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Alterations in the molecular species of plasmalogen phospholipids and glycolipids due to peroxisomal dysfunction in Chinese hamster ovary-mutant Z65 cells by FABMS method

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Abstract

Changes in the molecular species of lipids associated with Pex2 gene-mutation were investigated to elucidate the pathogeneses of peroxisome biogenesis disorders. Although no differences were observed in the concentrations of cholesterol and phosphatidyl choline between mutated Z65 and control CHO-K1 cells, the amounts of cholesterol esters and glycolipids in Z65 cells were twice those in CHO-K1 cells, but phosphatidyl ethanolamine (PE), particularly 1-*O*-octadec-1′-enyl-2-oleoyl PE, was absent in Z65 cells by FABMS. Enhanced synthesis of glycolipids in Z65 cells was associated with an abundance of lignoceric acid-containing ones, suggesting a role of glycolipids in the retention of longer saturated fatty acids.

Keywords: Peroxisome biogenesis disorder; Pex2-mutation; Ganglioside GM3; Lignoceric acid; Cholesterol esters; Negative ion FABMS

1. Introduction

Peroxisomes are intracellular organelles that participate in oxidative reactions, and their dysfunction in peroxisome biogenesis disorders (PBD) gives rise to severe neurological symptoms, mainly due to the altered lipid composition of neuronal cells [1,2]. The peroxisomal enzymes for the breakdown of fatty acids through α- and β-oxidation, and for the formation of plasmalogens have been known to be retarded in PBD, such as Zellweger syndrome, resulting in the accumulation of very long chain fatty acids and a lack of plasmalogens in nerve cells, followed by abnormal neuronal migration and an increased number of apoptotic cells [3]. Mutations or truncation of the peroxin (Pex) gene-products in PBD has been shown to interfere with the biogenesis of peroxisomes, resulting in empty peroxisomes or an impaired function of peroxisomes in PBD. However, the inability of peroxisomal β-oxidation itself is not the cause of a defect in neuronal migration in Pex5-null mice, Zellweger model mice [4]. The Pex5 protein binds to newly synthesized proteins ending in peroxisome targeting signal 1, and targets them to the peroxisomal membrane, indicating a low correlation of metabolic alterations and pathological changes in PBD. Although the altered lipid composition caused by peroxisomal dysfunction is apparently implicated in the impaired differentiation and migration of neural cells in PBD, it remains unclear what changes are induced by peroxisomal dysfunction that disturb the ordinary organization and function of biomembranes. We previously reported the accumulation of glycosphingolipids in Pex2 gene-mutated Z65 cells and the fibroblasts of patients with PBD [5,6]. Since the synthesis of glycosphingolipids was recently shown to involve the neutralization of the ceramide-mediated

Abbreviations: PBD, peroxisome biogenesis disorders; Pex, peroxin; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; FABMS, fast atom bombardment mass spectrometry; GC–MS, gas liquid chromatography–mass spectrometry; CE, cholesterol esters; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; FCS, fetal calf serum; PBS, phosphate-buffered saline

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apoptotic signals in cells exposed to several stimuli including anticancer drugs and γ-irradiation, our observation also seems to be a molecular event that maintains the homeostasis of membrane structure and function in Z65 cells with Pex2-mutation [7,8]. In this paper, we report a new finding regarding striking differences in the molecular species, particularly of lipids exhibiting altered concentrations, and the finding is thought to be involved in the vulnerability to hydrogen peroxide.

2. Experimental

2.1. Materials

Glycolipids, glucosyl ceramide (GlcCer), lactosyl ceramide (LacCer), ganglioside GM3, and sphingomyelin were purified from human erythrocytes as described previously [9,12], and their *N*-stearoyl derivatives were prepared by deacylation with sphingolipid ceramide *N*-deacylase (*Pseudomonas* sp. TK4), followed by reacylation with stearoyl chloride [9]. Ceramides were prepared by treatment of sphingomyelin with *Clostridium perfringens* phospholipase C (Sigma–Aldrich, St. Louis, MO, USA). A monoclonal anti-GM3 antibody (M2590) was obtained from Seikagaku Co., Tokyo, and an anti-sphingomyelin antibody (VJ-41) was established in our laboratory [10]. Dioleoyl phosphatidyl ethanolamine (PE), glycerol (PG), choline (PC), and serine (PS) were provided by Aswell Co., Osaka, Japan. Fatty acids, fatty acid methyl esters, and cholesterol, cholesterol oleate and 3-(4,5-dimethylthazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Supelco, Bellefonte, PA, USA, and Sigma–Aldrich, respectively.

2.2. Cell lines

CHO-K1 and Z65 cells [11] were cultured in Dulbecco-modified MEM medium, supplemented with 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, in a humidified incubator at 37 °C under a 5% CO_2 atmosphere, and were used for experiments when they reached sub-confluence (80–90%).

2.3. Separation and quantitation of lipids

After lyophilization of cells, total lipids were extracted from the lyophilized powder (55–62 mg) with 2 ml of chloroform/methanol/water (20:10:1, 10:20:1 and 1:1, v/v) sequentially and the extracts were combined. Then the concentrations of cholesterol and lipid-bound phosphorus in the total lipid extracts were determined by gas chromatography with 5α-cholestane as an internal standard and Bartlett's method, respectively [12]. Cholesterol ester, ceramides and phospholipids were developed on TLC plates with *n*-hexane/diethyl ether/acetic acid (80:30:2, v/v), chloroform/methanol/acetic acid (94:1:5, v/v), and chloroform/methanol/water (60:35:8, v/v), respectively, and their concentrations were determined by TLC-densitometry at the analytical wavelength of 500 nm after visualization with cupric acetate-phosphoric acid reagent using cholesterol oleate, *N*-stearoyl sphingosine, dioleoyl phosphatidyl ethanolamine and choline, and *N*-stearoyl sphingosylphosphorylcholine as standards, respectively. Then, the lipid extracts were fractionated into neutral and acidic lipids on a DEAE–Sephadex column (A-25, acetate form; Amersham Bioscience, Uppsala, Sweden). The unabsorbed neutral lipid fraction were evaporated to dryness, and acetylated with 2 ml of acetic anhydride and 3 ml of pyridine at room temperature overnight. The acetylated glycolipids were purified by Florisil column chromatography and then deacetylated with 0.5 M NaOH in methanol at 37 °C for 1 h. After neutralization with 1 M acetic acid in methanol, salts were removed by dialysis [12]. On the other hand, the gangliosides were prepared from the absorbed acidic lipid fraction by cleavage of the ester-containing lipids, followed by dialysis as described above [12]. The gangliosides and neutral glycolipids thus obtained were developed on TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v) and chloroform/methanol/water (60:35:8, v/v), and then visualized with resorcinol–HCl and orcinol–H_2SO_4 reagent, respectively. The density of spots was determined at the analytical wavelengths of 580 nm for resorcinol–HCl-positive spots and 420 nm for orcinol–H_2SO_4-positive spots, respectively, using a dual-wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto). Standard glycolipids: *N*-stearoyl derivatives of GlcCer, LacCer and GM3 (0.1–1.5 μg), were developed on the same TLC plates for the preparation of standard curves for quantitation.

2.4. TLC-immunostaining

The total lipid extracts were applied to plastic-coated TLC plates, which were then developed as above. Each plate was incubated with a blocking buffer (1% polyvinylpyrrolidone (PVP) and 1% ovalbumin in PBS) at 4 °C overnight and then with anti-GM3 or anti-sphingomyelin antibodies in 3% PVP in PBS at 37 °C for 2 h. Afterwards, the plates were washed five times with 0.1% Tween 20 in PBS, and the antibodies bound to the TLC plates were detected using peroxidase-conjugated anti-mouse IgG+M antibodies (Cappel Laboratories, Cochranville, PA), diluted 1:1000 (v/v) with 3% PVP in PBS, and with enzyme substrates H_2O_2 and 4-chloro-1-naphthol, as described previously [9,10,12]. The density of spots was also determined using 10–100 ng of GM3 and sphingomyelin as standards for quantitation with a TLC-densitometer as described above, the limit of detection being 5 ng.

2.5. Structural analysis of lipids

The individual lipids were purified with a silica gel (Iatrobeads 6RS8060; Iatron Lab., Tokyo) column chromatography by gradient elution; *n*-hexane to *n*-hexane/diethyl ether (1:1, v/v), chloroform to chloroform/methanol (1:1, v/v), and chloroform/methanol (1:1, v/v) to methanol. The purified lipids were analyzed by negative ion FABMS (JMS-700TKM; JEOL Ltd., Tokyo) with triethanolamine as a matrix solvent, and by GLC–mass spectrometry (QP-5050A; Shimadzu, Kyoto) as the methyl esters of fatty acids after methanolysis with 1 N HCl in methanol. The amount of plasmalogen-type phospholipids was determined on the basis of the acid-labile property as follows. First, purified phospholipids were applied on a TLC plate, which

was then exposed to HCl vapor for 1 min, followed by development with chloroform/methanol/water (60:35:8, v/v, and were visualized with cupric acetate–phosphoric acid reagent. The densities of intact and lysophospholipids produced from plasmalogen were measured by TLC densitometry with dioleoyl and monooleoyl phospholipids as standards. Second, purified phospholipids (5 μg) were dissolved in chloroform/methanol (1:1, v/v) containing 0.05 M HCl and incubated at 37 °C for 40 min to cleave the alkenyl linkage in plasmalogen. After partitioning with water, the lower phase containing intact and lysophospholipids was analyzed by TLC as above.

2.6. Cell viability assaying with MTT

Z65 and CHO-K1 cells were cultured in 96 well plates at the density of 1×10^4 cells/well for 72 h in the ordinary medium and then exposed to various concentrations of H_2O_2 for 3 h. After exchange of the medium to the ordinary one, the cells were cultured for 24 h, followed by in medium containing 0.1 mg of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) for 4 h, and the resultant insoluble formazan product was dissolved in dimethylsulfoxide. The optical density of each well was measured with a microplate reader at 490 nm, and that for control cells cultured without H_2O_2 was taken as 100% viability.

3. Results

3.1. Lipid compositions of Z65 and CHO-K1 cells

Fig. 1 shows TLC and TLC-immunostaining of the total lipid extracts of Z65 and CHO-K1 cells. Although the amounts of cholesterol, PG and PC/PS in Z65 cells were the same as those in CHO-K1 cells, that of CE in Z65 cells was higher than that in CHO-K1 cells, but PE and sphingomyelin were present at lower concentrations in Z65 cells than in CHO-K1 cells. The results were confirmed by TLC immunostaining with anti-sphingomyelin antibodies, which cross-reacted with disaturated fatty acyl PC [15], the density of disaturated fatty acyl PC being the same in both types of cells, and that of sphingomyelin in CHO-K1 cells being stronger than that in Z65 cells (Fig. 1A and B). On the other hand, in accord with the previous findings [6], GlcCer, LacCer and GM3 in Z65 cells were present in higher concentrations than in CHO-K1 cells (Fig. 1C and D). Then, the

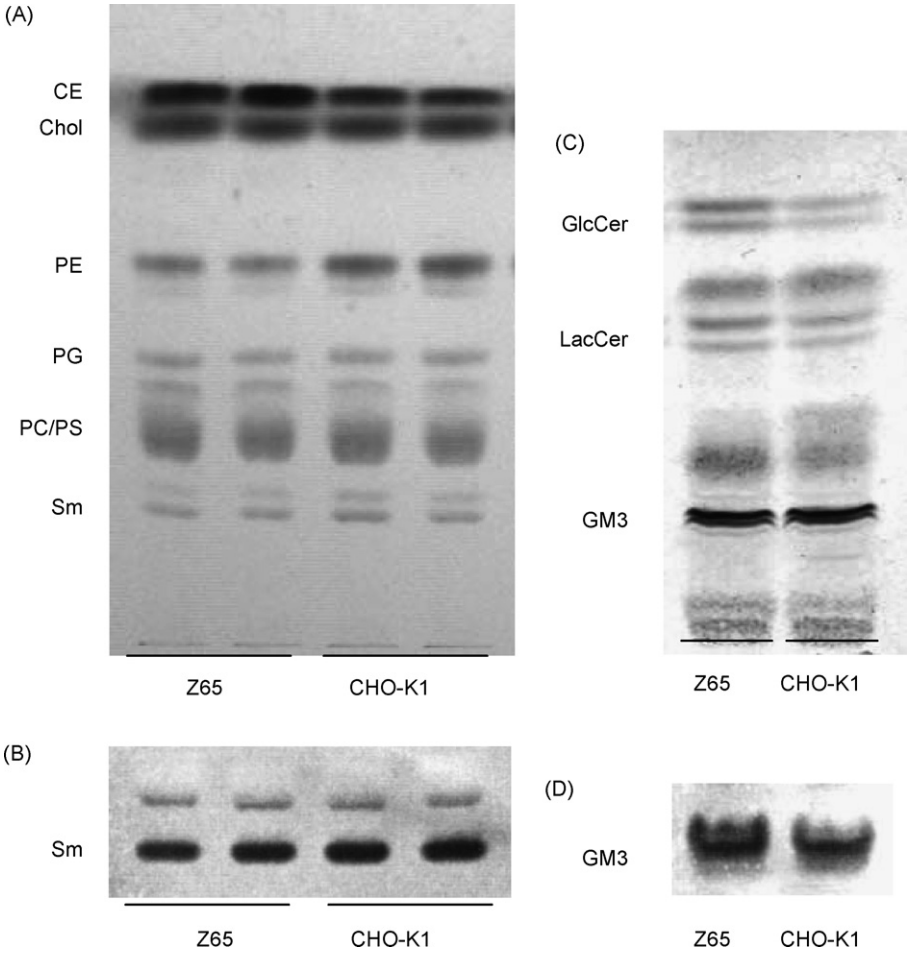

Fig. 1. TLC-and TLC-immunostaining of total lipid extracts of Z65 and CHO-K1 cells. The lipids, corresponding to 0.2 mg of dried cells, were developed on TLC plates with chloroform/methanol/water (60:35:8, v/v) for A and B, and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, v/v) for C and D, and were detected with cupric acetate-phosphoric acid reagent (A), anti-sphingomyelin antibodies (B), orcinol–sulfuric acid reagent (C), and anti-GM3 antibodies (D). Sm, sphingomyelin; Chol, cholesterol; CE, cholesterol ester.

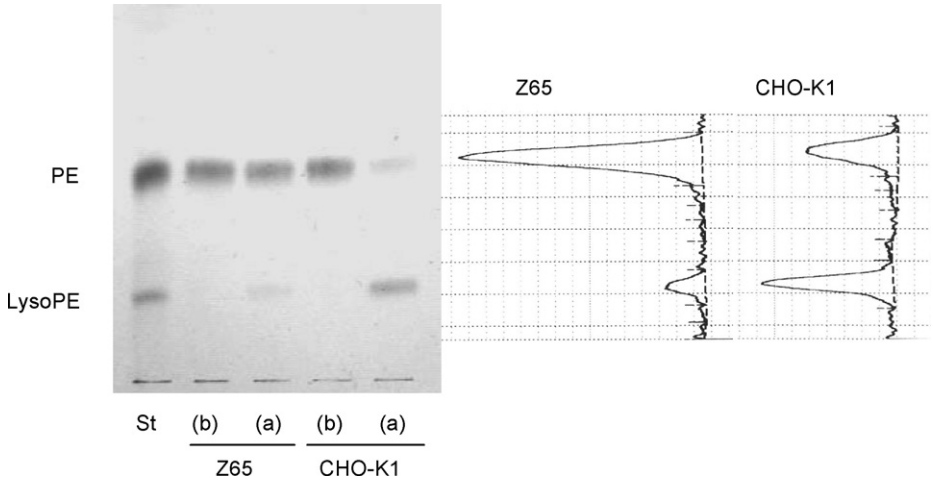

Fig. 2. TLC and TLC scanning of PE before (b) and after (a) treatment with HCl. PEs from Z65 and CHO-K1 cells were incubated in chloroform/methanol (1:1, v/v) containing 0.05 M HCl at 37 °C for 40 min, developed with chloroform/methanol/water (60:35:8, v/v), and then detected with cupric acetate-phosphoric acid reagent. TLC scanning was carried out of PEs after cleavage of the alkenyl linkage with HCl. St, standard mixture; PE, dioleoyl PE; Lyso PE, 1-monooleoyl PE.

proportion of plasmalogen-type in individual phospholipids was determined by acid-mediated cleavage of the alkenyl linkage in plasmalogen. The highest proportion of plasmalogen-type phospholipids in CHO-K1 cells was observed for PE, amounting to about half for PE (Fig. 2).

The concentrations of lipids determined on TLC-densitometry and TLC-immunostaining in Z65 and CHO-K1 cells are shown in Fig. 3. Although no change was observed in the amount of the nonplasmalogen-type of PE between Z65 and CHO-K1 cells, that of the plasmalogen-type of PE in Z65 cells was reduced to 13% of that in CHO-K1 cells. In this connection, the total amount of phospholipids including sphingomyelin in Z65 cells was 84% of that in CHO-K1 cells, and the reduced amounts of phospholipids in Z65 cells was compensated for by an increase in glycosphingolipid, GlcCer, LacCer and GM3, resulting in similar total amounts of phospholipids plus glycosphingolipids, i.e., 11.04 μg for Z65 cells and 11.82 μg for CHO-K1 cells per mg of dried cells. Among glycosphingolipids, the rate of increase of GlcCer was highest, probably due to enhanced transcription of ceramide glucosyltransferase, as reported previously [6]. The total amount of glycosphingolipids in Z65 cells was twice that in CHO-K1 cells, and GM3 comprised 76% of the glycosphingolipids in Z65 cells.

As to neutral lipids, although cholesterol and ceramides in Z65 cells were present in similar amounts to in CHO-K1 cells, the concentration of cholesterol esters in Z65 cells was 2.4 times higher than that in CHO-K1 cells.

3.2. Molecular species of cholesterol esters, GM3 and PE

The molecular species of cholesterol esters and GM3, whose amounts were increased in Z65 cells, were determined by GC–MS (Fig. 4) and FABMS (Fig. 5), respectively. As shown in Fig. 4, palmitic (16:0), oleic (18:1), and stearic (18:0) acids were the major fatty acids of cholesterol esters in both Z65 and CHO-K1 cells, whereas stearic (18:0) and longer chain fatty acids (20–24) were more abundant in Z65 cells than in CHO-K1 cells,

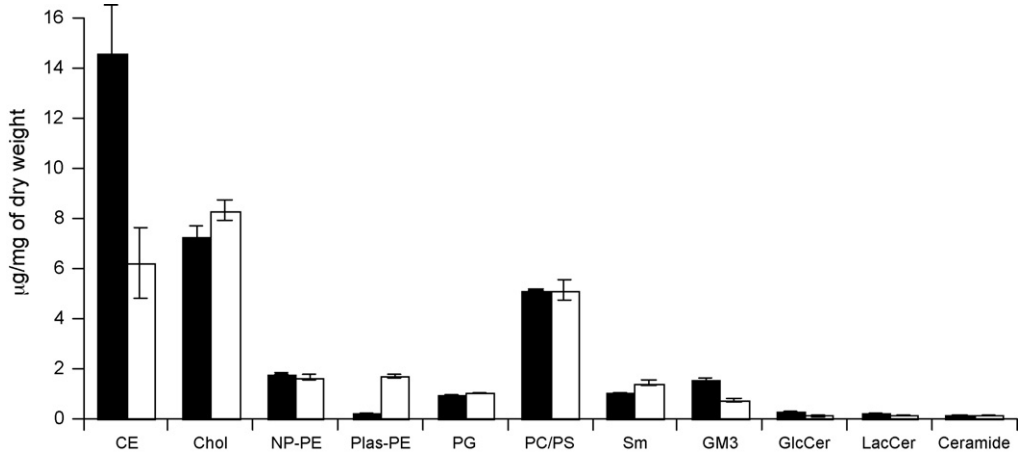

Fig. 3. Lipid compositions in Z65 and CHO-K1 cells. Values are means of individual lipids in three different experiments. Closed column, Z65 cells; open column, CHO-K1 cells; CE, cholesterol ester; Chol, cholesterol; NP-PE, nonplasmalogen type of PE; Plas-PE, plasmalogen type of PE; PG, phosphatidyl glycerol; PC, phosphatidyl choline; PS, phosphatidyl serine; Sm, sphingomyelin.

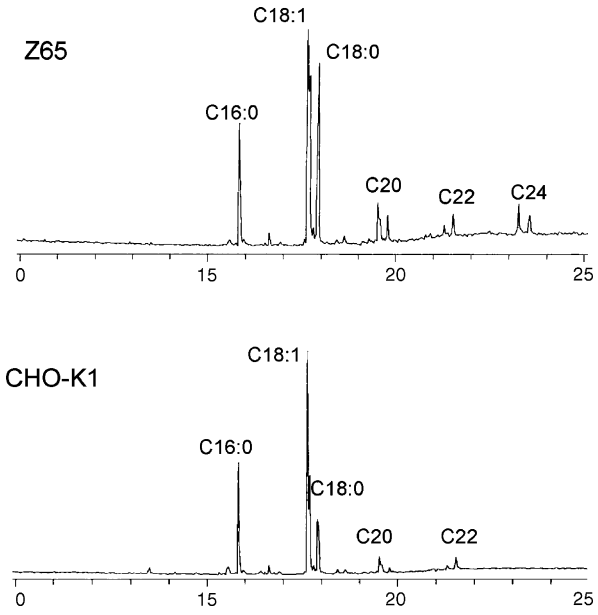

Fig. 4. Total ion chromatograms of fatty acid methyl esters derived from cholesterol esters. Cholesterol esters purified from Z65 and CHO-K1 cells were methanolyzed with HCl in methanol and the liberated fatty acid methyl esters were analyzed by GC–MS. The shoulder peak of oleic acid (18:1) represents the structural isomer as to the double bond.

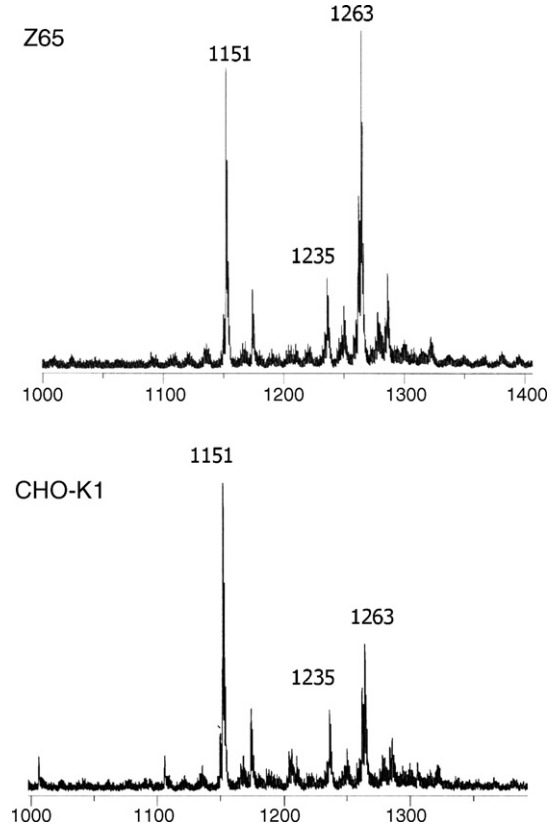

Fig. 5. Negative ion FABMS spectra of GM3 purified from Z65 and CHO-K1 cells. GM3 was analyzed by negative ion FABMS with triethanolamine as the matrix, the molecular ion regions being represented. The ions at *m/z* 1151, *m/z* 1235 and *m/z* 1263 correspond to GM3 with palmitoyl (16:0), behenoyl (22:0), and lignoceroyl (24:0) sphingosine, respectively.

amounting to 45% for Z65 and 21.5% for CHO-K1 cells of the total fatty acids. Also, the ceramide moieties of GM3 mainly comprised palmitoyl (16:0), behenoyl (22:0), and lignoceroyl (24:0) sphingosine, among which the 24:0- containing one was significantly abundant in Z65 cells in comparison with CHO-K1 cells, the content of 24:0 (*m/z* 1263) in GM3 from Z65 cells being twice than that for CHO-K1 cells (Fig. 5). Similar enrichment of lignoceric acid was observed in the molecular species of GlcCer and LacCer in Z65 cells.

On the other hand, the changes in the molecular species of PE in Z65 cells in comparison with those in CHO-K1 cells were dramatic, as shown in Fig. 6. Since oleic acid was found to be the predominant fatty acid of lyso PE yielded on cleavage of the alkenyl linkage on GC–MS, the intense molecular ions at *m/z* 700 and 728 for PE of CHO-K1 cells were identified as those of 1-*O*-hexadec-1′-enyl-2-oleoyl and 1-*O*-octadec-1′-enyl-2-oleoyl PE, but the relative intensities of the ions at *m/z* 700 and *m/z* 728 for PE of Z65 cells were greatly decreased, in particular, no ion at *m/z* 728 being yielded for PE of Z65 cells. As to the molecular species of the diacylated type of PE, when their relative intensities were compared with those of 16:0, 18:1-PE at *m/z* 716 and 18:0, 18:1-PE at *m/z* 744, both of which exhibited similar intensities in Z65 and CHO-K1 cells, the ions at *m/z* 746, 766 and 792 corresponding to 18:0, 18:0-PE, 18:0, 20:4-PE, and 18:0, 22:5-PE, respectively, from Z65 cells exhibited significantly higher intensities than for those from CHO-K1 cells. The fatty acid composition determined by GC–MS of PE remaining after treatment with HCl (Fig. 2) also revealed the higher concentrations of 18:0, 20:4 and 22:5 in PE of Z65 cells than for that of CHO-K1 cells, suggesting that the reduction of plasmalogen-type PE in Z65 cells is affected by the molecular species of PE with higher proportions of polyunsaturated fatty acids, probably for maintenance of the fluidity of biomembranes.

3.3. Viability of cells cultured in the presence of H_2O_2

As shown in Fig. 7, the concentrations of H_2O_2 at which the cell viability was 50%, compared with control cells cultured without H_2O_2, were 1.38 mM for CHO-K1 cells and 0.69 mM for Z65 cells, respectively, indicating that Z65 cells are more sensitive to H_2O_2 than CHO-K1 cells. The sensitivity of Z65 cells to H_2O_2 was thought to be partly correlated with the reduced amount of plasmalogen, which traps peroxide, and with the increase in polyunsaturated fatty acids sensitive to peroxide.

4. Discussion

The major function of amphipathic lipids, i.e., cholesterol, phospholipids and glycolipids, is to form the lipid bilayer, whose physicochemical properties, including membrane fluidity, are determined by the concentrations of membrane constituents and the molecular species of phospholipids and glycolipids, and they are essential to maintain several membrane-mediated processes, such as ion transport, signal transduction and cellular migration.

Peroxisomes are one of the main sites in the cell where oxygen-free radicals are both generated and scavenged. Hydrogen peroxide in peroxisomes is mainly formed by oxidases that

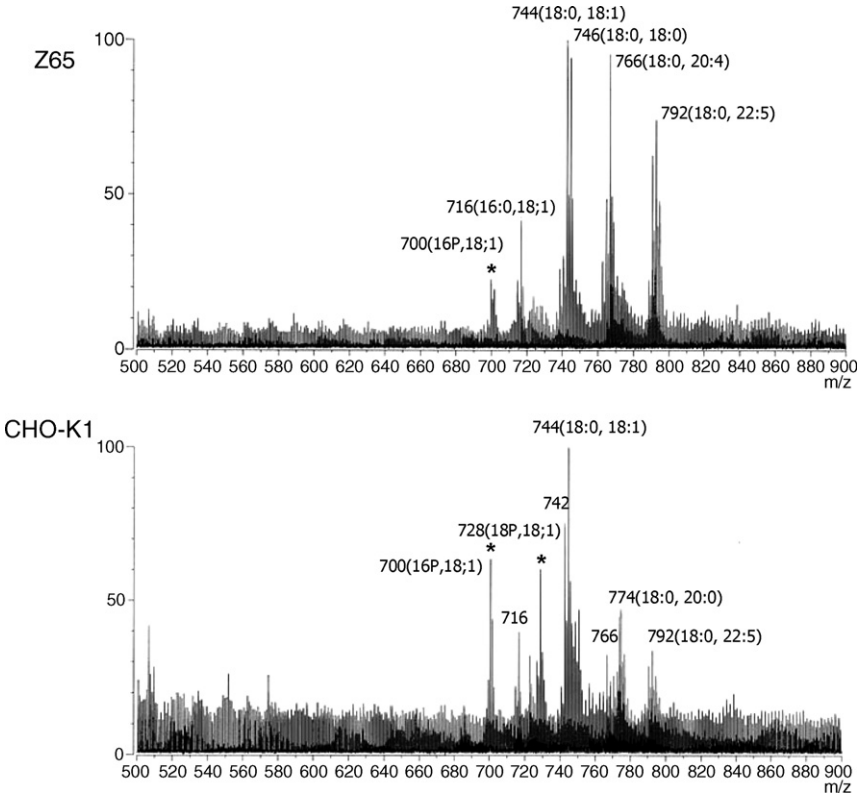

Fig. 6. Negative ion FABMS spectra of PE purified from Z65 and CHO-K1 cells. PEs were analyzed by negative ion FABMS with triethanolamine as the matrix, the molecular ion regions being represented. Asterisks denote the ions for plasmalogen type of PE, 1-O-hexadec-1′-enyl-2-oleoyl PE at *m/z* 700 (16P, 18:1) and 1-O-octadec-1′-enyl-2-oleoyl PE at 728 (18P, 18:1), respectively.

transfer hydrogen from metabolites to molecular oxygen. Peroxisomes contain a battery of anti-oxidant enzymes to prevent damage by free radicals derived from hydrogen peroxide. In PBD, both the generation and scavenging of oxygen-free radicals are impaired, which leads to a functional loss of various enzymes such as acyl CoA oxidase and catalase. Accordingly, failure in fatty acid metabolism due to peroxisomal dysfunction is deemed to bring about impaired physicochemical properties of biomembranes as a membrane disorder in the neuronal cells in PBD.

The present research was undertaken to disclose the effects of Pex2-mutation on the concentrations and molecular species of lipids to elucidate the pathogeneses of PBD. As shown in Fig. 3, reduced amounts of phospholipids, particularly of plasmalogen-type PE, were evidently observed in Pex2-mutated Z65 cells, and were compensated for by increased amounts of glycosphingolipids, resulting in similar amounts of phospholipids plus glycolipids in Z65 and CHO-K1 cells. Increases in the relative concentrations of glycolipids were proven to be due to the enhanced transcription of ceramide glucosyltransferase, an enzyme involved in the initial step of glycolipid synthesis, probably in response to the retarded synthesis of phospholipids [6]. The metabolism of glycolipids is known to be utilized for the regulation of various cellular functions. For example, an increase in the transcription of ceramide glucosyltransferase was shown to be closely associated with resistance to anticancer drugs through removal of ceramide to escape from ceramide-mediated apoptosis [13]. In Z65 cells, enhanced synthesis of glycolipids, together with that of cholesterol esters [17], might be useful for removing saturated longer chain fatty acids, which accumulate on impaired oxidation due to peroxisomal dysfunction, and perturbed lipid bilayer packing and functioning of acylated proteins [14–16]. Since the amount of lignoceric acid (24:0) in glycolipids was higher than that in cholesterol esters in Z65 cells, the elevated syntheses of GlcCer, LacCer and GM3 with a higher

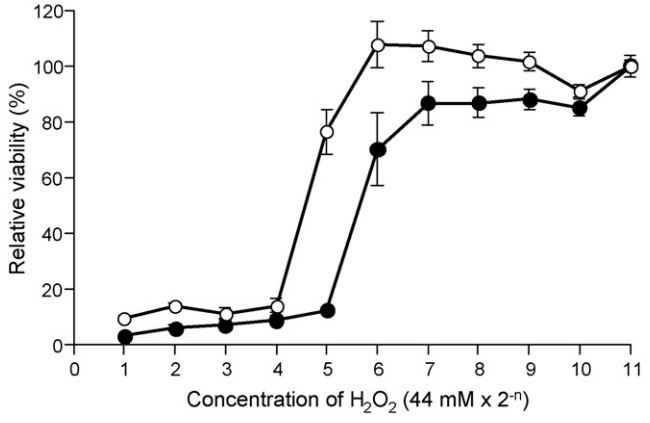

Fig. 7. MTT assaying of viable cells cultured in the presence of hydrogen peroxide. Cell viability was expressed in comparison with the optical densities of Z65 and CHO-K1 cells cultured in the ordinary medium without H_2O_2. ○, CHO-K1; ●, Z65 cells.

proportion of lignoceric acid were quite effective for retaining it as conjugated molecules in membrane constituents. A slight decrease in sphingomyelin was considered to be due to enhanced glycosphingolipid metabolism, as reported previously [5,6,18].

On the other hand, as it is well known for cells in PBD, the amount of plasmalogen-type PE in Z65 cells was 13% of that in CHO-K1 cells [19,20]. Among the molecular species, 1-*O*-octadec-1′-enyl-2-oleoyl PE, which was abundant in PE of CHO-K1 cells, disappeared from PE of Z65 cells. In addition, the amounts of the nonplasmalogen-type of PE were similar, but the molecular species were found to be strikingly different between Z65 and CHO-K1 cells. A significant increase in polyunsaturated fatty acid-containing molecular species, 18:0, 20:4-PE and 18:0, 22:5-PE, in Z65 cells was noticeable in addition to an increase in a disaturated fatty acyl PE, 18:0, 18:0-PE. The dien bonds in arachidonic (20:4) and docosapentaenoic (22:5) acids belonging to the omega 6- polyunsaturated fatty acid family are generally susceptible to attack by free radicals, and accordingly the decreased amount of plasmalogen was thought to be compensated for by an increase in polyunsaturated fatty acids-containing molecular species. Also, since the relative amount of arachidonic acid in the total fatty acids obtained on methanolysis of all lipid classes in Z65 cells was decreased [18], it might be highly concentrated in PE present in the inner leaflet of the lipid bilayer of the plasma membrane. Peroxidation of polyunsaturated fatty acids was shown to produce very reactive species, the activity modifying several proteins and lipids [21,22], suggesting that oxidative stress, and subsequent modification of membrane proteins and lipids may be relevant to the cellular disorders in PBD. In fact, antioxidant vitamin E was shown to restore the oxidative imbalance and to normalize peroxisomal functions in fibroblasts in patients with milder forms of PBD [23]. As described above, the plasmalogen type of PE was an effective endogeneous antioxidant in the plasma and peroxisomal membranes, and played a role in preventing the oxidation of polyunsaturated fatty acids in membranes [24]. Thus, the vulnerability of Z65 cells to oxidative stress due to hydrogen peroxide is partly implicated the alteration of the molecular species of PE with loss of plasmalogen and enrichment of polyunsaturated fatty acids. Further studies should be performed to determine whether or not there are increases of lipid peroxides in the membranes of Z65 cells. Also, an increase in the polyunsaturated fatty acids in membrane phospholipids has been reported to modify the affinity of receptors toward their signaling molecules and the activity of membrane enzymes [25–27]. Consequently, the alteration in the molecular species of membrane lipids brought about by the peroxisomal dysfunction in cultured cells is probably relevant to the pathogeneses of PBD, and the analyses of the molecular species of membrane lipids in Pex-gene-mutated mice is now in progress to clarify the biochemical events *in vivo* in our laboratory.

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