Accumulation of Unique Globo-series Glycolipids in PC 12h Pheochromocytoma Cells*

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In a previous paper, we reported the presence of a unique globo-series glycolipid as one of the major neural glycolipids: Galα1-3Galα1-4Glcβ1-1′Cer, in the subcloned PC 12h pheochromocytoma cells (Ariga, T., Yu, R. K., Scarsdale, J. N., Suzuki, M., Kuroda, Y., Kitagawa, H., and Miyatake, T. (1988) Biochemistry 27, 5335–5340). Recently we found that the subcloned PC 12h cells accumulated other unusual neutral glycolipids. In order to characterize these glycolipids, PC 12h cells were subcutaneously transplanted into rats. The induced tumor tissue accumulated four minor neutral glycolipids, which were purified by droplet counter-current, Iatrobeads column, and preparative thin-layer chromatographies. These glycolipid structures were determined by fast atom bombardment-mass spectrometry, proton nuclear magnetic resonance spectroscopy, permethylation study, and sequential degradation with various exoglycosidases to be as follows: A, Fucα1-2Galα1-3Galα1-4Galβ1-1′Glcβ1-1′Cer; B, GalNAcβ1-3Galα1-4Galβ1-1′Glcβ1-1′Cer; C, Galα1-3Galα1-4Galβ1-1′Glcβ1-1′Cer; and D, Galα1-3Galα1-4Galβ1-1′Glcβ1-1′Cer. Glycolipids A and B were tentatively characterized in normal rat small intestine (Breimer, M. E., Hansson, G. C., Karlsson, K.-A., and Leffler, H. (1982) J. Biol. Chem. 257, 557–568; Angstrom, J., Breimer, M. E., Falk, K.-E., Hansson, G. C., Karlsson, K.-A., and Leffler, H. (1982) J. Biol. Chem. 257, 682–688). Glycolipids C and D have not been reported in the literature.

It is well known that the glycolipid composition changes dramatically during cell differentiation and oncogenic transformation (2, 3). In recent years, many tumor-associated glycolipids have been characterized in several tumor tissues as well as established tumor cell lines (4). The PC 12 pheochromocytoma cells are a clonal line of rat adrenal medullary chromaffin cells and display many properties associated with normal adrenal chromaffin cells (5, 6). We recently reported that PC 12 cells contained many fucosylated gangliosides (7) and these fucosylated gangliosides were purified and characterized from an induced tumor, which was produced by subcutaneous transplantation of the subcloned PC 12h cells into rats. These unusual gangliosides were characterized as fucosyl-GM1, fucosyl-GD1b, and the corresponding fucosylated gangliosides with blood group B determinant (8). In addition, we have reported that the PC 12 cells contained predominantly globo-side in the neutral glycolipid fraction (7). Schwanitz et al. (9) also found globo-side as the major neutral glycolipid in the PC 12 h cells maintained in culture. In addition, we reported that glycolipid composition changed dramatically during subcloning and that the subcloned PC 12h cells accumulated another unusual neutral glycolipid: Galα1-3Galα1-4Galβ1-4Glcβ1-1′Cer† (10). We also found that the subcloned PC 12h cells contained many complex neutral glycolipids having unusual chromatographic behavior.

We have isolated four unique glycolipids from an induced tumor and characterized their structures. These unique glycolipids are characteristic of the presence of repetitive Galα1-3 residues in the molecule. Breimer et al. (11) first reported complex neutral glycolipids carrying repetitive Galα1-3 residues in normal rat small intestine and they suggested that these unique glycolipids accumulated in the subcloned PC 12h cells were also present in normal rat small intestine (11, 12).

EXPERIMENTAL PROCEDURES

Cell Culture and Induced Tumor Tissue—PC 12h cells were subcloned by Dr. Hatanaka, Mitsubishi Kasei Institute of Life Science, Machida, Japan, in which the tyrosine hydrolase activity was significantly increased in the presence of the physiological concentration of nerve growth factor (13). In order to obtain sufficient tissues to isolate and characterize the neutral glycolipids, the PC 12h cells were subcutaneously transplanted into New England Deaconess Hospital rats. Conditions of the cell culture and induced tumor tissue were described previously (7, 8).

Isolation of Complex Neutral Glycolipids—The isolation procedures for the neutral glycolipids in the cells were described previously (7, 14).

The tumor tissues, 100 g, were homogenized in 1 liter of chloroform:methanol (2:1, v/v), followed by successive extractions with 1 liter each of chloroform:methanol (1:1 and 1:2, v/v) and methanol. The combined extracts were evaporated, dissolved in 2 liters of chloroform:methanol:water (8:4:3, v/v) and then partitioned. The lower chloroform layer was washed twice with Folch's theoretical upper phase (15). The combined upper phase lipids were evaporated and dialyzed against distilled water for 3 days, followed by lyophilization. The residue was then dissolved in 200 ml of chloroform:methanol:water (90:6:4.5, v/v) and applied to a DEAE-Sepha-
dx A-25 column (acetate form; 100-ml bed volume). Neutral lipids containing the complex neutral glycolipids were eluted with 600 ml of methanol and evaporated to dryness in vacuo, and then subjected to droplet counter-current chromatography (DCC). The DCC was performed with 250 glass columns (2 mm inner diameter × 40 cm) by the following method using the upper layer of a solvent mixture of chloroform:methanol:water (50:25:65:30, v/v) as the mobile phase and the lower phase of the same solvent mixture as the stationary phase (16, 17). The glycolipid sample was dissolved in 10 ml of the lower phase of the mixture and applied to the DCC column. The effluent was collected in 3.5-ml fractions. In a separate experiment, acetone-insoluble lipids obtained from the lower phase on Folch’s partitioning were also applied to the DCC column to yield small amounts of the complex neutral glycolipids. The neutral glycolipid fraction containing complex glycolipids were combined, evaporated to dryness, and then applied to an Iatrobeads column (42 g). The lipids were recovered in the chloroform:methanol (1:4, v/v) fraction. The lipids were also combined, evaporated to dryness in vacuo, and then subjected to thin-layer chromatography using two different solvent systems. Briefly, the TLC plates were developed with 1-propanol, 28% ammonium hydroxide, water (70:25:20, v/v) and then the bands were scraped separately from the plates and glycolipids were isolated as described above. Final purification of neutral glycolipids A to D was achieved by the Sephadex LH-20 column (20 ml × 48 cm) with methanol as the eluting solvent (18).

Analytical Procedures—Composite analysis was carried out by gas-liquid chromatography (GLC) and nuclear magnetic resonance (NMR) spectroscopy. Neutral sugars, amino sugars, fatty acids, and long-chain bases were analyzed as described previously (7, 18).

**RESULTS**

The neutral glycolipid patterns of the subcloned PC 12h cells and tumor tissues induced by PC 12h cell transplantation are shown in Fig. 1. The glycolipid patterns in the tumor tissue were quite similar with that of the PC 12h cells (Fig. 1, lanes 2 and 3). As compared with the parent PC 12 cells, the PC 12h cells contained the complex neutral glycolipids, which might be expressed during cellular subcloning (10). In the present study, we have isolated these complex glycolipids from the induced tumor tissues. As shown in Fig. 1, the glycolipids, A to D, were found to be homogeneous on TLC with both neutral and basic solvent systems. The yields of these glycolipids, A to D, starting from 100 g of tumor tissue, were 1.0, 0.5, 0.7, and 0.9 mg, respectively. The amount of these glycolipids was approximately 8% of the total neutral glycolipids.

Table I shows compositional analysis of the carbohydrates, fatty acids, and long-chain bases. Glycolipid A was found to contain glucose, galactose, fucose, and long-chain base in the ratio of 1:3:1:1. Glycolipid B contained 1 mol of N-acetylgalactosamine instead of the fucose in glycolipid A. Glycolipids C and D were found to contain glucose, galactose, and long-chain base in the molar ratios of 1:4:1 and 1:5:1, respectively, and they did not contain any fucose or hexosamine. These glycolipids contained mainly palmitic, stearic, behenic, lignoceric, and nervonic acid, but not α-hydroxy fatty acids. They were found to contain C18 sphinganine (over 92%) as the major long-chain base, and they did not contain any phytosphingosine.

Negative ion FAB mass spectra of glycolipids A to D showed prominent dehydrogenated molecular ion (M-H)− which corresponded to glycolipid molecular species containing fatty acids with chain lengths ranging from C16:0 to C24:0 and C18 sphinganine (Fig. 2). All glycolipids showed the same fragment ions corresponding to ceramide (a), and ceramide mono- (b), di-, and tri-acetate derivatives of sugars were analyzed by GLC-electron impact ionization (EI) mass spectrometry (Shimadzu QP-1000) on a fused silica capillary column (0.32 mm × 25 m) of 5% phenylmethylsilicone (Hewlett Packard Co., Palo Alto, CA) or DB-225 (J & W Scientific, Inc., Cordova, CA), with the temperature programmed at a rate of 5 °C/min from 180 to 220 °C (10).

Fig. 1. Thin-layer chromatograms of neutral glycolipids from the subcloned PC 12h cells and rat tumor tissues induced by PC 12h cell transplantation, and of isolated neutral glycolipids A to D from induced tumor tissues. Lane 1, neutral glycolipid mixtures from pig erythrocyte membranes; lanes 2 and 3, neutral glycolipid fractions from the PC 12h cells and tumor tissues, respectively; lanes 4–7, isolated glycolipids, A, B, C, and D, from induced tumor tissues. The glycolipid bands were stained with orcinol-sulfuric acid reagent. Plate A was developed with 1-propanol, water (25:75, v/v) and plate B with 1-propanol, 28% ammonium hydroxide, water (70:10:20, v/v).
TABLE I

| Compositional analysis of isolated neutral glycolipids A to D |
|-------------------------------------------------------------|
|                  | A    | B    | C    | D    |
|------------------|------|------|------|------|
| molar ratio Glc  | 1.00 | 1.00 | 1.00 | 1.00 |
| Glu 1.00–2.95    | 3.08 | 3.79 | 5.17 |
| Fuc 1.11–3.08    | 1.19 | 1.82 | 0.87 |
| Long-chain base  | 0.84 | 1.19 | 1.92 |

| Fatty acid | Percent |
|------------|---------|
| C16:0      | 30.3    |
| C18:0      | 25.6    |
| C20:0      | 10.9    |
| C22:0      | 15.5    |
| C23:0      | 3.0     |
| C24:0      | 13.9    |
| Others     | 4.6     |

Fig. 2. Negative ion FAB mass spectra and fragmentation dia-
grams for glycolipids, A to D.

Fig. 3 shows the TLC of glycolipid products from these isolated unknown glycolipids, A to D, after digestion with various exoglycosidases in the presence of sodium taurocholate. As shown in Fig. 3A, glycolipid A was digested by the treatment of a-fucosidase to yield Galα1-3Galα1-4Galβ1-1'Cer (lane 4), which was already characterized in the PC 12h cells as previously described (10). Glycolipid B was also found to produce the same glycolipid product following the treatment of β-N-acetylgalactosaminidase (Fig. 3B, lane 6). Glycolipids C and D were found to produce lactosyl ceramide, globotriaosyl ceramide, and Galα1-3Galα1-4Galβ1-1'Cer following the treatment of α-galactosidase for 30 min (Fig. 3C, lanes 8 and 11). These glycolipids, C and D, produced lactosyl ceramide after the incubation for 12 h with α-galactosidase (Fig. 3C, lanes 9 and 12).

Analysis by capillary GLC-MS mass spectrometry of these glycolipids, A to D, revealed the presence of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylgalactitol (3Gall-), 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylgalactitol (4Gall-), and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol (4Glcl-). In addition, glycolipid A yielded 1,2,5-tri-O-acetyl-3,4,6-tri-O-methylgalactitol (2Gal-1-) and 1,5-di-O-acetyl-2,3,4-tri-O-methylfucositol (OFuc1-). In glycolipid B, 1,5-di-O-acetyl-3,4,6-tri-O-methyl-2N-meth-

ceramide di- (c), ceramide tri- (d), and ceramide tetrasaccharide (e). However, in glycolipids A, B, and C the terminal sugar was found to be deoxyhexose (fucose) (m/z 146), N-acetylgalactosamine (m/z 203), and hexose (m/z 162), respectively. In the case of glycolipid D, an additional terminal hexose should be attached to glycolipid C. FAB mass data suggested that glycolipids A to C were ceramide pentasaccharide having different terminal sugar residues, and glycolipid D was ceramide hexasaccharide having a terminal hexose, as: A, ceramide-Hex-Hex-Hex-deoxyHex; B, ceramide-Hex-Hex-Hex-Hex-HexNAc; C, ceramide-Hex-Hex-Hex-Hex; and D, ceramide-Hex-Hex-Hex-Hex-Hex.
pig erythrocyte membranes; beans) treatment; treatment; various exoglycosidases.

glycolipid products from glycolipid A after α-fucosidase (C. lampus) treatment; lane 4, Galα1-3Galα1-4Galβ1-1′Cer from the PC 12h cells (10); lane 5, isolated glycolipid B; lane 6, glycolipid products from glycolipid B after β-N-acetylgalactosaminidase (green coffee beans) treatment; lane 7, isolated glycolipid C in this study; lanes 8 and 9, glycolipid products from glycolipid C after α-galactosidase (green coffee beans) digestion for 30 min and 12 h, respectively; lane 10, isolated glycolipid D; lanes 11 and 12, glycolipid products from glycolipid D after α-galactosidase (green coffee beans) digestion for 30 min and 12 h, respectively. The fast-running band in lanes 3, 6, 8, 9, 11, and 12 was sodium taurocholate. The plates were developed 30 min and 12 h, respectively. The fast-running band in glycolipid A was sodium taurocholate. The plates were developed 30 min and 12 h, respectively. The fast-running band in glycolipid A was sodium taurocholate. The plates were developed 30 min and 12 h, respectively.

The glycolipid bands were stained with orcinol-sulfuric acid reagent.

| TABLE II |
|-----------------|-----------------|-----------------|-----------------|
| GLC-EI mass spectrometry of partially methylated alditol acetates obtained from permethylated glycolipids A to D |
| 0Puc1- | 0Galα1- | 0GalNAc1- | -3Galα1- | -3Galα1- | -4Galα1- | -4Glcα1- |
| A | + | + | + | + | + | + | + |
| B | - | - | - | + | + | + | + |
| C | + | - | - | + | + | + | + |
| D | - | + | + | + | + | + | + |

The combined data suggest that these glycolipids, A to D, have the following structures; A, Fucα1-2Galα1-3Galα1-4Galβ1-1′Cer; B, GalNAcβ1-3Galα1-3Galα1-4Galβ1-1′Cer; C, Galα1-3Galα1-3Galα1-4Galβ1-1′Cer; and D, Galα1-3Galα1-3Galα1-4Galβ1-1′Cer.

**DISCUSSION**

In a previous paper we have described that a new globo-series glycolipid, Galα1-3Galα1-4Galβ1-1′Cer, is accumulated in the subcloned PC 12h cells. PC 12h cells also accumulated other complex neutral glycolipids. In order to obtain these glycolipids from the tumor tissues induced on the PC 12h cells, we used the combination of Folch’s partitioning method and an anion exchange column chromatography. Most of these complex neutral glycolipids were recovered with the upper phase upon Folch’s partitioning. In addition, the DCC method should be valuable, because the contamination of phospholipids and the concentration of simple glycolipids, such as ceramide mono-, di-, and trisaccharides are quite low in the complex glycolipid fraction (16, 17). Attempts to isolate the complex neutral glycolipids A to D from the induced tumor tissues by Iatrobeads column chromatography or by TLC with the solvent systems using chloroform:methanol or by TLC with the one-dimensional solvent system were not successful. Final separation of glycolipids A to D was achieved by preparative high performance TLC with two developments using both neutral and ammonium-containing solvent systems. For the analyses of glycolipids by FAB mass spectrometry and NMR spectroscopy, the Sephadex LH-20 column proves to be useful to remove contaminants, such as lower molecular substances and silica gels, which are derived from TLC or Iatrobeads column chromatography (18).

These glycolipids A to D had a same core structure, Galα1-3Galα1-4Galβ1-1′Cer, which was confirmed by digestion with various exoglycosidases (Fig. 3). The terminal sugars of glycolipids A to C were found to be α-fucose, β-N-acetylgalactosamine, and α-galactose, respectively. Glycolipid D had 2 mol of α-galactose in the nonreducing terminus. Exhaustive digestion of glycolipids C and D by α-galactosidase suggests the presence of lactosyl ceramide core structure. Negative ion FAB mass spectrometry of the glycolipids provides information directly on their molecular weights and sugar sequence. In addition, their fatty acid and long-chain base compositions can be obtained (19, 24). NMR spectroscopy can provide information on the assignments of anomeric proton in each sugar residue on the basis of their chemical shifts and coupling constant. We have assigned all ring protons in a globo-series glycolipid; Galα1-3Galα1-4Galβ1-1′Cer, which is ac-
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TABLE III

Glycosyl H-1 chemical shifts (ppm) and J1,2 coupling constants

NMR spectra were recorded by 500 MHz NMR spectrometry at 27 °C. The values are parts/million from tetramethylsilane. The numbers in parentheses are hertz.

| IV  | Galα1-3 | Galα1-4 | Galβ1-4 | Glcβ1-1’Cer (Ref. 10) |
|-----|---------|---------|---------|----------------------|
| A   |         |         |         |                      |
| V   | (3.7)   | (3.9)   | (4.2)   | (4.7)                |
| Fucα1-2Galα1-3 | (4.863) | (4.852) | (4.843) | (4.872)              |
| VI  | (3.7)   | (3.7)   | (2.9)   | (8.1)                |
| B   |         |         |         |                      |
| V   | (4.588) | (4.924) | (4.867) | (4.273)              |
| C   |         |         |         |                      |
| VIII | (2.9)  | (2.9)   | (2.9)   | (2.9)                |
| D   | (3.7)   | (4.4)   | (2.9)   | (2.9)                |

*a Glycosyl H-1 signals were tentatively assigned from NMR data of Refs. 10 and 25 (globoside).
*b Glycosyl H-1 signals corresponding to repetitive Galα1-3 residues were not identified.

cumulated in the subcloned PC 12h cells, by two-dimensional NMR techniques (10). Inagaki et al. (25, 26) have recently reported the NMR spectra of globoside and Forssman glycolipid by the two-dimensional homonuclear Hartmann-Hahn technique. These data provide information on the assignment of anomeric protons in the unusual glycolipids A, B, and C (Table III). The glycolipid H-1 protons and all ring protons in glycolipids A and C were assigned by two-dimensional NMR and homonuclear Hartmann-Hahn techniques.* The GLC-EI mass spectrometry of the methylated alditol acetates is useful to decide the sugar linkages in the glycolipids using a fused silica capillary column coated with 5% phenylmethylsilicone (8, 10). However, peaks corresponding to -2Gal1- and -3Gal1-in glycolipid A failed to be separated from each other by this column. Finally, we succeeded in identifying the peak of -2Gal1- by the use of a fused silica capillary column coated with DB-225.

Breimer et al. (11) and Ångstrom et al. (12) suggested the presence of these unusual glycolipids A, C, and D in epithelial cells and glycolipid B in non-epithelial cells in normal rat intestine. They further found that these intestinal glycolipids contained α-hydroxy fatty acids and phytosphingosine. However, they failed to isolate glycolipids A to D in pure form and to characterize them fully. In this study, we have purified these glycolipids and provided evidence on the proposed structure. These glycolipids were also found to contain C18-sphingosine and normal fatty acids ranging from C16:0 to C24:0 (see Table I), but not phytosphingosines and α-hydroxy fatty acids.

We (7) and Schwarting et al. (9) have reported that globoside is a major glycolipid in the PC 12 cells. We also reported that the subcloned PC 12h cells accumulated an unusual globo-series glycolipid: Galα1-3Galβ1-4-Glcβ1-1’Cer in addition to globoside (10). We assumed that this unusual glycolipid should be present in much low concentrations in the parent PC 12 cells. In addition, we reported that the concentration of globotriaosylceramide was increased in the PC 12h cells (10). The accumulation of the unusual globo-series glycolipids might be due to the induction of α-galactosyltransferase in the subcloned PC 12h cells and solid tumor cells (10). Schwarting et al. also reported that the metabolic labeling of the original PC 12 cells with [3H]-fucose and/or [3H]-galactose revealed the incorporation of unknown complex neutral glycolipids (N1 and N2) and that these glycolipids were increased following the treatment of nerve growth factor (9). They were tentatively identified as fucosyl and galactosyl derivatives of globoside for N1 and N2, respectively. In this study, we have proved that these unique glycolipids have a same core structure, Galα1-3Galα1-4Galβ1-4-Glcβ1-1’Cer, but not globoside. It is possible that the accumulation of these unique glycolipids, A to D, in the subcloned PC 12h cells or solid tumor cells may be the consequence of the activation of specific glycosyltransferases during cellular differentiation and/or the neurite outgrowth.

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