Enhanced expression of a-series gangliosides in fibroblasts of patients with peroxisome biogenesis disorders

K. Tatsumi a, M. Saito a,*, B. Lin b, M. Iwamori c, H. Ichiseki a, N. Shimozawa d, S. Kamoshita e, T. Igarashi a, Y. Sakakihara a

a Department of Pediatrics, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
b Department of Biochemistry, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
c Department of Biochemistry, Faculty of Science and Technology, Kinki University, Kinki, Japan
d Department of Pediatrics, Gifu University, School of Medicine, Gifu, Japan
e International Medical Center of Japan, Tokyo, Japan

Received 5 December 2000; accepted 1 February 2001

Abstract

Peroxisome biogenesis disorders (PBD) are classified into Zellweger syndrome (ZS), infantile Refsum disease (IRD) and neonatal adrenoleukodystrophy. Disturbances in the differentiation of neural cells such as migration arrest are characteristic of PBD. So far the pathogenesis of these disturbances is not clearly understood. We describe an altered metabolism of glycosphingolipids in PBD which has not yet been investigated. We observed an increased amount of a-series gangliosides, GM2, GM1 and GD1a, in the fibroblasts of patients with ZS and IRD. Gangliosides GM1 and GD1a were not present in detectable amounts in normal subjects. A key step in the synthesis of a-series gangliosides is a transfer of GalNAc to ganglioside GM3, so we determined the level of ganglioside GM3 by immunohistochemical methods. We found a granular structure, which was positive toward anti-ganglioside GM3 antibody in the cytoplasm of the patients' fibroblasts. In control cells, the cell membrane was slightly positive toward anti-GM3 antibody. These results may help to clarify the pathogenesis of PBD with respect to the functional roles of glycosphingolipids in cell differentiation, proliferation and apoptosis. ß 2001 Elsevier Science B.V. All rights reserved.

Keywords: Zellweger syndrome; Infantile Refsum disease; Ganglioside; Fibroblast

1. Introduction

Peroxisome biogenesis disorders (PBD) are characterized by a dysfunction in the formation of peroxisomes. Zellweger syndrome (ZS) is the most severe form of PBD, in which peroxisomes are entirely absent. There are numerous biochemical abnormalities, such as an accumulation of very long free fatty acids,
and a decrease in plasmalogen levels in patients with ZS [1]. Infantile Refsum disease (IRD), the mildest form of PBD, is also characterized by the absence of peroxisomes and the presence of biochemical abnormalities. Pathologically, various migration anomalies, such as polymicrogyria and a heterotropic gray matter, were reported in the brains of patients with ZS and ZS model mice [2–7]. Although the exact mechanism underlying these pathological changes in the central nervous system (CNS) is as yet unknown, Evrard et al. suggested the possibility of toxicity of a PAS-positive inclusion body to developing neural tissues in the CNS of patients with ZS [3]. The inclusion body was likely to be composed of abnormal gangliosides although it was not accurately identified [8].

The functional roles of glycosphingolipids in cell growth, differentiation, proliferation and cell recognition have been clarified in recent years [9,10]. In the developing CNS, glycosphingolipids such as ceramide monohexoside (CMH) and gangliosides play an important role in neural cell differentiation [11–13]. Therefore, the analysis of the glycosphingolipid composition of peroxisome-deficient cells may help to understand the pathogenesis of PBD. Previously, we analyzed the glycolipid composition of model cells (Z65) which were derived from wild Chinese hamster ovary cells (CHO-K1 cells) with a mutagen, ethyl methanesulfonate, and exhibited defective peroxisomal biogenesis. We observed the accumulation of CMH and ganglioside GM3 in Z65 cells [14]. The purpose of the present study was to investigate the changes in glycosphingolipid metabolism in peroxisome-deficient cells of PBD patients. Thus, the glycolipid composition and localization of ganglioside GM3 in fibroblasts from patients with ZS and IRD were examined.

2. Patients and methods

2.1. Patients

Patient F-04 presented classical features of ZS, i.e., the patient manifested craniofacial dysmorphism, cardiopathy, hepatosplenomegaly and a typical clinical course. Patient F-04 had an accumulation of very long chain fatty acids (VLCFA) in the fatty acid moiety of serum sphingomyelin and had no peroxisomes in the skin fibroblasts. Patient F-04 died of heart failure at 3 months old. Patient F-05 presented at 1 year of age with delayed developmental milestones and extreme joint laxity. Later, retinal pigmentation was found and biochemical investigations showed an accumulation of VLCFA. A diagnosis of IRD was made and she died at 3 years of age following an episode of bronchiolitis. Both patients carried the PEX2 gene mutation. The details of the mutation analysis of the PEX2 gene in both patients were previously reported by one of the authors (N.S.) [15,16]. Control subjects (C1 and C2) of normal individuals aged 3 months and 1 month, respectively, were obtained from the cell bank (Riken Cell Bank, Japan).

2.2. Lipid extraction and thin layer chromatography (TLC)

Fibroblasts from patients F-04 and F-05 were cultured in α minimal essential medium supplemented with 10% fetal calf serum in 5% CO₂. After the fibroblasts were harvested, they were lyophilized. Total lipids were extracted from the fibroblasts successively with 2 ml of chloroform/methanol/water solution (1:1:0, 10:20:1, 20:10:1, v/v) at 40°C for 20 min. The combined crude lipids were fractionated into neutral and acidic portions using DEAE-Sepharose (A-25, acetate form). Isolation of neutral glycosphingolipids by acetylation and Florisil column chromatography was carried out according to the methods previously reported [14]. The purified acidic and neutral glycosphingolipids were applied on each glass-coated TLC plate (Silica gel 60, Merck, Germany). Both fractions were developed with chloroform/methanol/water (55:45:10, v/v). The spots were located by spraying with orcinol-H₂SO₄ reagent. The mobilities on TLC of individual glycolipids were compared with those of standard glycolipids. The densities of the spots were quantified at 500 nm with a dual-wavelength TLC densitometer (CS-9000, Shimadzu Co., Kyoto, Japan). The standard curve was linear from 0.1 to 1.5 μg of each standard glycolipid. Furthermore, we attempted to identify each lipid by TLC immunostaining according to the methods previously reported [17]. Briefly, acidic lipids were applied on a plastic plate (Sigma, St. Louis, MO, USA) with chloroform/methanol/water.
After overnight incubation of the plates with blocking buffer at 4°C, the plates were incubated with rabbit polyclonal anti-asialoGM1 (GA1), anti-asialoGM2 (GA2), anti-gangliosides GM1 and mouse monoclonal anti-ganglioside GM2 (YHD06) antibodies as the first antibodies. Polyclonal antibodies exhibiting monospecificity toward the carbohydrate moieties of GA1, GA2 and GM1 were prepared by immunizing rabbits and YHD06 antibodies were generated by the conventional procedure by one of the authors (M.I.) [18]. After washing and re-incubation with blocking buffer, the first antibodies bound on the plates were detected using 4-chloro-1-naphthol and H2O2 with a peroxidase-conjugated goat anti-mouse or anti-rabbit IgM+G (H+L) antibody (Jackson Immuno Research Labs., Westgrove, PA, USA).

2.3. Structural characterization of acidic lipids by negative ion fast atom bombardment mass spectrometry (FAB-MS)

Further identification of acidic glycolipids was performed by negative ion FAB-MS, as follows. The individual gangliosides were purified by high performance liquid chromatography with Iatrobeads (Iatron Lab, 6RB-8010, Tokyo, Japan), and the elution was carried out with chloroform/methanol/water (55:45:2, v/v). About 5 µg of the purified lipid from fibroblasts of patient F-05 in 5 µl of chloroform/methanol (1:1, v/v) was mixed with 5 µl of triethanolamine, and the resultant mixture was put on a stainless steel sample holder of FAB-MS. Analysis was performed by bombardment with a neutral xenon beam, with a kinetic energy of 4 keV, and detection of the negative ion using a mass spectrometer (JMS HX-110; Jeol, Tokyo, Japan). Perfluoroalkyl phosphazine was used as the mass marker.

2.4. Immunohistochemical staining of fibroblasts of PBD patients with anti-ganglioside GM3 antibody

The expression of ganglioside GM3 in fibroblasts of patients with PBD was immunohistochemically analyzed by indirect immunoperoxidase staining with a Vectastain ABC Kit (Vector Lab., Burlingame, CA, USA). Fibroblasts were cultured directly on a glass slide with a chamber. When the subconfluent stage (70% of the glass slide) was reached, the culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Then the chamber was removed and the glass slide was immersed in acetone at 4°C for 5 min. After the cells were washed with PBS, they were incubated with anti-mouse ganglioside GM3 antibody (M-2590, obtained from Seikagaku-Kougou, Tokyo, Japan). Then, they were incubated with biotinylated second antibodies (Vector Lab., Burlingame, CA, USA) and avidin-conjugated peroxidase. Labelled gangliosides GM3 were visualized with 0.02% diaminobenzidine and 0.005% hydrogen peroxide in 0.05 M Tris buffer (pH 7.6). The nuclei were also stained with hematoxylin.

3. Results

3.1. Glycolipids purified from the fibroblasts of patients with PBD

Fig. 1 shows the TLC analyses of neutral (A) and acidic (B) glycolipids from the fibroblasts of patient F-04 with ZS and patient F-05 with IRD and those from fibroblasts (C1 and C2) of normal controls. The position of glycolipids migrating on a TLC plate was compared with those of standards. In the case of neutral glycolipids, CMH, ceramide dihexoside (CDH), ceramide trihexoside (CTH) and globoside (Gb4Cer) were detected. Although the amount of neutral glycolipids was varied, the amount of acidic glycolipids, particularly GM3, was increased in the fibroblasts of patients with PBD. The detailed composition of glycolipids is shown in Table 1.

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>C1</th>
<th>C2</th>
<th>F-04</th>
<th>F-05</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcCer</td>
<td>0.20</td>
<td>0.34</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>LacCer</td>
<td>0.29</td>
<td>0.29</td>
<td>0.58</td>
<td>0.48</td>
</tr>
<tr>
<td>Gb2Cer</td>
<td>1.55</td>
<td>1.60</td>
<td>1.61</td>
<td>2.79</td>
</tr>
<tr>
<td>Gb4Cer</td>
<td>0.78</td>
<td>0.70</td>
<td>0.79</td>
<td>0.82</td>
</tr>
<tr>
<td>GM3</td>
<td>1.81</td>
<td>1.95</td>
<td>2.52</td>
<td>2.18</td>
</tr>
<tr>
<td>GM2</td>
<td>0.03</td>
<td>0.01</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>GM1</td>
<td>tr</td>
<td>tr</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>GD3</td>
<td>0.15</td>
<td>0.20</td>
<td>0.11</td>
<td>0.43</td>
</tr>
<tr>
<td>GD1a</td>
<td>tr</td>
<td>tr</td>
<td>0.09</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Values are µg/mg dry weight of each glycolipid in the fibroblasts of controls (C1, C2), the patient with ZS (F-04) and the patient with IRD (F-05). tr = trace.
CDH increased slightly in the PBD patients, no significant differences in the concentration of neutral glycolipids were observed between the patients and controls (Table 1). In the case of acidic glycolipids, the amounts of gangliosides GM2, GM1 and GD1a were increased in patients with PBD. The amounts of GM2 in patients were about 6–10-fold higher than in the controls. The amount of ganglioside GD3 in patient F-05 with IRD was about twice higher than that in the controls. Gangliosides GM1 and GD1a
were not detected in control cells, which contained gangliosides GM3 and GD3 with a shorter carbohydrate chain. On the other hand, synthesis of a-series gangliosides, i.e., GM2, GM1 and GD1a, was significantly accelerated in the PBD patients. Further characterization of gangliosides GM2, GM1 and GD1a was carried out as follows.

3.2. TLC immunostaining assay

Fig. 2 shows the TLC immunostaining analysis using anti-ganglioside GM2 (A) and the anti-ganglioside GM1 (B) antibodies. Bands that were positively stained with anti-ganglioside GM2 antibody were found in both the PBD patients and normal controls. Ganglioside GM1 was detected only in the PBD patients.

![TLC immunostaining analysis](image)

Fig. 2. TLC immunostaining of acidic lipids with anti-ganglioside GM2 (A) and GM1 (B) antibodies. (A) In both normal controls and PBD patients, immunoreactive fractions were detected. The amount of ganglioside GM2 was increased in the PBD patients. (B) No fraction immunoreactive with anti-ganglioside GM1 was found in the normal controls. Ganglioside GM1 was detected only in the PBD patients.

3.2. TLC immunostaining assay

Fig. 2 shows the TLC immunostaining analysis using anti-ganglioside GM2 (A) and the anti-ganglioside GM1 (B) antibodies. Bands that were positively stained with anti-ganglioside GM2 antibody were found in both the PBD patients and normal controls.

![Negative ion FAB-MS spectrum](image)

Fig. 3. Negative ion FAB-MS spectrum of GD1a isolated from fibroblasts of patient F-05 with IRD. About 5 μg of GD1a was analyzed using FAB-MS with triethanolamine as a matrix.
The concentration of ganglioside GM2 in the PBD patients was higher than that in the controls. There was no band immunoreactive with anti-GM1 antibody in the normal controls. It was revealed that the concentrations of gangliosides GM2 and GM1 increased in PBD patients with the specific antibodies. There was no band immunoreactive with anti-GA1 and anti-GA2 antibodies in both normal controls and PBD patients (data not shown).

3.3. Negative ion FAB-MS of acidic lipids

Negative ion FAB-MS spectrum of GD1a isolated from fibroblasts of PBD patient F-05 is shown in Fig. 3. Negative ion FAB-MS yielded the molecular ions [M−H]− at m/z 1807 and m/z 1891, and the fragment ions corresponding to the sequential cleavage at glycosidic linkages were [ceramide]−, [CMH]−, [CDH]−, [GM3]−, [GM2]− and [GM1]−, indicating that the ganglioside was GD1a and the ions at m/z 1807 and m/z 1891 were due to the difference in fatty acid composition, corresponding to lignoceric and stearic acids, respectively. Further characterization of GD1a was done by TLC immunostaining with anti-GM1 antibody after treatment of the TLC plate with Vibrio cholerae neuraminidase at 37°C for 2 h (data not shown). Thus fibroblasts from the PBD patients were proven to express gangliosides GM2, GM1 and GD1a, that is, α-series gangliosides. Since the transfer of GalNAc to ganglioside GM3 initiates the synthetic pathway of α-series gangliosides, we then examined its expression in the cells by immunohistochemical staining with anti-ganglioside GM3 antibody.

3.4. Immunohistochemical staining with anti-ganglioside GM3 antibody of the fibroblasts from the patients with PBD

Fig. 4 shows fibroblasts of control (A), patient F-04 (B) and patient F-05 (C) stained with anti-ganglioside GM3 antibody. In control cells, the cell membrane was slightly positive toward anti-ganglioside GM3 antibody. On the other hand, the granular structure inside the fibroblasts from the PBD patients was strongly stained with the antibody.
4. Discussion

One of the characteristic findings in the CNS of PBD patients is an impaired neuronal migration. Powers [8] reported that focal losses of the cortical radial pattern associated with micropolygyric ripples, thin cortical plates and subcortical heterotopia can be identified in an affected fetus at 14–24 weeks gestation. These findings were associated with abnormal lipid inclusions at the ultrastructural level. Astrocytes, brain macrophages, meningeal, interstitial testicular, adrenocortical and renal tubular cells also display cytoplasmic accumulation of an electron-opaque slightly lamellated structure [19]. The chemical nature of these inclusions is as yet unknown, but they are speculated to represent abnormal ganglioside [8]. To our knowledge, the present study is the first description of an increase in ganglioside levels in the fibroblasts of PBD patients. Although much attention has been paid to the pathogenic role of VLCFA accumulation in PBD, little has been given to the behavior of glycolipids in PBD. Glycosphingolipids are abundant in the CNS. Among this lipid family, gangliosides are the most abundant particularly in the mammalian CNS and display a characteristic distribution pattern [20,21]. Their component levels change during development. At gestation week 10, b-series gangliosides are the dominant gangliosides, with the major ganglioside being GT1b, contributing 40% of the total ganglioside sialic acid. The proportion of ganglioside GD1a, which belongs to the a-series gangliosides, begins to increase around gestation week 12 [22]. These lipids are believed to participate in various neuronal events, including differentiation and survival, signal transduction, synaptic transmission and neural plasticity [13]. Therefore, the appropriate regulation of ganglioside synthesis is thought to be important at various developmental phases in the CNS. In this study, we found increased levels of gangliosides of the a-series, i.e., ganglioside GM2, GM1 and GD1a. Ganglioside GM2 accumulates in the CNS of patients with Tay-Sachs and Sandhoff diseases. Apoptotic neuronal death in mouse models of this GM2 gangliosidosis has been reported, and the accumulated ganglioside GM2 or a derivative is suggested to trigger the apoptotic cascade [23]. Ganglioside GD1a is restricted to Purkinje cells in the cerebellum and associated with dendrite growth and survival of Purkinje cells [24,25]. Those observations imply that the accumulation of gangliosides in PBD is associated with the pathological cause of PBD. In general, the distribution patterns of gangliosides are different between various tissues. In the CNS, the amount of gangliosides is much higher than that in fibroblasts and gangliosides of more complicated structures are contained in the CNS [26,27]. It seems that the change in ganglioside composition in the CNS of PBD patients is more significant and complicated than that in fibroblasts. The analysis of glycolipid composition in the CNS of patients with PBD is required to confirm it.

It has not been identified whether the accumulation of glycosphingolipids is a result of primary peroxisomal dysfunction or that of a secondary event followed by metabolic abnormalities such as VLCFA accumulation and plasmalogen deficiency. Previously, we demonstrated the increased incorporation of [3-14C]serine into sphingolipids and the accumulation of CMH and GM3 in peroxisome-deficient Z65 cells [14]. We also measured the metabolic rate of glycosphingolipids using [3-14C]serine as a tracer in patients' fibroblasts. The incorporation of [3-14C]serine to phosphatidylserine, phosphatidylethanolamine and sphingomyelin in the patients' fibroblasts was greater than that in normal subjects in addition to an accelerated metabolic rate of CMH (data not shown). The expression of ceramide glucosyltransferase, which catalyzes the first glycosylation step of glycosphingolipid synthesis, increased in Z65 cells compared with that in control CHO-K1 cells (in preparation). In PBD patients, the activities of sialytransferase and N-acetylgalactosaminyltransferase, which are involved in the synthesis of a-series gangliosides, are expected to enhance the activity. In our next study we will investigate the relation between peroxisomal dysfunction and the acceleration of ganglioside synthesis in peroxisome-deficient cells.

In conclusion, we demonstrated the accumulation of a-series gangliosides in fibroblasts of patients with PBD due to the PEX2 gene mutation. This finding may help to elucidate the pathogenesis of impaired neuronal cell migration and other abnormalities in the CNS of patients with PBD.
Acknowledgements

This study was supported by Grant 11670743 from the National Institute of Science and Education.

References


