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Characterization of SSEA-1 Glycolipids from the Brain of a Patient with Fucosidosis

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Neutral glycolipids from the brain of a patient with Fucosidosis were analyzed and two complex glycolipids containing five and eight sugars were isolated from the cortical grey matter. These two glycolipids reacted with antibodies recognizing the SSEA-1 [Le^x(X)] carbohydrate determinant. SSEA-1 glycolipids are normally expressed in human embryonic brain but are found in only small amounts in postnatal human brain. The accumulation of the two SSEA-1 glycolipids in Fucosidosis brain thus represents a defect which affects the normal developmentally regulated decrease in postnatal expression of these glycolipids, and may be a contributing factor in the abnormal brain development associated with the disease. Chemical characterization of the two isolated glycolipids by gas chromatographic and mass spectrometric analyses has identified the two glycolipids as lacto-*N*-fucopentaosylceramide (III) and difucosyl-neolactonorhexaosylceramide.

Fucosidosis is a lysosomal storage disease characterized by the deficiency of α-fucosidase activity and the accumulation of fucose-containing glycolipids, oligosaccharides and glycopeptides. Dawson and Spranger [1] characterized the glycolipids stored in the liver of a Fucosidosis patient and reported the accumulation of an H blood group glycolipid. Tsay and Dawson [2] reported the minor storage of H antigen glycolipid in the brain of a Fucosidosis patient. They also detected a fucoganglioside that migrated similar to GD1a in this brain sample. Oligosaccharide analysis of Fucosidosis brain revealed the accumulation of a fucose-containing decasaccharide along with smaller amounts of a disaccharide containing fucose. In a preliminary report, Kolodny *et al.* [3] described a series of complex neutral glycolipids from a Fucosidosis brain, which were likely to represent ceramide fucosyloligosaccharides. We report here the isolation and structural characterization of the two major complex neutral glycolipids from the same Fucosidosis brain, using gas chromatographic and mass spectrometric techniques.

Abbreviations: DCI, direct chemical ionization; FAB, fast atom bombardment; GC, gas chromatography; GSLs, glycosphingolipids; MS, mass spectrometry; SSEA-1, stage specific embryonic antigen-1; TLC, thin layer chromatography.

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Materials and Methods

Tissue

Brain tissue was obtained from a female patient who died at 4 years, 10 months, having been diagnosed with Fucosidosis [3]. Previous examination had shown the absence of plasma and leukocyte α-fucosidase activity with 4-methylumbelliferyl-α-L-fucoside. Analysis of urine revealed several accumulating oligosaccharides and glycopeptides. Control brain tissue was obtained at autopsy from a 4 year, 6 month old child with no neurological involvement.

Glycosphingolipid Extraction

Grey matter [10 g] from the Fucosidosis brain, and from the control brain (10 g) was extracted twice in 10 volumes of chloroform/methanol, 1/1 by vol, followed by a second extraction in 10 volumes of chloroform/methanol/water, 10/10/3 by vol. The combined extracts were dried by rotary evaporation. Extracts were desalted by C18 reversed-phase chromatography [4], and neutral and acidic glycosphingolipids (GSLs) were separated by DEAE-Sephadex A-25 (Pharmacia, Piscataway, NJ, USA) ion exchange chromatography [5]. Neutral GSLs were treated with 0.6 N sodium hydroxide in methanol for 1 h, neutralized with 0.6 N HCl in methanol and desalted. Neutral glycolipids were separated from cholesterol and fatty acids on a silicic acid column as previously described [6]. After removal of solvent by evaporation under nitrogen, the GSLs were redissolved in chloroform/methanol, 1/1 by vol.

The neutral glycolipid fraction was chromatographed on a 50 x 2 cm Iatrobead (Iatron Labs, Tokyo, Japan) column in chloroform/methanol/water, 60/35/5 by vol. The glycolipids were eluted in the same solvent at 1 ml/min. Fractions (10 ml) were collected and monitored by thin layer chromatography (TLC) with orcinol spray reagent.

Sugar and Fatty Acid Analysis

For sugar and fatty acid analysis, the glycolipids were subjected to methanolysis in anhydrous 0.75 M methanolic HCl [7]. The fatty acid methyl esters were analyzed by GLC on a DB-1 0.25 mm x 30 m fused silica capillary column (J & W Scientific, Inc.) with helium as the carrier gas at 20 psi. A Hewlett-Packard model 5890 chromatograph equipped with a flame ionization detector was used and the column oven was programmed from 140-275°C at 10°C/min. The sugar methyl glycosides were analyzed as their trimethylsilyl ethers on the same DB-1 column as described [7]. The temperature was programmed from 140-250°C at 10°C/min.

Permethylation Analysis

Approximately 100 μg of purified glycolipid was permethylated according to the method of Larson *et al.* [8]. One tenth of the permethylated antigen was then taken for hydrolysis and subsequent mass spectrometric analysis of the partially *O*-methylated alditol acetates [7]. The remainder of the sample was used for the mass spectrometric analysis of the intact glycolipid.

Mass Spectrometry

Partially methylated alditol acetates were analyzed by GC-MS using a Finnigan 4500 mass spectrometer interfaced to a Hewlett-Packard 5890 GC and a Teknivent Vector/One GC-MS data system. A DB-1 0.25 mm x 30 m fused silica capillary column (J & W Scientific, Inc.) was used with helium as the carrier gas at 20 psi. The column temperature was initially held at 170°C for 2 min after injection then programmed to 192°C at 2°C/min and finally to 232°C at 10°C/min where it was held for 1 min. Injection was by a "dropping needle type" solventless injector (Chrompak). Electron impact ionization (EI) was used at 70 eV with the ion source at 170°C.

Fast atom bombardment (FAB) and direct chemical ionization (DCI) MS were performed on the intact permethylated glycolipids with a VG 70-250 SE high resolution mass spectrometer. DCI was performed in the positive ion mode under ammonia CI conditions. From 1 to 5 μg of permethylated glycolipid was dried onto the DCI filament and desorbed at 1.5 A. Scans were acquired from m/z 100 to m/z 2,500 amu at 1 sec/decade, 3,000 resolution and 7 kv ion acceleration. FAB spectra were acquired with 3-nitrobenzyl alcohol as the matrix and a xenon fast atom source (Ion Tech, Ltd.) operated at 1.2 mA and 8 kV. Sodium acetate was added to the sample on the probe tip to promote formation of sodium adduct ions. Spectra were acquired from m/z 100 to m/z 2,500 amu with resolution of 3,000 at 10 sec/decade and 7 kV ion acceleration.

Because the FAB spectra persisted for 10 to 20 scans, they were averaged to improve the signal to noise ratio.

TLC Immunostaining

TLC immunostaining, with antibodies reactive with SSEA-1 glycolipids, was performed essentially as described by Yamamoto *et al.* [10]. Glycolipids were separated by TLC on aluminium backed silica gel 60 high performance TLC plates (E Merck, Applied Analytical Industries, Wilmington, NC, USA). Plates were developed in chloroform/methanol/water, 60/35/8 by vol. The standard lane was cut from the plate and visualized with orcinol reagent (0.2%). Plates were then coated with 0.05% polyisobutyl methacrylate (PolyScience, Inc., Warrington, PA, USA) in hexane, dried thoroughly, and soaked in 0.05 M PBS, pH 7.4, containing 1% BSA for 1 h as described [9]. The plates were incubated with 7A antibody (undiluted hybridoma supernatant) for 2 h at 4°C, then with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG/IgM (Boehringer Mannheim, Indianapolis, IN, USA) at a 1:400 dilution, for 1 h at room temperature. Plates were rinsed with PBS, and developed with 33 mM 4-chloro-1-naphthol (Sigma, St. Louis, MO, USA) in 0.02 M Tris-HCl buffer, 0.5 M NaCl, pH 7.5, containing 20% methanol and 0.02% H_2O_2.

Results

Grey matter (10 g) from the temporal lobe of a brain obtained at autopsy from a four year old patient with Fucosidosis was analyzed for its composition of neutral glycolipids. The brain tissue was extracted with chloroform/methanol/water, treated with 0.5 N methanolic

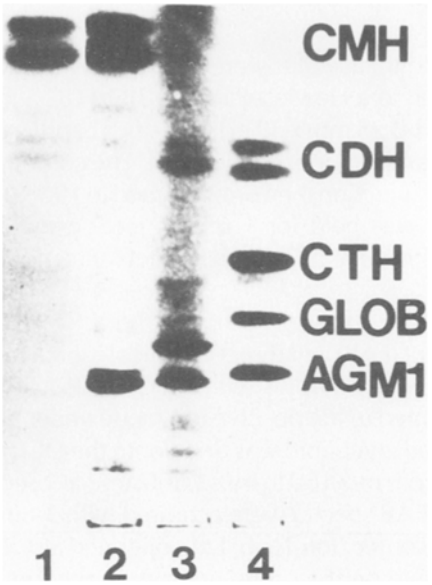

Figure 1. Thin layer chromatogram of the neutral glycolipids from human brain. The glycolipids fractions were obtained from the cortex of a four year old control brain (lane 1); the cortex of a four year old patient with Fucosidosis (lane 2) and the forebrain of a first trimester fetus (lane 3). Standards are in lane 4. Visualization was with orcinol spray reagent.

sodium hydroxide to destroy ester lipids, chromatographed on DEAE-Sephadex to remove acidic glycolipids, and subjected to Unisil™ chromatography to remove fatty acids and cholesterol. The resulting neutral glycolipids were analyzed by TLC (Fig. 1), and compared to brain glycolipids from an age matched control and from a first trimester fetus. Fucosidosis brain contained a large amount of a glycolipid which was seen on TLC just below asialo-GM1 as detected with orcinol spray. A glycolipid with the same mobility was present in the embryonic brain, but was nearly undetectable in the age matched control brain sample. The Fucosidosis brain also contained a more complex glycolipid which was seen half way between the more prominent glycolipid and the origin. Again, the embryonic brain contains a similar component, while the control brain does not. The Fucosidosis brain glycolipids were fractionated on a column of Iatrobeads™ with a chloroform/methanol/water mixture as the mobile phase. The two major neutral glycolipids were obtained in a pure form as demonstrated by orcinol detection on TLC (Fig. 2). The smaller glycolipid was termed SSEA-1 (V) and the larger one SSEA-1 (VIII). In order to aid in the identification of the two Fucosidosis glycolipids, they were subjected to TLC-immunostaining with several monoclonal antibodies which specifically react with fucose containing structures. One of these reagents, monoclonal antibody 7A, which reacts with the SSEA-1 (Le^x) determinant, also reacts with each of the Fucosidosis brain glycolipids (Fig. 3). As has been shown previously [10], 7A antibody shows a stronger reactivity with the more complex glycolipid SSEA-1 (VIII), than with the smaller one, SSEA-1 (V) (Fig. 3).

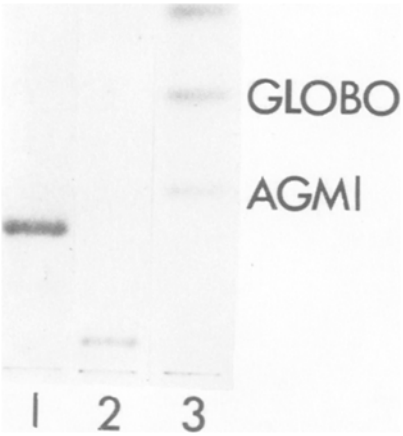

Figure 2. Thin layer chromatogram of purified neutral glycolipids from Fucosidosis brain. SSEA-1 (V) is in lane 1; SSEA-1 (VIII) is in lane 2. Standards are in lane 3. Visualization was with orcinol spray reagent.

The permethylated glycolipids were hydrolyzed and the partially methylated alditol acetates examined by electron impact GC-MS with selected ion monitoring to identify and measure the molar ratios between the resulting PMAAs by their spectral and retention characteristics. Ion plots for characteristic ions were used to identify tri-*O*-methyl fucose, tetra-*O*-methyl galactose, 2,3,6-tri-*O*-methyl glucose, 2,4,6-tri-*O*-methyl galactose and 3,6-di-*O*-methyl *N*-acetylglucosamine in both SSEA-1 (V) and SSEA-1 (VIII). A SSEA-1 positive GSL of known structure from C57BL/6J mouse kidney [11-13] was used as a standard to determine the response ratios between these various *O*-methyl hexoses and to infer the molar ratios in GSLs under study here. With this standard the respective peak area ratios between the above *O*-methyl hexoses were measured and the results normalized to one mole of tetra-*O*-methyl galactose are shown in Table 1. These data are interpreted to indicate that SSEA-1 (V) contains one mole of each of the identified components and that SSEA-1 (VIII) contains an additional mole of terminal fucose, 3-substituted galactose and a 3,4-disubstituted *N*-acetylglucosamine.

The intact permethyl GSLs were examined by mass spectrometry with both ammonia DCI and FAB ionization. SSEA-1 (V) showed an ion in the DCI spectrum at m/z 1,639 identified as MH^+ for the molecular species containing stearic acid, and 4-sphingenine. The FAB spectrum of this compound shows an ion at m/z 1,661 that is calculated to be $[M+Na]^+$ for this same species. Both the FAB and DCI spectra show ions at m/z 638 and 842 for the non-

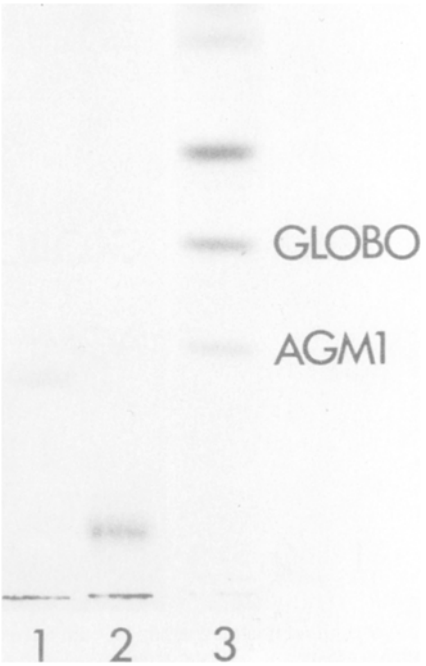

Figure 3. TLC-immunostaining of purified Fucosidosis brain neutral glycolipids, using monoclonal antibody 7A. SSEA-1 (V) in lane 1 and SSEA-1 (VIII) in lane 2, both react with the 7A antibody. Neutral glycolipid standards detected by orcinol spray reagent are in lane 3.

reducing terminal trisaccharide and pentasaccharide shown in the proposed structure (Fig. 4). Fig. 5 shows our proposed structure for SSEA-1 (VIII) along with some of the ions that were observed in both the DCI and FAB spectra. The molecular weight is established from an $[M+Na]^+$ ion at m/z 2,284 in the FAB spectrum. This molecular weight (2,261) is consistent with the proposed structure being a stearic acid, 4-sphingenine-containing species. Ions at m/z 638, 842 and 1,261 were observed in both DCI and FAB spectra and are interpreted to be tri-, tetra-, and hexasaccharides from the oligosaccharide of the proposed structure. The ion at m/z 576 is indicative of a stearic acid, 4-sphingenine-containing ceramide, and the ion at m/z 1,418 derives from the fragment Fuc-GlcNAc-Gal-Glc-ceramide, as illustrated in Fig. 5.

Discussion

Fucosidosis is characterized by the onset of psychomotor retardation at about one year of age. Growth retardation and neurologic deterioration are rapidly progressive. Blood group H antigen was reported to be the major glycolipid accumulating in the brain of a Fucosidosis patient [2]. In addition, many fucose-containing oligosaccharides have been identified from

Table 1. Molar ratios of partially methylated alditol acetates identified from SSEA-1 (V) and SSEA-1 (VIII).

PMAA	m/z[a]	SSEA-1 (V)[b]	SSEA-1 (VIII)[b]
tri-*O*-methyl fucose	175	0.5	2.1
tetra-*O*-methyl galactose	117	1.0	1.0
2,3,6-tri-*O*-methyl glucose	117	1.2	1.1
2,4,6-tri-*O*-methyl galactose	117	1.0	2.0
3,6-di-*O*-methyl *N*-acetylglucosamine	158	1.4	2.0

[a] Ion used to measure peak area for this PMAA by GC-MS.
[b] Mole ratios were calculated using a GSL of known structure to correct for response differences between PMAAs as described in the Methods section.

human Fucosidosis urine, including a disaccharide and a decasaccharide with terminal fucose. Tsay and Dawson [2] reported accumulation of GM1 and GM2 gangliosides in a Fucosidosis brain sample, whereas Kolodny *et al.* [3] reported the increased concentration of a ganglioside (presumably a fucoganglioside) in the polysialoganglioside fraction from Fucosidosis brain. This fucoganglioside has not been further characterized; however, it was demonstrated that there is no accumulation of SSEA-1 ganglioside in the brain of this patient (data not shown). The tissue utilized in the studies reported here were from the case of a patient with Fucosidosis presenting as a leukodystrophy [3]. As previously reported, the patient developed normally until age six months. By 30 months linear growth was below the third percentile. The EEG at 2 1/2 years showed excessive slowing; at 3 1/2 years paroxysmal sharp slow discharges were present. The CT scan at 3 3/4 years revealed slightly enlarged ventricles with decreased white matter density. A skin biopsy demonstrated enlargement of axonal terminals due to accumulation of numerous electron-dense multilayered bodies, multiple intracytoplasmic inclusions in fibroblasts and endothelial cells, and vacuolization of epithelial and endothelial cells. Plasma and leukocyte α-fucosidase activity, as measured using 4-methylumbelliferyl-α-L-fucoside, was absent. This patient, both clinically and pathologically, resembled the two Fucosidosis cases described by Durand *et al.* [14], and died at 4 years 10 months of age.

Results presented here show that the major neutral glycolipids accumulating in Fucosidosis brain express the SSEA-1 antigenic determinant. The structures of SSEA-1 glycolipids from several sources have been characterized previously. Hakomori *et al.* [15] determined the structure of SSEA-1 glycolipids in adenocarcinoma tissue. Using FAB-MS techniques, Svennerholm *et al.* [16] recently described similar SSEA-1 glycolipids, which were isolated from brains of cases of neural ceroid lipofuscinosis, a degenerative brain disorder. All of these glycolipids contained neolacto internal structures. Recently however, Sekine *et al.* [11, 12] described a kidney SSEA-1 glycolipid which contained a globoside internal

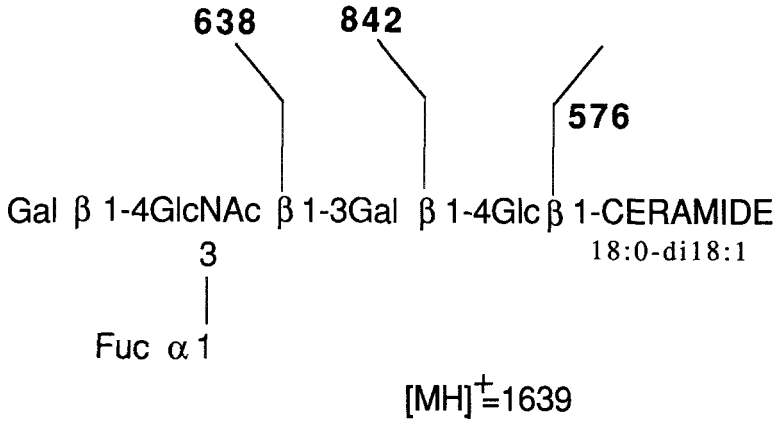

Figure 4. Structure and fragmentation of SSEA-1 (V) from the FAB and DCI mass spectra of the permethylated glycolipid.

structure. Antibodies to SSEA-1 cannot distinguish between the neolacto- and globo- forms of SSEA-1, therefore isolation and structure determination of the glycolipids from Fucosidosis brain was necessary to characterize these fucolipids definitively. The results presented here demonstrate that neolacto, SSEA-1 glycolipids are the predominant glycolipids accumulating in the brain of this Fucosidosis patient. The results of our mass spectrometric analyses produced nearly identical spectra to those published by Svennerholm *et al.* [16]. No globo SSEA-1 glycolipids or blood group H glycolipids [17] could be detected. The differences seen between these data and previous studies of Fucosidosis brain could be due to the variable accumulation of different substrates as a result of individual differences in the site and nature of the mutation.

Svennerholm *et al.* [16] suggested that the accumulation of neutral fucoglycolipids was mostly derived from the increased number and volume of astrocytes in neural ceroid lipofuscinosis brains. Gliosis was also present in the Fucosidosis brain reported here. Thus it is possible that the neutral glycolipids accumulating in this brain were concentrated in glial cells. However, lectin binding studies of Fucosidosis brains have shown that the fucose-specific lectin, *Ulex europeus*, reacts strongly with some neurons but not with glial cells [18]. This suggests that neurons may be the major source of fucosylated glycoconjugate accumulation in the CNS of human Fucosidosis patients. The data presented here, however, do not address the specific site of accumulation of neutral fucoglycolipids.

It seems likely that the accumulation of fucoglycolipids is in part due to the inability of the developing Fucosidosis brain tissue to metabolize SSEA-1 glycolipids, which are prominent constituents in the normal embryonic and early postnatal CNS. In rodents, SSEA-1 glycolipids are expressed on the proliferating neuronal precursors which line the ventrical of the cerebral cortex prior to their migration towards the cortical plate. Postnatally, SSEA-1 glycolipids diminish significantly in concentration and are nearly undetectable in the post-

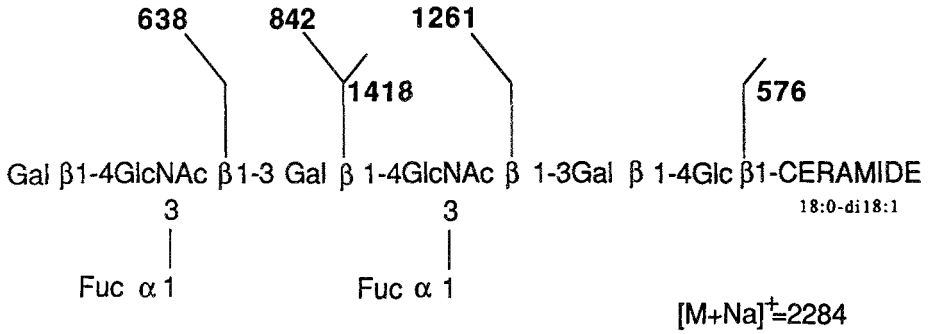

Figure 5. Structure and fragmentation pattern of SSEA-1 (VIII) from the FAB-MS spectrum of the permethylated glycolipid.

migratory cells of the cortex. Other mammals, including pigs and cows express similar profiles of SSEA-1 glycolipids during embryonic and perinatal development of the brain (unpublished results). In humans however, this pattern may be slightly altered. Vanier *et al.* [19] have shown that III^3-fucosyllactoneotetraosylceramide (LNF III-Cer), the smallest of the SSEA-1 glycolipids, is a minor component of normal postnatal human brain, suggesting that this glycolipid does not disappear altogether postnatally. Although we have not quantified the amounts of SSEA-1 glycolipids in our human brain samples, comparison of normal embryonic and postnatal brain tissue by TLC (Fig. 1) or by TLC-immunostaining methods (data not shown) indicates that LNF III-Cer does not disappear completely from normal postnatal human brain tissue, just as Vanier *et al.* [19] have suggested, but these studies also show that there are many times more SSEA-1-reactive glycolipids in embryonic human brain than in postnatal human brain. Data presented here thus suggests that SSEA-1 glycolipids are temporally regulated embryonic brain antigens in humans which are programmed to diminish rapidly from late prenatal or early postnatal brains. It is possible that any disruption in this programmed disappearance of fucoglycolipids could affect the differentiation, maturation or the cellular interactions in those areas of the brain where fucoglycolipids accumulate. Thus, in Fucosidosis, the continued presence of large amounts of fucoglycolipids throughout the postnatal period may contribute to the neurological deterioration of the cerebral cortex.

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