These Le^x glycolipids were found to be located not only on the cell bodies but also on short cellular processes, suggesting that they may play a role in controlling cellular recognition during contact formation and development in this cell line.

Materials and methods

Materials

Neutral glycolipids used as references were isolated from erythrocyte membranes and human brains in our laboratories. Le^x, dimeric Le^x, and sialyl-Le^x as references were purified from human lenses as previously described [13]. GM3 and GD3 were purified from bovine adrenal medulla [14] and bovine butter milk [15], respectively. The monoclonal antibodies against Le^x and sialyl-Le^x (SH-3) were generous gifts of Dr A. Suzuki, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan and Dr R. Kannagi, Aichi Cancer Center, Okazaki, Aichi, Japan, respectively. All reagents used were of analytical grade or higher. Solvents were freshly redistilled before use.

Cell culture

The N-370 FG glioma cells were placed on Falcon dishes (60 mm, Falcon, Franklin Lake, NJ) and grown in Dulbecco's Modified Eagle Medium (GIBCO, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD), and antibiotics (GIBCO). They were kept in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C.

Isolation of total neutral glycolipids and gangliosides

The isolation procedures for the total lipids and neutral glycolipids were described previously [13]. Briefly, total lipids were extracted from the N-370 FG cells with chloroform:methanol (2:1, 1:1, v/v) and chloroform:methanol:water (30:60:8, v/v; Solvent A), successively. The combined extracts were then applied to a DEAE-Sephadex A-25 column (acetate form, 3 ml bed volume; Pharmacia, Uppsala, Sweden), and further eluted with 15 ml of Solvent A. Neutral glycolipids were eluted in this fraction. The neutral glycolipid fraction was evaporated and redissolved in 0.5 ml of 0.3 M NaOH in methanol and then incubated at 40 °C for 2 h in order to destroy alkali-labile phospholipids. The reaction mixture was passed through a Sephadex LH-20 with Solvent A for desalting. This glycolipid fraction was then evaporated to dryness and redissolved in a small volume of chloroform:methanol (1:1, v/v), and an aliquot of this glycolipid fraction was examined by high-performance thin-layer chromatography (HPTLC) with two different solvent systems: (A) chloroform:methanol:water (60:40:5, v/v), and (B) chloroform:methanol:2.5 M ammonium hydroxide (60:40:10, v/v). Neutral glycolipids were visualized by spraying with the orcinol-sulfuric acid reagent and heating at 120 °C.

After recovery of the neutral glycolipid fraction, the acidic glycolipids were eluted from the DEAE-Sephadex A-25 column as described above with chloroform:methanol:0.8 M sodium acetate (30:60:8, v/v; Solvent B). The acidic lipid fraction was evaporated to dryness, and the sample was desalted by a Sephadex LH-20 column as described above. An aliquot of this acidic glycolipid fraction was examined by HPTLC with two different solvent systems: (C) chloroform:methanol:0.2% $CaCl_2$·2-H_2O (50:45:10, v/v), and (D) chloroform:methanol:0.4 M ammonium hydroxide:0.4% $CaCl_2$·2H_2O (50:40:5:4, v/v). Gangliosides were visualized by spraying with the resorcinol-hydrochloric acid reagent followed by heating at 95 °C.

Quantitation of neutral glycolipids and gangliosides was carried out by gas-liquid chromatography (GLC) of the trimethylsilyl derivatives of the sugars with myoinositol as an internal standard according to the procedure of Vance and Sweeley [16].

Isolation of major neutral glycolipids and gangliosides

In order to characterize the major neutral glycolipids and gangliosides, lipids were extracted from large batches of N-370 FG cells (wet weight: 15 g) with 20 volumes each of chloroform:methanol (2:1, 1:1, v/v) and Solvent A. The lipid extracts were evaporated to dryness, dissolved in 100 ml of Solvent A, and then applied to a DEAE-Sephadex A-25 column (100 ml bed volume, 2 × 30 cm). The neutral glycolipid fraction was eluted with 300 ml of Solvent A and 100 ml methanol. The neutral glycolipid fraction was evaporated to dryness and the sample was dissolved in 10 ml of 0.3 M NaOH in methanol and then incubated at 40 °C for 2 h. After neutralization with 4 M acetic acid, the sample was evaporated and dialysed against distilled water for 2 days. The retentate was evaporated to dryness under vacuum. The residue was dissolved in 5 ml of chloroform:methanol (7:3, v/v) and applied to an Iatrobeads column (70 ml bed volume, 0.7 × 140 cm). The neutral glycolipids were fractionated and purified by linear gradient elution with 500 ml each of

chloroform:methanol:2.5 M ammonium hydroxide (70:30:1 and 40:60:5, v/v). Final purification of each neutral glycolipid was achieved by preparative HPTLC using two different solvent systems (A and B).

After removal of the neutral glycolipids, the acidic lipid fraction containing gangliosides was recovered from the DEAE-Sephadex A-25 column described above with 400 ml of Solvent B. The acidic lipid fraction was evaporated and the residue was suspended into 20 ml of distilled water and then dialysed against water for 2 days. The retentate was evaporated to dryness under vacuum. The residue was dissolved in 5 ml of methanol and applied to another DEAE-Sephadex A-25 column (20 ml bed volume, 1.5 × 20 cm). Mono-, di-, and polysialogangliosides were recovered with stepwise elution of 100 ml each of 20 mM, 60 mM, and 100 mM ammonium acetate in methanol, respectively. After desalting using a Sephadex LH-20 column as described above, each ganglioside was purified by preparative HPTLC using two different solvent systems (C and D).

Compositional analyses

Compositional analyses for carbohydrates, fatty acids, and long-chain bases (LCB) were carried out by GLC as described previously [13].

High-performance thin-layer chromatography (HPTLC)-overlay method

HPTLC-overlay for glycolipids using specific anti-glycolipid antibodies was carried out as described previously [17, 18]. The neutral glycolipids and gangliosides isolated from the N-370 FG cells were developed on HPTLC plates. After the plates were dipped in a 0.2% polyisobutylmethacrylate solution in n-hexane, the bands were overlaid with an appropriate anti-glycolipid polyclonal or monoclonal antibody at a dilution of 1:50 in 1% bovine serum albumin (BSA)/phosphated buffer saline (PBS, pH 7.3) for 2 h at room temperature. After washing the plates thoroughly with PBS, the bands were covered with peroxidase-conjugated anti-rabbit IgG or anti-mouse IgM (Cappel, Cooper Biomedical, Malvern, PA) at a dilution of 1:500 in 1% BSA/PBS and incubated for 2 h at room temperature. After washing with PBS, the plates were then dipped in a solution of 4-chloro-1-naphthol (15 mg per 25 ml PBS containing 0.02% hydrogen peroxide) for a few minutes.

Liquid-secondary ion (LSI) mass spectrometry

LSI mass spectra were recorded in the negative ion mode on a TSQ 70 triple-stage quadruple mass spectrometer (Finnigan MAT, Inc., San Jose, CA), equipped with a cesium ion gun (Finnigan MAT). The accelerating voltage of the primary beam was maintained at 20 kV. Approximately 1 μg of sample, dissolved in 0.4 μl of chloroform:methanol (1:1, v/v), was loaded onto the LSI mass spectrometry target with 0.5 μl of triethanolamine as the matrix [19].

Preparation of polyclonal and monoclonal antibodies against glycolipids

Rabbit polyclonal antibodies against Gb4, and nLc4 were prepared by immunization of New Zealand white rabbits in complete Freund adjuvant with glycolipids as follows. A glycolipid sample, 500 μg, was mixed with 0.5 ml of complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis* (10 mg ml^{-1}). An equal volume of keyhole limpet haemocyanin (2 mg ml^{-1}) in PBS was added, and the mixture was then emulsified as described previously [20]. The emulsion (1 ml) was injected into an adult female rabbit intradermally. Booster injections were performed 2 weeks later in the same way but without *M. tuberculosis*. The animal then received a booster injection 2 weeks later. One month later, the animal was bled and sera were prepared. Mouse monoclonal antibodies against GalCer, GD1a, and GM3 were prepared as follows [21]: Female mice of C3H/HeN inbred strain were immunized with the purified glycolipid, GalCer, GD1a, or GM3 (100 μg, total amount per mouse) adsorbed to acid-treated *S. minnesota* (250 μg, total amount per mouse). Each mouse received the intravenous injections of antigen-bacteria complex on days 0, 4, 7, 11, and 21. Sera were obtained 3 days after the last injection. Titres of the sera were determined by an enzyme-linked immunosorbent assay (ELISA). The hybridoma cells were screened against the glycolipid used for immunization. Antibody titres in hybridoma supernatant were determined by ELISA. Positive hybridoma cells were cloned by limiting dilution.

Immunocytochemistry of GalCer, GD1a and Le^x glycolipids in N-370 FG cells

Cultured N-370 FG cells on 35-mm dishes were fixed with 4% paraformaldehyde for 15 min and washed with PBS for at least three times. After incubation in PBS with 3% non-immunized mouse serum, a mouse monoclonal anti-antibody (GalCer, GD1a, Le^x, or sialyl-Le^x), diluted 1:100 in PBS, was applied at 4 °C for 72 h. The dishes were washed three times and FITC-conjugated anti-mouse immunoglobulin (Cappel, 1:200 dilution) was applied for 1 h at room temperature to visualize the labelled sites. The fluorescent secondary antibody site was detected by a Nikon microscope, model UFX-IIA (Nikon Inc., New York, NY) [22].

Results

I. NEUTRAL GLYCOLIPIDS

Figure 1 shows the neutral glycolipid pattern of the N-370 FG cells. The cells were found to contain GlcCer (11.4 ± 0.7% SEM, of total neutral glycolipids; $n = 4$), GalCer (21.5 ± 4.3%), LacCer (21.4 ± 2.9%), Gb4

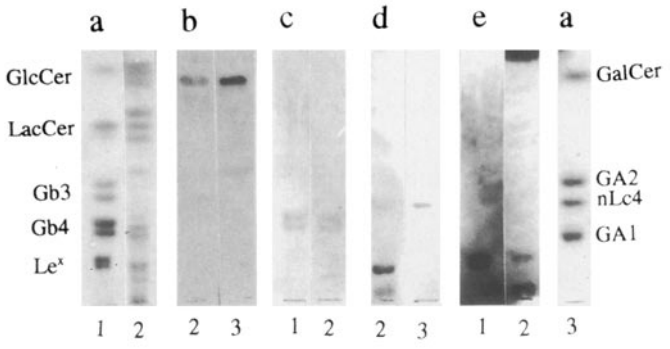

Figure 1. High-performance thin-layer chromatogram (HPT-LC) of neutral glycolipids in N-370 FG cells. Lane 1, neutral glycolipid standards (from top to bottom): GlcCer (from Gaucher's disease), LacCer, Gb3, and Gb4 (from pig erythrocyte membrane), and Le^x (from human cataractous lens [13]); 2, total neutral glycolipids of N-370 FG cells; 3, neutral glycolipid standards: GalCer (from human brain), GA2 and GA1 (prepared by the method of Kasai *et al.* [45] from human brain GM2 and GM1, respectively), and nLc4 (from guinea pig erythrocyte membrane). The plates were developed with the solvent system A. The bands of plate a were visualized with the orcinol-sulfuric acid reagent. The bands on plates b–e were immunostained with anti-GalCer, -Gb4, -nLc4, and -Le^x, respectively. In plate d, lane 2, the two slow migrating bands which reacted strongly with the anti-nLc4 antibody may represent nLc6 and nLc8.

(21.1 ± 2.4%), Le^x (14.2 ± 4.0%) as the major neutral glycolipids, and Gb3 (1.2 ± 0.9%), nLc6 (2.3 ± 1.2%), and dimeric Le^x (3.1 ± 1.0) as the minor species. The amount of neutral glycolipids in the 370 FG cells was estimated to be 1.58 ± 0.88 μg (SEM) lipid-bound glucose/galactose per mg protein. Figure 1 also shows the HPTLC immunostaining pattern using various antibodies against glycolipids. The results confirmed the presence of GalCer, Gb4, and Le^x (plates b, c, and e, respectively). Interestingly, the antibody against neolactonortetraosyl ceramide (nLc4) reacted more strongly with two slow migrating glycolipids near the origin of TLC than with nLc4 (plate d, lane 2). This observation suggests that the two slower migrating bands might correspond to neolactonorhexaosyl ceramide (nLc6) and neolactonoroctaosyl ceramide (nLc8) which, by virtue of their longer oligosaccharide chains, are more exposed to the antibody on the surface of the TLC plate following the treatment with the organic polymer. Since the amount of these glycolipids was low, this assignment can only be regarded as tentative. The monoclonal antibody against Le^x reacted with another band migrated near the origin (plate e, lane 2), whose structure is also unknown.

Neutral glycolipids in the N-370 FG cells were further isolated and characterized. Figure 2a and 2b show the elution profiles of the neutral glycolipids extracted from

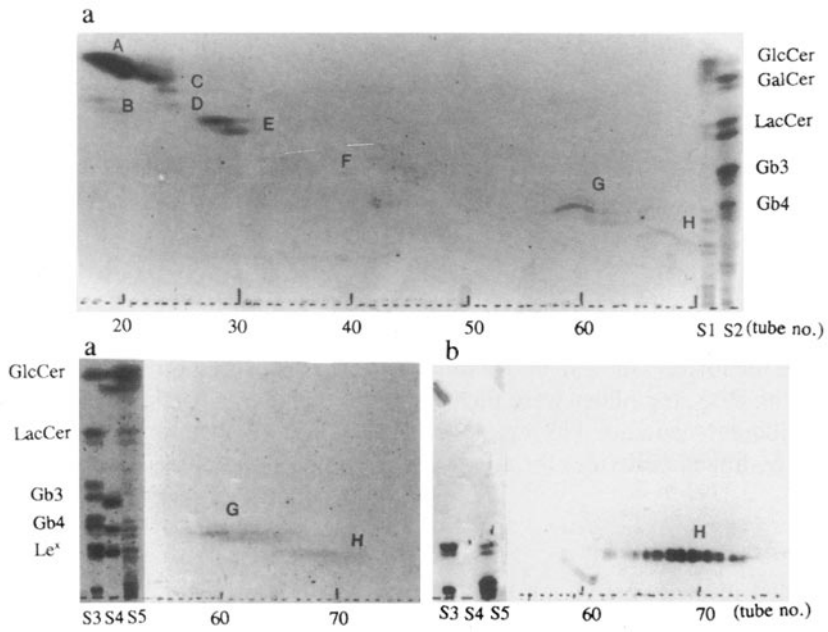

Figure 2. The elution profile of the neutral glycolipid fraction of N-370 FG cells from Iatrobeads column chromatography. Plates a and b were developed with the solvent system B. Lanes S1 and S5, total neutral glycolipids from N-370 FG cells; S2, neutral glycolipid standards of GlcCer, GalCer, LacCer, Gb3, and Gb4; S3, neutral glycolipid standards of GlcCer, LacCer, Gb3, Gb4, and Le^x; S4, neutral glycolipid standards of GalCer, GA2, nLc4, and GA1. The bands on plates a were stained with the orcinol-sulfuric acid reagent. The bands on plates b were immunostained with monoclonal antibody against Le^x.

the cells from Iatrobeads column chromatography using the basic solvent system B. Eight neutral glycolipid fractions (named as A, B, C, D, E, F, G, and H) were eluted. Fractions corresponding to each glycolipid were pooled and subjected to preparative HPTLC using two different solvent systems, A and B. Neutral glycolipids A and B, and C and D were tentatively identified as GlcCer and GalCer containing normal fatty acids, and GlcCer and GalCer containing α-hydroxy fatty acids, respectively. Neutral glycolipids E, F, and G were tentatively identified as LacCer, Gb3, and Gb4, respectively, based on comparison of their migratory rates with those of authentic neutral glycolipids. Only neutral glycolipid H reacted strongly with the monoclonal antibody against Le^x (Fig. 2b). Fractions over number 80 were pooled and band I, which appeared near the origin of TLC and reacted with anti-Le^x antibody, was further purified by preparative HPTLC using the developing solvent system C (Fig. 3, lane 4).

Compositional analysis

Neutral glycolipids A and B contained only glucose (Glc), and C and D only galactose (Gal); E contained Glc, and Gal in the molar ratio of 1.00:1.12; G contained Glc, Gal, and *N*-acetyl galactosamine (GalNAc) in the molar ratios of 1.00:1.96:0.81; H and I contained Glc, Gal, *N*-acetyl glucosamine (GlcNAc), and fucose (Fuc) in the molar ratio of 1.00:2.98:0.76:0.71, and 1.00:3.78:1.60:0.67, respectively. The amount of glycolipid F was too low for carbohydrate analysis. The major long-chain base for all these glycolipids was C18 sphingosine (> 92% of the total LCB). These glycolipids contain mainly C16:0, C20:0, C22:0, C22:1, C24:0 and C24:1 as well as smaller amounts of α-hydroxy fatty acids (5–10% of the total fatty acids).

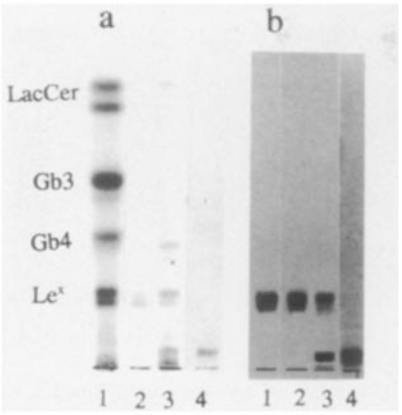

Figure 3. HPTLC-overlay of Le^x glycolipids in N-370 FG cells. Lane 1, neutral glycolipid standards: GlcCer, LacCer, Gb3, Gb4, and Le^x; 2, isolated Le^x from N-370 FG cells; 3, total neutral glycolipids from N-370 FG cells; 4, isolated dimeric Le^x. The bands of plate a were stained with the orcinol-sulfuric acid reagent. The bands of plate b were immunostained with the mouse monoclonal antibody against Le^x. The plates were developed with the solvent system A.

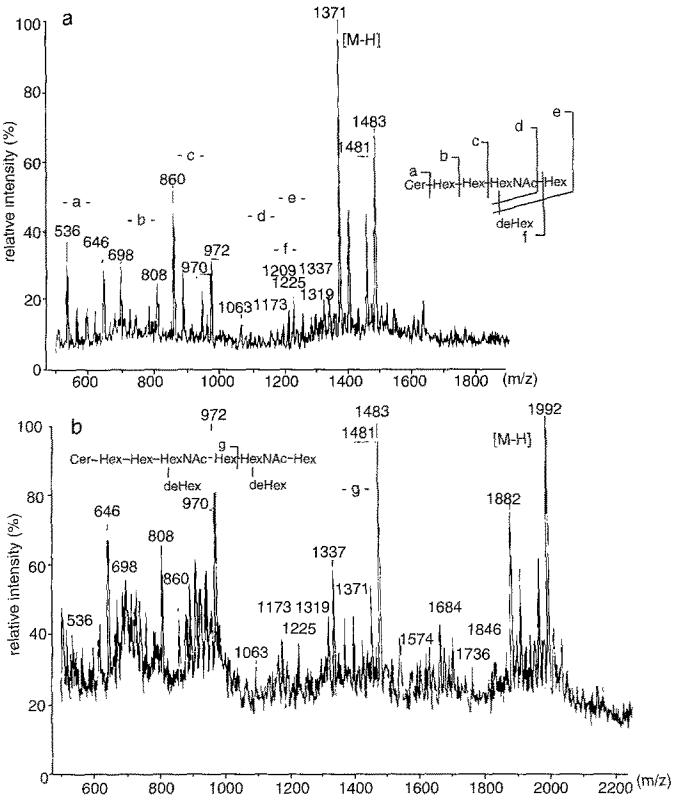

Figure 4. LSI mass spectra of Le^x glycolipids from N-370 FG cells. a, glycolipid H; b, glycolipid I.

Liquid-secondary ion (LSI) mass spectrometry

The negative-ion LSI mass spectra of neutral glycolipids revealed prominent dehydrogenated molecular ions *m/z* $[M–H]^-$ and fragment ions having C18-sphingosine and various fatty acids with chain lengths ranging from C16:0 to C24:0 and C24:1, and C16h:0 to C24h:0 and C24h:1, with α-hydroxy fatty acids accounting for 5 to 10% of the total fatty acids. For example, the two prominent dehydrogenated molecular ions in glycolipids A and B, and C and D were detected at *m/z* 754 and 810, and *m/z* 772 and 828, corresponding to Ceramide(Cer)-hexose(-Hex) having C20:0 and C24:0, and C20h:0 and C24h:0, respectively. In the case of glycolipids E and F, the prominent dehydrogenated molecular ions appeared at *m/z* 860 and 972, and *m/z* 1022 and 1134, corresponding to Cer-Hex-Hex, and Cer-Hex-Hex-Hex having normal fatty acids C16:0 and C24:0, respectively. The mass spectrum of glycolipid G demonstrated the presence of four sugars as indicated by the presence of five major ion groups corresponding to the molecular species of Cer [*m/z* 536 and 646], Cer-Hex [*m/z* 698 and 808], Cer-Hex-Hex [*m/z*

860 and 970], Cer-Hex-Hex-Hex [*m/z* 1022 and 1132], and Cer-Hex-Hex-Hex-HexNAc [*m/z* 1225 and 1335]. The prominent dehydrogenated molecular ions $[M–H]^-$ at *m/z* 1225 and 1335 corresponded to glycolipid molecular species containing C18 sphingosine, and C16:0 and C24:1 fatty acids, respectively (data not shown). Figure 4 shows the LSI mass spectra of glycolipids H and I. The mass spectrum of glycolipid H revealed the presence of five sugars as indicated by the presence of seven major ions corresponding to the molecular species of Cer [*m/z* 536 and 646], Cer-Hex [*m/z* 698 and 808], Cer-Hex-Hex [*m/z* 860 and 970], Cer-Hex-Hex-HexNAc [*m/z* 1063 and 1173], Cer-Hex-Hex-HexNAc-Hex [*m/z* 1225 and 1335], Cer-Hex-Hex-HexNAc-deoxyhexose(deHex) [*m/z* 1209 and 1319] and Cer-Hex-Hex-HexNAc(-deHex)-Hex [*m/z* 1371 and 1481]. The prominent dehydrogenated molecular ions $[M–H]^-$ at *m/z* 1371 and 1481 corresponded to glycolipid molecular species containing C18 sphingosine, and C16:0 and C24:1 fatty acids, respectively (Fig. 4a). Mass spectra of glycolipid I revealed two prominent dehydrogenated molecular ions at *m/z* 1882 and 1992, which corresponded to Cer-Hex-Hex-HexNAc(-dexHex)-Hex-HexNAc(-deHex)-Hex. The fragment ion group g ranging from *m/z* 1371 to 1481 should represent the molecular species of Le^x (Fig. 4b). The combined data suggest that these glycolipids in N-370 FG cells have the following structures: A and B, GlcCer; C and D, GalCer; E, LacCer; F, Gb3; G, Gb4; H, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer (Le^x, $III^3FucnLc_4$)nLc_4; and I, difucosylneolactonorhexaosyl ceramide, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer ($V^3FucIII^3FucnLc_6$).

II. GANGLIOSIDES

The content of gangliosides in N-370 FG cells was 0.62 ± 0.05 μg lipid-bound sialic acid per mg protein. NeuAc was the only sialic acid species found and no *N*-glycolyl neuraminic (NeuGc) could be detected based on mass spectrometric analysis. The cells contained an unusually large proportion of GD1a (named as N, $82.7 \pm 4.4\%$ of total gangliosides). Other gangliosides included GM3 (named as J, $5.4 \pm 0.9\%$), GM2 (named as K, $6.1 \pm 0.7\%$), and GM1 (named as L, $2.0 \pm 0.5\%$). All of these ganglioside species co-migrated with authentic standards as shown in Fig. 5. In Fig. 5c, the major band reacted with a monoclonal antibody against GD1a, which confirmed its assignment.

Further structural analysis of each ganglioside, isolated and purified from the mono- or di-sialoganglioside fraction by preparative HPTLC using the solvent system C, was performed using the HPTLC-overlay method as well as LSI mass spectrometry. Figure 6 shows the results of HPTLC-overlay pattern of the monosialoganglioside fraction. Ganglioside M appeared as two bands with an intense band (Fig. 6, lanes 3 and 4), which migrated slightly lower than GM1, reacting strongly with a mouse monoclonal antibody against sialyl-Le^x (SH-3). The bands migrated slightly lower than GM1 are tentatively assigned as sialyl-Le^x. In addition, several complex gangliosides in the monosialoganglioside fraction reacted with this antibody (Fig. 6b, lane 3). Further structural analysis by LSI mass spectrometry of the monosialogangliosides, GM3, GM2, GM1, the unknown ganglioside, and GD1a are described below.

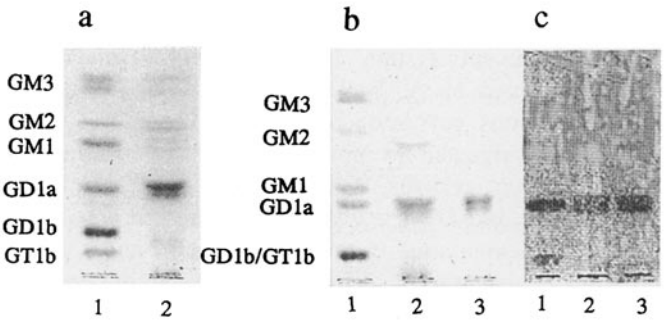

Figure 5. HPTLC-overlay of gangliosides in N-370 FG cells using monoclonal antibody against GD1a. Lane 1, authentic standards: GM3, GM2, GM1, GD1a, GD1b, and GT1b; 2, gangliosides from N-370 FG cells; 3, isolated GD1a from N-370 FG cells. The bands of plates a and b were stained with the resorcinol-hydrochloric acid reagent. The bands of plate c were immunostained with the monoclonal antibody against GD1a. Plate a was developed with the solvent system C and b and c with the solvent system D.

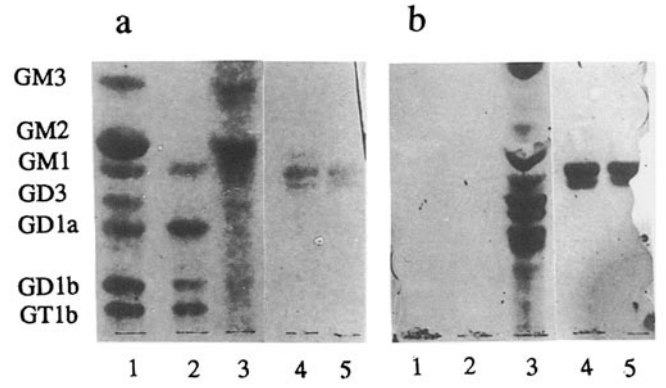

Figure 6. HPTLC-overlay of monosialogangliosides in N-370 FG cells using monoclonal antibody against sialyl-Le^x. Lane 1, authentic standard: GM3, GM2, GM1, GD3, GD1a, GD1b, and GT1b; 2, gangliosides from bovine brain grey matter; 3, total monosialoganglioside fraction from N-370 FG cells; 4, isolated ganglioside M from N-370 FG cells; 5, authentic standard of sialyl-Le^x (from human lens). The bands of plate a were stained with the resorcinol-hydrochloric acid reagent. The bands of plate b were immunostained with the mouse monoclonal antibody against sialyl-Le^x. The plates were developed with the solvent system B.

Compositional analysis

The monosialoganglioside J contained Glc, Gal, and *N*-acetylneuraminic acid (NeuAc) in a molar ratio of 1.00:1.21:0.89, and the gangliosides K and L contained Glc, Gal, GalNAc, and NeuAc in the molar ratio of 1.00:1.12:0.93:0.91 and 1.00:2.17:0.87:0.89, respectively. The disialoganglioside N contained Glc, Gal, GalNAc, and NeuAc in a molar ratio of 1.00:2.04:0.87:2.09. The major fatty acids were found to be C16:0, C22:1, C24:0, and C24:1, and C18 sphingosine was the predominant LCB species.

Liquid-secondary ion (LSI) mass spectrometry

The negative-ion LSI mass spectra of gangliosides revealed the prominent dehydrogenated molecular ions *m/z* $[M–H]^-$ and fragment ions having C18 sphingosine and various fatty acids with chain lengths ranging from C16:0 to C24:0. Figure 7 shows the molecular ions of monosialogangliosides J, K, L, and an unknown ganglioside M by LSI mass spectrometry. In the case of J, the quasi-molecular ions at *m/z* 1151, 1235 and 1261 corresponded to GM3 having C16:0, C22:1, and C24:1 fatty acids, and C18 sphingosine (Fig. 7a). In the cases of K and L, these ions were shifted by *m/z* 203 (HexNAc) and *m/z* 365 (Hex plus HexNAc), respectively (Fig. 7b and c), suggesting that they possess the structures of GM2 and GM1, respectively. The mass spectra of ganglioside M revealed the presence of dehydrogenated molecular ions *m/z* 1663 and 1773, which corresponded to Cer-Hex-Hex-HexNAc(-dexHex)-Hex-NeuAc. Thus, ganglioside M appeared to be a mixture containing two components, one of which was GM1 having C16:0 fatty acid and C18 sphingosine based on the detection of the peak at *m/z* 1516 (see also Fig. 6a, lane 4). Figure 8 shows the mass spectra of the disialoganglioside N. In the case of N, the two prominent quasi-molecular ions at $[M–H]^-$ and $[M + Na–2H]^-$ were detected at *m/z* 1807 and 1919, and 1829 and 1941, respectively, which corresponded to GD1a having C16:0 and C24:1 fatty acids and C18 sphingosine. The ion groups ranging from *m/z* 1516 to 1628 corresponded to a substructure of GM1 in this ganglioside. Other fragment ion groups indicating Cer (a), Cer-Hex (b), Cer-Hex-Hex (c), Cer-Hex-Hex-HexNAc (d), Cer-Hex-Hex(-NeuAc) (e) and Cer-Hex-Hex(-NeuAc)-HexNAc (f) were also clearly demonstrated (Fig. 8). The combined data suggest that these gangliosides in the N-370 FG cells have the following structures: J, GM3; K, GM2; L, GM1; N, GD1a. The ganglioside M was a mixture of sialyl-Le^x and a small amount of GM1 containing predominantly C16:0 fatty acid.

Immunocytochemical localization of glycolipids

Figure 9 shows the immunocytochemical localization of GD1a and Le^x glycolipids. Anti-GD1a immunoreactivities were confined to the cell body. Figure 10 reveals the

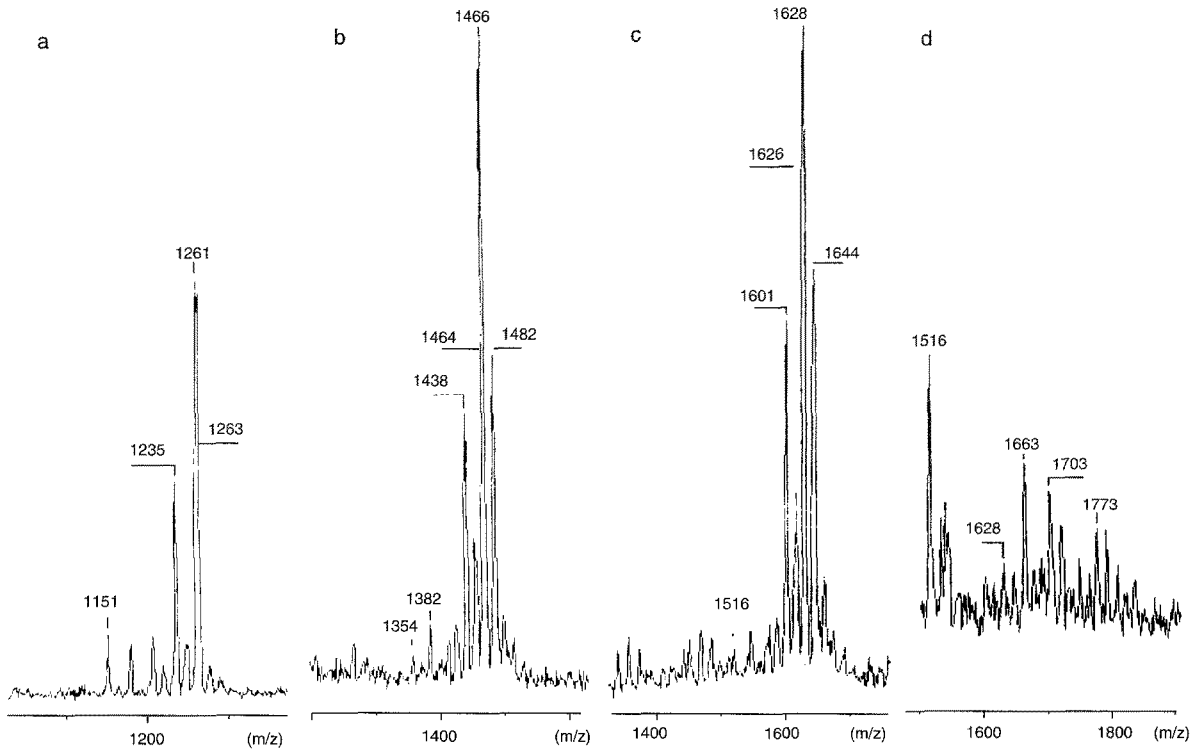

Figure 7. LSI mass spectra of isolated monosialogangliosides, GM3, GM2, GM1, and ganglioside M. (a) GM3, (b) GM2, (c) GM1, and (d) ganglioside M.

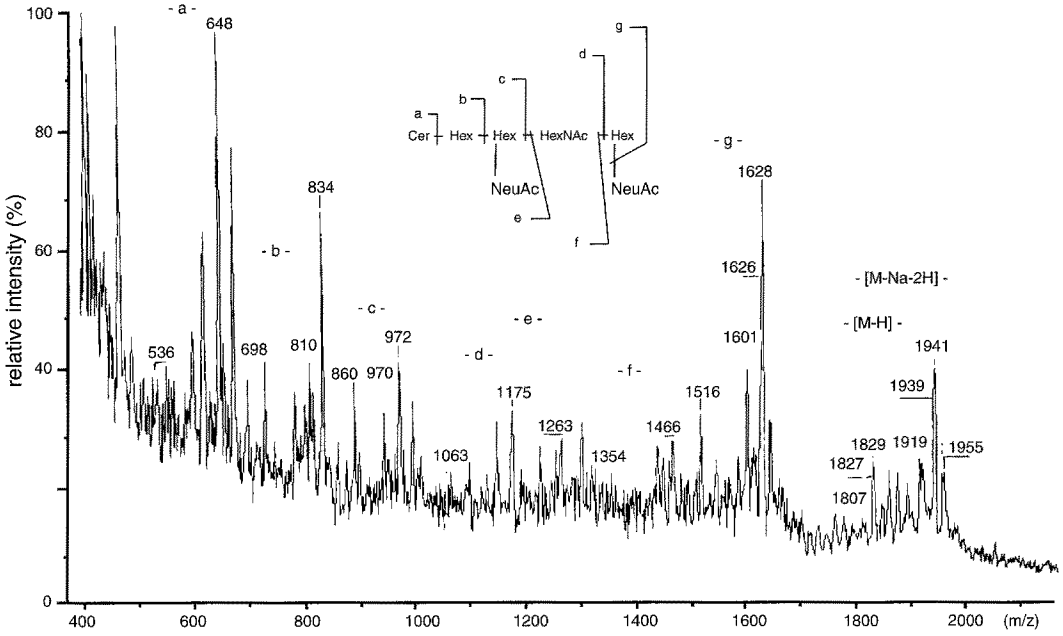

Figure 8. LSI mass spectra of isolated ganglioside GD1a.

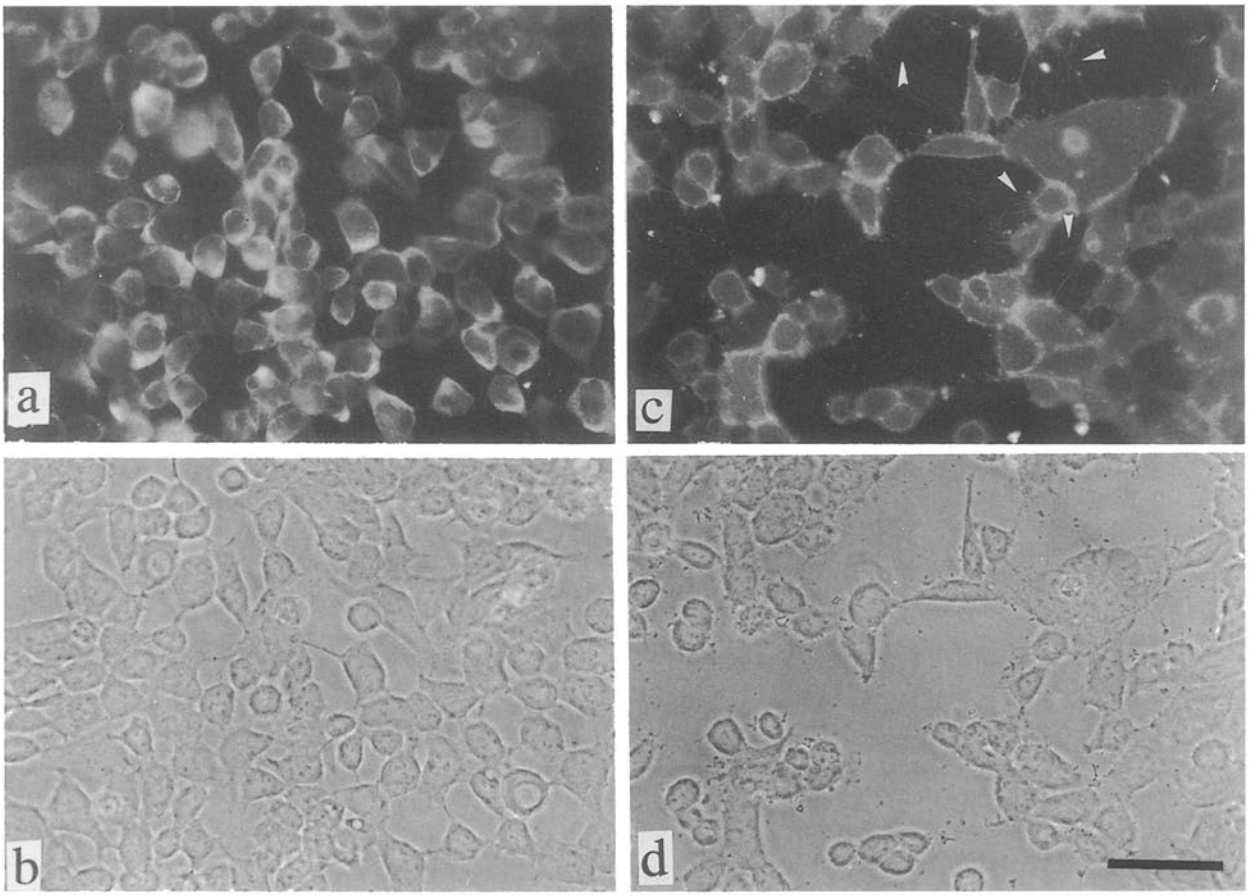

Figure 9. Immunocytochemical localization of GD1a (a and b) and Le^x glycolipid (c and d). a and c, immunocytochemistry; b and d, phase contrast microscopy. Bar = 50 μm.

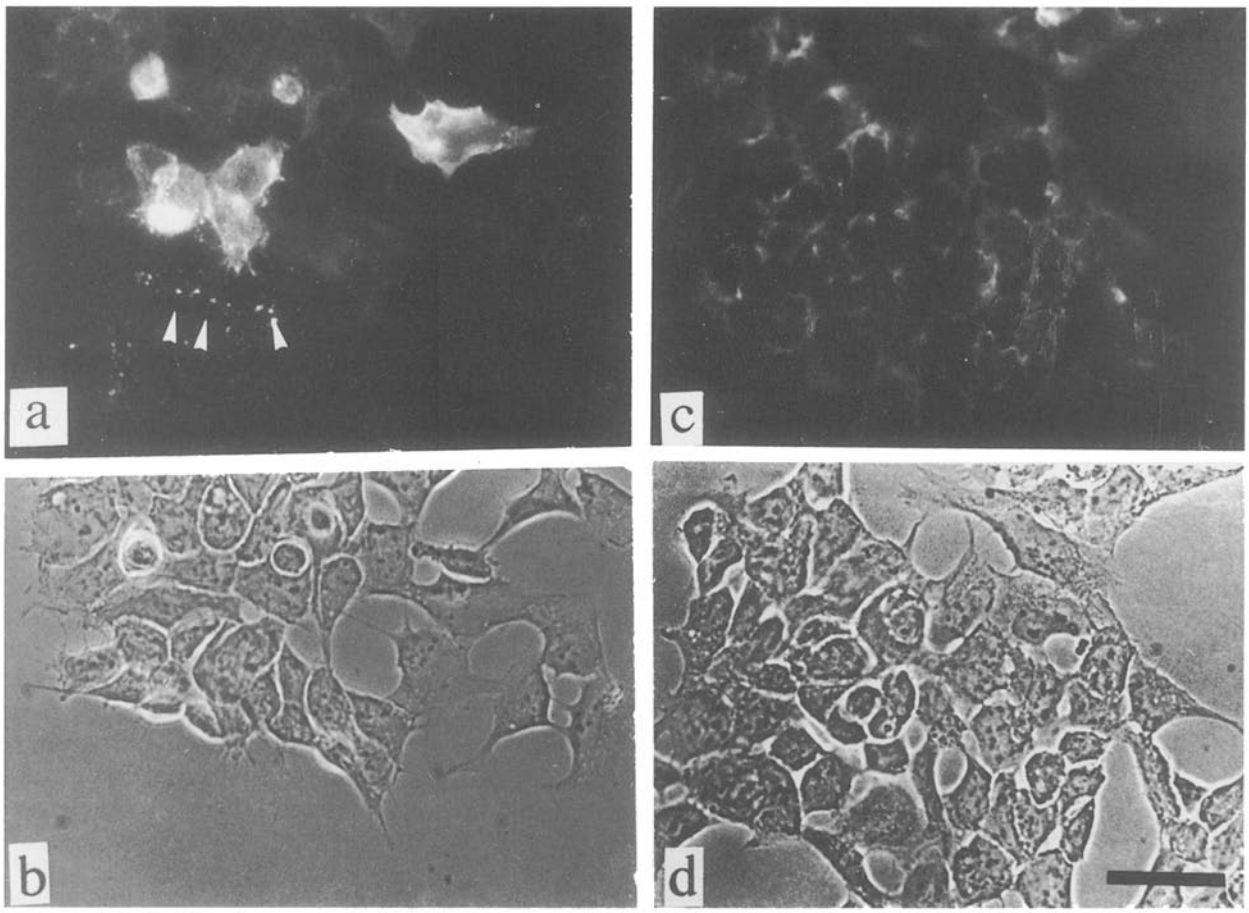

Figure 10. Immunocytochemical localization of sialyl-Le^x (a and b) and GalCer (c and d). a and c, immunocytochemistry; b and d, phase contrast microscopy. Bar = 50 µm.

Table 1. Neutral glycolipid composition of N-370 FG cells (% of total neutral glycolipids; $n = 4$)

GlcCer	11.4 ± 0.7	
GalCer	21.5 ± 4.3	
LacCer	21.4 ± 2.9	
UK1	3.6 ± 0.3	
Gb3	1.2 ± 0.9	
Gb4	21.1 ± 2.4	
UK2	14.2 ± 4.0	Le^x
UK3	2.3 ± 1.2	nLc6?
UK4	3.1 ± 1.0	dimeric Le^x

The content of neutral glycolipids was 1.58 µg lipid-bound glucose per galactose per mg protein.

Table 2. Ganglioside composition of N-370 FG cells

Ganglioside composition (% of total gangliosides; n = *4)*	
GM3	5.4 ± 0.9
GM2	6.1 ± 0.7
GM1	2.0 ± 0.5
GD1a	82.7 ± 4.4
others	3.8 ± 0.9

The content of gangliosides was 0.62 µg lipid-bound sialic acid per mg protein.

immunocytochemical localization of sialyl-Le^x and GalCer. Anti-Le^x immunoreactivities were observed at the fine, small cellular processes, in addition to the cell body (Fig. 9c, arrow heads), whereas anti-GalCer immunoreactivities located mainly at the cell body. Intense sialyl-Le^x immunopositivities were also found to be located on the tip of fine cellular processes (Fig. 10a, arrow heads).

Discussion

It is well-known that alteration in the biosynthesis of the sugar moieties of glycoconjugates and the ensuing changes in their expression is a hallmark of transformed cells as well as malignant tumours [1–3]. Numerous studies have focused on defining specific structural changes of cell surface glycoconjugates with greater attention to the underlying mechanisms of aberrant glycosylation in these cells [2, 3, 7]. In the present study, we have analysed the glycolipid composition in N-370 FG cells, a hybrid tumour cell line derived from human glioma and oligodendroglial cells. The N-370 FG cells were shown to contain a high concentration of GalCer,

LacCer, and globoside (Gb4). The expression of GalCer, a characteristic myelin-associated glycolipid, may reflect the oligodendroglial origin of this cell line. Among the neutral glycolipids, Gb4 constitutes the predominant neutral glycolipid species, accounting for 21% of the total neutral glycolipids. It is well-known that Gb4, or P-antigen, is a stage specific embryonic carbohydrate antigens (SSEA-3) and this glycolipid has been shown to be located on the primordial germ cells in mouse embryos [23, 24] and is synthesized in early chick embryos [25]. Our present finding is consistent with the concept that Gb4 may serve as an oncofetal glycolipid antigen which may play an important role in events involved in early stages of cellular differentiation.

Studies from many laboratories have indicated a common feature of increased expression of lacto-series gangliosides, GM3 and GD3, with concomitant decreased expression of gangliotetraose-series gangliosides in malignant brain tumours such as gliomas, neuroblastomas, meningiomas, astrocytomas, and medulloblastomas [26–34]. In some cases, the increased expression of GD2 and several other tumour-associated gangliosides can also occur. Thus, Fredman *et al.* [35, 27] reported that GD3 and 3′-isoLM1 (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer) were both associated with human malignant gliomas. In particular 3′-isoLM1 was advocated as a potential marker of gliomas as well as medulloblastomas by these investigators [37]. These investigators also found that the expression of GD3 ganglioside was correlated with monosomy chromosome 22 in human meningiomas [36]. In addition, the ganglioside composition of various glioma cell lines, e.g. D-263 MG, D-54 MG, U-118 MG, U-251 MG, U-373 MG were reported to contain GM3, GM2, GD3, and 3′-LM1, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1′Cer, with 3′-LM1 found to be a common major ganglioside in these cell lines [37, 38]. Large variations in ganglioside concentration and patterns, however, were observed in the cells grown in culture and GM3 and GD3 did not dominate in some glioma cell lines [37, 38]. Fredman and her collaborators also reported that the solid tumours induced by transplanting D-54 MG cells into nude mice expressed 3′-isoLM1 containing *N*-glycolyl neuraminic acid (NeuGc) as the major gangliosides, but this ganglioside was not expressed in the cells grown in culture [36–38], raising the possibility that this ganglioside may derive from mouse tissues. Interestingly, D-54 MG cells themselves expressed GM2, GD1a, and 3′-LM1 as the major gangliosides [37]. In the present study, we found an unusually high concentration of GD1a (83% of the total gangliosides) in addition to appreciable amounts of GM2. Such a high concentration of GD1a has never been reported in any other cells, and suggests an enhanced synthesis of GD1a in this cell line (data not shown). Interestingly, N-370 FG cells did not express GD3 and 3′-LM1 as judged by the HPTLC-overlay method using specific monoclonal antibodies against these gangliosides (data not shown). Additionally, other complex types of neolacto-series glycolipids are also present in these cells (Fig. 1, plate E, lane 2). Clearly, heterogeneity in glycolipid expression is a common feature in neural tumour cells which may reflect the divergence in their cellular origin, morphology, and growth characteristics. A similar view was recently expressed by Yates' group based on their extensive study of human astrocytomas and primitive neuroectodermal tumours [34].

Another noteworthy finding of the present investigation was that N-370 FG cells expressed high levels of Lewis^x glycolipids, which are known to be tumour-associated glycolipids and are also expressed in earlier stages of embryogenesis. At least three Le^x glycolipids were characterized in this cell line, namely, fucosylneolactonorpentaosyl ceramide (III^{3}FucnLc$_4$, Le^x), difucosylneolactonorhexaosyl ceramide (V^{3}FucIII^{3}FucnLc$_6$), and sialylated fucosylneolactonorpentaosyl ceramide (III^{3}FucIV^{3}NeuAcnLc$_4$, sialyl-Le^x). Neutral glycolipids bearing the Le^x determinant (lacto-*N*-fucopentaose III structure) and fucosyl-lactoneonorpentaosyl ceramide (III^{3}FucnLc$_4$) were initially found in human adenocarcinomas and characterized by Yang and Hakomori [39]. Difucosylneolactonorhexaosyl ceramide (V^{3}FucIII^{3}FucnLc$_4$; dimeric Le^x) was also characterized by Hakomori and associates in human adenocarcinoma [40]. A glycolipid with a sialylα2-3 residue of the lacto-*N*-fucopentaose III structure (sialyl-Le^x) was originally isolated and characterized from human kidney [41]. These Le^x active glycolipids are known to be expressed in human colon cancer, liver adenocarcinomas, most lung adenocarcinoma, myelogenous leukaemia cells, small cell lung cancer, and human cataractous lens [41–44]. A possible role of surface carbohydrate in embryonic cell interactions has been suggested, particularly for the Le^x determinant which is highly expressed during early mammalian development [8–11]. These glycolipids bearing the Le^x determinant have been reported to be associated with differentiation, development, and organogenesis [12, 42–44]. Interestingly, Le^x expression was relatively abundant in the neutral glycolipid fraction in N-370 FG cells, accounting for 14% of the total neutral glycolipids. In the present study, we have shown that these glycolipids were located in the fine cellular processes. In particular, sialyl-Le^x was found to be located on the tip of cellular processes. This is in contrast to GalCer and GD1a, which are the major glycolipids in N-370 FG cells and are mostly localized on the cell body. The biological role of the differential localization of the various glycolipids in N-370 FG cells is still a subject of speculation. Because of their specific localization, it is a reasonable assumption that the Le^x glycolipids may play some role in controlling cell recognition/adhesion during cellular differentiation and

process outgrowth in this cell line. Further studies are needed to substantiate this putative function.

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