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K Inui, … , S Okada, H Yabuuchi

Research Article

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Metabolism of Cerebroside Sulfate and Subcellular Distribution of Its Metabolites in Cultured Skin Fibroblasts from Controls, Metachromatic Leukodystrophy, and Globoid Cell Leukodystrophy

Koji Inui, Masumi Furukawa, Shintaro Okada, and Hyakuji Yabuuchi
Department of Pediatrics, Osaka University Medical School, Osaka 553, Japan

Abstract

With pulse-chase study of 1-[^{14}C]stearic acid-labeled cerebroside sulfate (^{14}C-CS) and subsequent subcellular fractionation by Percoll gradient, the metabolism of CS and translocation of its metabolites in human skin fibroblasts from controls, metachromatic leukodystrophy (MLD), and globoid cell leukodystrophy (GLD) were studied. In control skin fibroblasts, CS was transported to lysosome and metabolized there to galactosylceramide (GalCer) and ceramide (Cer) within 1 h. During the chase period, radioactivity was increased at plasma membrane plus Golgi as phospholipids and no accumulation of GalCer or Cer was found in lysosome. In MLD fibroblasts, 95% of ^{14}C-CS taken up was unhydrolyzed at 24 h-chase and accumulated at not only lysosome but also plasma membrane. In GLD fibroblasts, GalCer was accumulated throughout the subcellular fractions and more accumulated mainly at plasma membrane plus Golgi with longer pulse. This translocation of lipid from lysosome seems to have considerable function, even in lipidosis, which may result in an imbalance of the sphingolipid pattern on the cell surface and these changes might be one of causes of neuronal dysfunction in sphingolipidosis.

Introduction

Sphingolipids are important membrane lipids and particularly abundant in neuronal membranes. As outer surface membrane constituents of animal cells, they play important roles, including membrane stability, nerve conduction, receptor action, oncogenic transformation and other functions that are necessary for growth, differentiation, and survival (1–3). Cerebroside sulfate (CS),[1] galactocerebroside with a sulfate esterifying carbon-3 of the galactosyl moiety, is one of myelin constituents and has an important role during developmental period encompassing myelination (4). The metabolic pathway of this lipid is well known and genetic defects of the catabolism at

each step result in metachromatic leukodystrophy (MLD), globoid cell leukodystrophy (GLD), and Farber disease. Due to enzyme deficiencies, massive lysosomal storage of lipids is demonstrated in MLD (4) and Farber disease (5). In GLD it is well known that there is no accumulation of galactosylceramide and that the major abnormalities are the presence of a large number of globoid cells, a severe lack of myelin and astrogliosis in the nervous system (6).

The enzymic defects of most lysosomal storage disorders have been clarified, but the molecular mechanisms that lead to the clinical and pathological manifestations remain largely obscure. Recent morphological studies of neurons from humans (7), cats (8), and dogs (9) with gangliosidoses have shown meganeurites, inappropriate proliferation of secondary neurites, aberrant synaptogenesis, somatic swelling, and abnormal somatic processes. Biochemically, impaired neurotransmitter metabolism was suggested in cerebral cortical and cerebellar synaptosomes from cats with GM$_1$ gangliosidosis (10) and increased amount of ganglioside in synaptosomal membranes from feline GM$_1$ and GM$_2$ gangliosidosis was presented (11). These studies suggested that altered membrane structure could be one of causes of neuronal dysfunction.

To understand the mechanism causing neurological dysfunction in sphingolipidoses, clarification of the synthesis, translocation and insertion of sphingolipids in the different subcellular components in normal and pathological states is essential. In the present study, using 1-[^{14}C]stearic acid-labeled cerebroside sulfate (^{14}C-CS), we studied the metabolism of CS and the subcellular distribution of its metabolites in cultured skin fibroblasts from normal control, MLD, and GLD. Our findings indicated that enzyme-deficient cells stored lipid in both lysosomes and plasma membranes, suggesting that the altered lipid composition of membrane is a possible pathogenesis of neuronal dysfunctions in sphingolipidoses.

Methods

Materials. Chemicals were purchased from the indicated sources: Eagle's minimum essential medium (Nissui Seiyaku Co., Tokyo, Japan); HRP-wheat germ agglutinin (Seikagaku Co., Tokyo, Japan); ovalbumin grade V (Sigma Chemical Co., St. Louis, MO); Percoll and density marker beads (Pharmacia Fine Chemicals, Uppsala, Sweden); cerebroside sulfate (Supelco, Inc., Bellefonte, PA); phospholipid kit (Serdary Research Laboratories, Inc., Ontario, Canada); choline-methyl-[^{14}C]sphingomyelin, 1-[^{14}C]stearic acid and UDP-[^{14}C(U)]-galactose (New England Nuclear, Boston, MA); 4-methylumbelliferyl glycosides (Koch-Light Laboratories, Colnbrook, United Kingdom).

All other reagents were purchased from standard commercial suppliers.

Preparation of lipids. ^{14}C-CS was prepared according to the method of Dubois et al. (12) and purified by silica gel column chromatography and preparative TLC. The radiopurity was 99% checked by TLC.

Cell culture. Human skin fibroblasts were grown from forearm skin

1. *Abbreviations used in this paper:* Cer, ceramide; CS, cerebroside sulfate; ^{14}C-CS, 1-[^{14}C]stearic acid-labeled cerebroside sulfate; FFA, stearic acid; GalCer, galactosylceramide; GLD, globoid cell leukodystrophy; MLD, metachromatic leukodystrophy; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

biopsies with Eagle's minimum essential medium supplemented with 10% fetal calf serum, glutamine (5 mM), and fungizone (25 μg/ml) (complete medium) and maintained in a 5% CO_2 incubator at 37°C. The cell lines from two patients with infantile GLD were supplied by Dr. K. Suzuki, Albert Einstein College (New York) and Dr. D. A. Wenger, University of Colorado Health Sciences Center (Denver, CO). The other cell lines from control subjects, patients with late infantile MLD and infantile GLD were established in this laboratory with informed consents.

CS feeding experiments. ^{14}C-CS was dissolved in the medium as described by Kudoh et al. (13) with minor modification (lipid concentration changed to 10 nmol/ml). In some CS feeding experiments, FCS was omitted. For pulse and chase studies, cells were incubated with 4 ml of complete medium (40 nmol, specific activity of ^{14}C-CS; 15,000 dpm/nmol) for 1 h and the feeding was terminated by changing the medium to 10 ml of fresh complete medium without ^{14}C-CS. Sterile condition was maintained throughout the experiments. After the chase period, the cells were washed three times with PBS, then detached by incubation with 0.25% trypsin for 3 min at 37°C. To neutralize trypsin, 1% BSA in PBS was added. The cells were harvested by centrifugation and washed three times with PBS. Without this procedure, about 1.3 times higher radioactivity was detected in the cells. The increased radioactivity was found to be solely unhydrolyzed ^{14}C-CS detected in plasma membrane plus Golgi fraction. This washing procedure effectively removed the attached ^{14}C-CS from cell surface.

Subcellular fractionation. Fractionation was conducted using the self-forming gradient medium (Percoll). Cell pellets obtained by the method described above were suspended in 0.5 ml of 0.25 M sucrose solution containing 1 mM EDTA adjusted to pH 7.0 with 1 M KOH and homogenized with 10 strokes in a Duall homogenizer (Kontes Co., Vineland, NJ). Aliquots were taken for determination of protein and radioactivity to calculate the amount of lipids taken up by the cells. Then the homogenates were centrifuged at 800 g for 10 min, the supernatant reserved and the pellet was rehomogenized with five strokes in the same way. After recentrifugation at 800 g for 10 min, both supernatants were combined (0.9 ml) and layered on 8 ml of Percoll solution in 0.25 M sucrose solution with a density of 1.07 g/ml. Then the samples were subjected to centrifugation at 33,000 g for 50 min at 4°C with a RP-65 rotor in an ultracentrifuge (55P-7S; Hitachi, Ltd., Tokyo, Japan). After centrifugation, 24 fractions (10 drops, ∼ 0.4 ml for a fraction) were collected from top by a density gradient fractionator (Advantech Toyo, Ltd., Tokyo, Japan). By these procedures, ∼ 15% of total β-glucuronidase activity was found in fractions 1–3 (cytosol fraction) and almost the same percentage of β-glucosidase was found in fractions 4–7 (plasma membrane plus Golgi fraction). Therefore these enzymes leaked due to the rupture of intact lysosomes. In some experiments aliquots (0.25 ml) of fractions 4–7 of the first Percoll gradient were combined and subjected to the second Percoll gradient by the methods of Merion et al. (14) with minor modification. Briefly, the combined sample (1.0 ml) was layered on 8 ml of a Percoll solution in 0.25 M sucrose solution (density 1.044 g/ml) and centrifuged at 33,000 g for 20 min. 24 fractions were collected as described above.

Enzyme and plasma membrane marker assays. Galactosyltransferase was measured as a Golgi apparatus marker enzyme as described by Lipsky et al. (15). The lysosomal enzyme, β-hexosaminidase or β-glucuronidase, was assayed as described previously (16). To determine a plasma membrane fraction, monolayer cultures were incubated for 30 min at 2°C with HRP-labeled wheat germ agglutinin instead of ^{125}I-wheat germ agglutinin used by Lipsky et al. (15), washed three times with PBS and then subjected to the subcellular fractionation by the first Percoll gradient. The HRP activity was measured using 4-aminoantipyrine (0.2 mg/ml) and phenol (0.1 mg/ml) containing 0.015% hydrogen peroxide as substrate.

Extraction and analysis of lipids. To extract lipids, pellets obtained by trypsinization were homogenized with 0.2 ml of distilled water in a small Duall homogenizer, and added 0.8 ml of chloroform/methanol (2:1, by volume). The mixture was vortexed, centrifuged at 1,000 g for 5 min and the lower phase was transferred to a small tube. After evaporation under nitrogen stream, cellular lipid extracts were analyzed by silica gel TLC (HPTLC; Merck & Co., Rahway, NJ) with chloroform/methanol/water (70:30:4, by volume) and the plate exposed to x-ray film (Fuji RX) for 7–14 d as described by Kudoh et al. (13). For lipid extract from subcellular fractions, every three fractions starting from a light density were combined to make eight fractions after the assay of marker enzymes. These eight fractions were ultracentrifuged at 100,000 g for 90 min at 4°C to remove Percoll. The supernatant was taken into a glass tube and the surface of pellet was rinsed with small amount of distilled water. Then 4 ml of chloroform-methanol (2:1, by volume) was added, well vortexed and kept standing overnight. Then the upper phase was removed by centrifugation, 2 ml of the theoretical upper phase was added. After mixing and sitting overnight, the lower phase was taken to a small tube and evaporated under nitrogen stream. The recovery of radioactivity by this procedure was consistently > 95%. Extracted lipids were analyzed by the method described above.

For the quantification of lipid, each radioactive area on the plate was carefully scraped by tracing the autoradiogram and the radioactivity (expressed by disintegrations per minute) was measured using a scintillation counter (Mark III; Tracor Analytic, Chicago, IL) after addition of 4 ml of ACN II (Amersham Corp., Ontario, Canada). The amount of each lipid is expressed as a percentage of total radioactivity taken up by the cells.

For the identification of radioactive lipid, each radioactive spot on the plate was scraped, eluted from silica gel and cochromatographed with standard lipids in two-dimensional TLC. After exposure to an x-ray film, the radioactive spot was compared to the standard lipids visualized with 50% sulfuric acid spray.
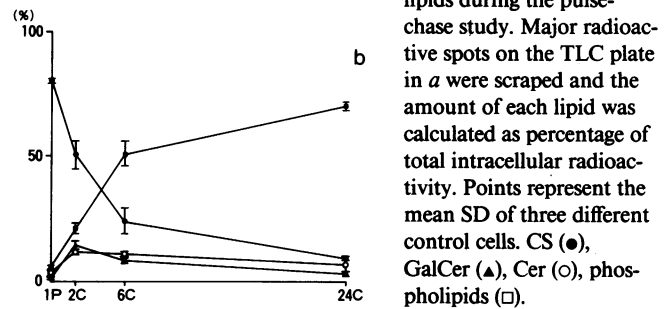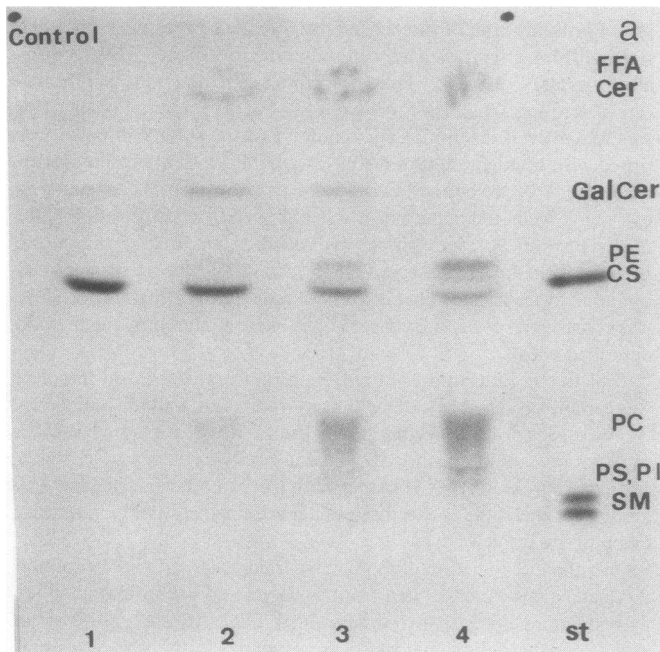
Protein determination. Protein was estimated by the method of Lowry et al. with BSA as standard (17).

Results

Uptake of ^{14}C-CS. In the pulse studies the uptake of ^{14}C-CS by cultured cells from controls and patients with sphingolipidoses ranged 8.3 to 14 nmol/mg cell protein in 24 h and 0.3 to 2.3 nmol/mg cell protein in 1 h. The amount of ^{14}C-CS taken up varied with cell growth, but not with the pathological condition.

[14]C-CS metabolism in control skin fibroblasts. The metabolic fate of ^{14}C-CS in 1 h-pulse and following 2-, 6- and 24-h-chase experiment was examined by TLC (Fig. 1 a). Major radioactive metabolites of CS were identified as stearic acid (FFA), ceramide (Cer), galactosylceramide (GalCer), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS) by two-dimensional TLC. These results were compatible with those reported by Kudoh et al. (13). ^{14}C-CS taken up by the cells in 1-h pulse (1P) (mean 0.41 nmol/mg cell protein) was hydrolyzed at the rate of 0.07 nmol/h per mg protein in the initial 2-h chase (2C) (Fig. 1 b). The decrease of radioactivity of CS is parallel to the increase of radioactivity of total phospholipids (PE, PC, PI, and PS). Neither GalCer nor Cer was accumulated (Fig. 1 b).

To investigate the subcellular distribution of radioactive lipids, cultured skin fibroblasts were incubated with ^{14}C-CS for 24 h and then homogenates of cells were fractionated in an isoosmotic Percoll gradient. By this method, fractions containing lysosomal marker enzymes (i.e., β-glucuronidase) could be clearly separated from a membrane fraction (HRP-wheat germ agglutinin as plasma membrane marker) and a Golgi fraction (galactosyltransferase) (Fig. 2 a). Radioactivity was mainly found in plasma membrane or Golgi fraction and identified as phospholipids by TLC. To examine precise sub-

Figure 1. (*a*) Autoradiogram of lipids extracted from the control cultured skin fibroblasts after the pulse-chase study of ^{14}C-CS: cells were pulsed with 40 nmol of ^{14}C-CS for 1 h (lane *1*) and chased for 2 h (lane *2*), 6 h (lane *3*), and 24 h (lane *4*). At each time point, lipids were extracted, separated and visualized by autoradiogram described in Methods. st: standard radioactive CS and sphingomyelin. (*b*) The amount of radioactivities of major lipids during the pulse-chase study. Major radioactive spots on the TLC plate in *a* were scraped and the amount of each lipid was calculated as percentage of total intracellular radioactivity. Points represent the mean SD of three different control cells. CS (●), GalCer (▲), Cer (○), phospholipids (□).

cellular location of radioactivity, the second Percoll gradient was performed with the pooled fractions from 4 to 7 of the first Percoll gradient, and the main radioactive peak was cofractionated with the plasma membrane marker (Fig. 2 *b*).

For the study on the intracellular translocation of lipids, Percoll gradient method following the pulse-chase experiment was introduced. ^{14}C-CS was loaded for 1 h and chased for 2, 6, 24, and 72 h. At each time point cell homogenates were sub-
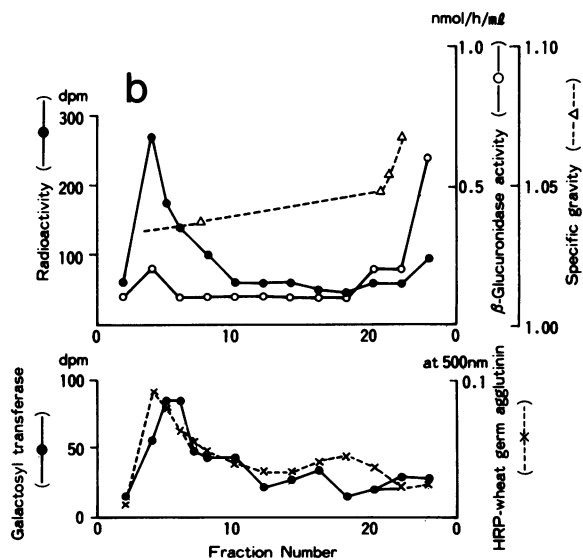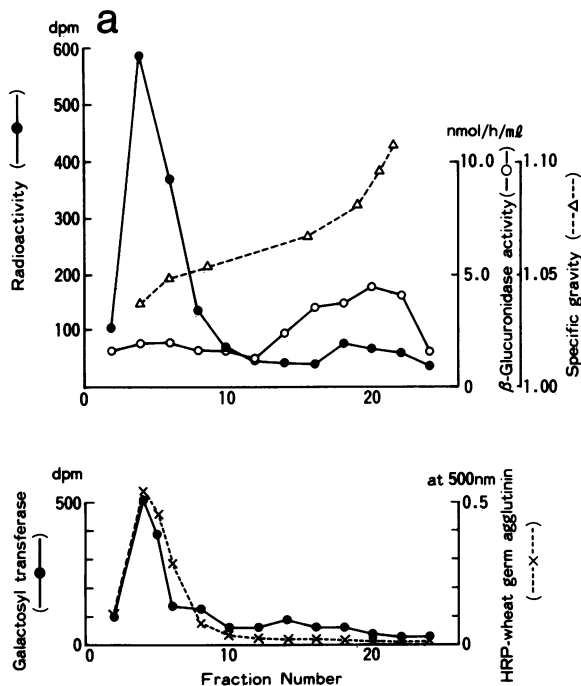

Figure 2. Percoll gradient profiles of radioactivity, enzymic activity and markers of subcellular fractions: control cells were pulsed for 24 h with 40 nmol of ^{14}C-CS and subjected to the first Percoll gradient (density 1.07 g/ml) (*a*) and then fractions 4–7 (plasma membrane plus Golgi fraction) in the first gradient was subjected to the second Percoll gradient (density 1.044 g/ml) (*b*) as described in Methods. Fractions were collected from top and activities were determined. Radioactivity (●), β-glucuronidase (○), specific gravity (△), galactosyltransferase (Golgi marker) (●), HRP-wheat germ agglutinin (plasma membrane marker) (×).
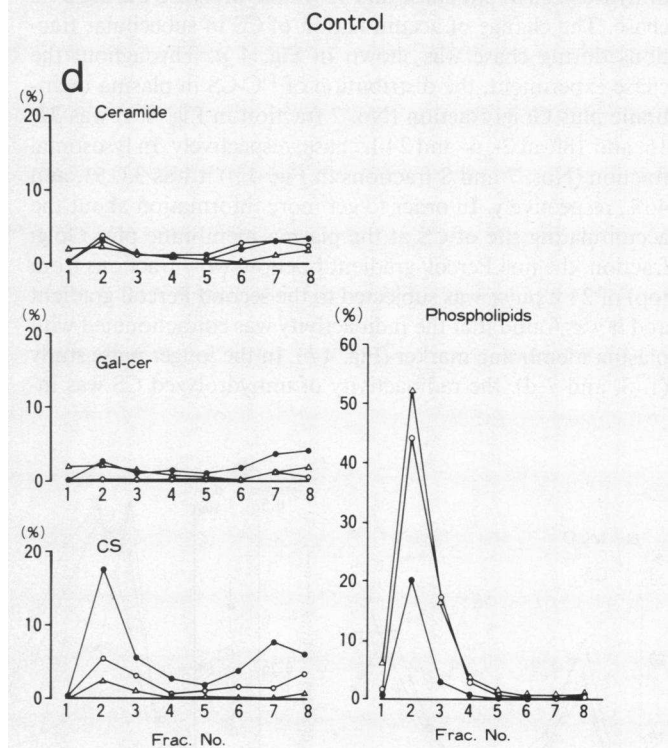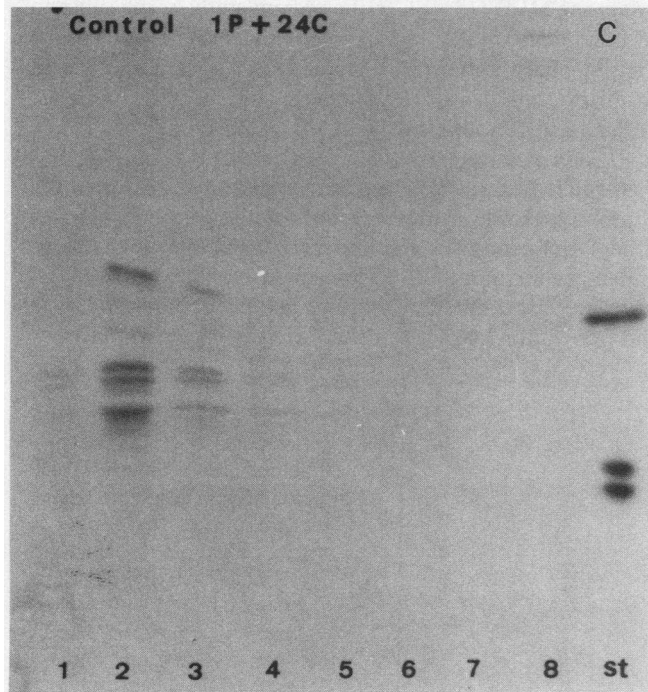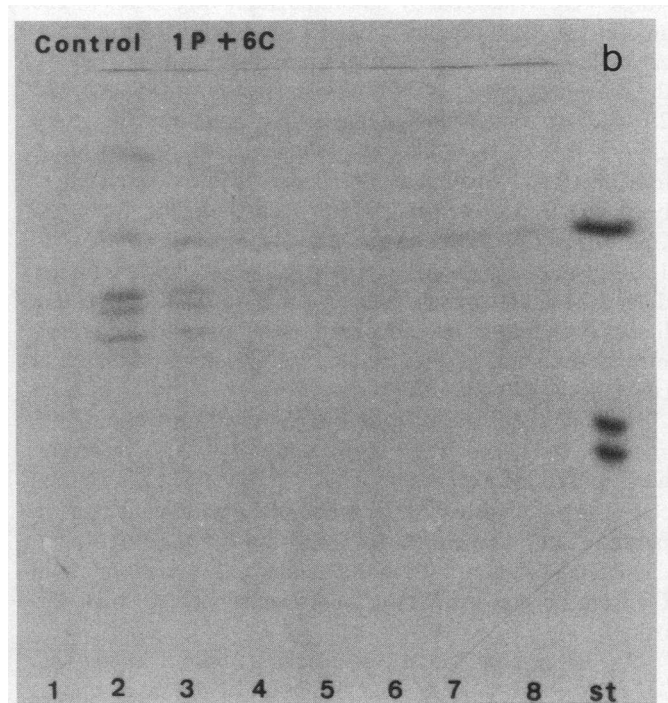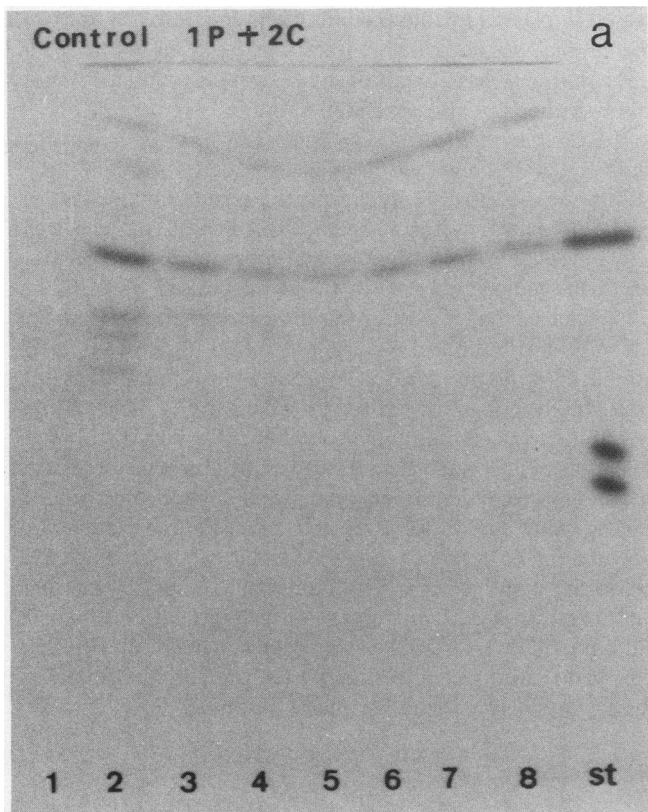
Figure 3. Autoradiogram (*a–c*) and distribution pattern of radioactivity of major lipids extracted from the subcellular fractions (*d*) in control cultured skin fibroblasts after the pulse-chase study of ¹⁴C-CS: cells were pulsed for 1 h with 40 nmol of ¹⁴C-CS and chased for 2 h (*a*, ● in *d*), 6 h (*b*, ○ in *d*) and 24 h (*c*, △ in *d*). At each time point cell homogenates were subjected to the first Percoll gradient. After fractions were collected from top and marker enzymes were assayed, every three fractions starting from a light density were combined to make eight fractions. Lipids were extracted, separated and visualized as described in Methods (*a–c*). Each radioactive area on the plate was scraped and the amount of the individual lipid in subcellular fractions was calculated as percentage of total radioactivity (*d*). No. 2 fraction in *a–d* was corresponded to plasma membrane plus Golgi and No. 7 and No. 8 in *a–d* corresponded to lysosome. *st;* standard radioactive CS and sphingomyelin.

jected to the first Percoll gradient. After lysosomal enzymic activities were assayed to confirm the gradient, every three fractions from a light density were combined to extract lipids, which were visualized by autoradiograph (Fig. 3 a–c). The amount of the individual lipid in subcellular fractions was calculated as percentage of total intracellular radioactivity. In the chase experiment, ^{14}C-CS was decreased from 18% (2 h) to 2.5% (24 h), whereas total phospholipids were increased from 20% (2 h) to 52% (24 h) in plasma membrane plus Golgi fraction (No. 2 fraction in Fig. 3 d). On the other hand in lysosomal fraction (Nos. 7 and 8 fractions in Fig. 3 d), CS, GalCer, and Cer were decreased from 14, 7.6, and 6.0% (2 h) to 1.1, 0.4, and 2.8% (24 h), respectively. Phospholipids were not detected in lysosomal fraction. The pattern of radioactive lipids was found to be unchanged in the longer chase experiment than 24 h. This lipid pattern was also identical to that in control cells without CS feeding.

When ^{14}C-CS was dissolved in the medium without FCS and added to the control cells, the amount of ^{14}C-CS taken up was same to the experiment with FCS, but this CS remained completely unhydrolyzed. Its accumulation was elucidated in plasma membrane plus Golgi fraction using the Percoll gradient. Thus, without FCS in the medium, the translocation of CS from plasma membrane to lysosome seemed to be impaired.

^{14}C-CS metabolism in skin fibroblasts from a patient with MLD. After 1 h-pulse of ^{14}C-CS, 97% of ^{14}C-CS taken up was unhydrolyzed at 2 h-chase and 95% unhydrolyzed during 24 h chase. The change of accumulation of CS in subcellular fractions during chase was shown in Fig. 4 a. Throughout the chase experiment, the distribution of ^{14}C-CS in plasma membrane plus Golgi fraction (No. 2 fraction in Fig. 4 a) was 22, 16, and 18% at 2-, 6- and 24-h chase, respectively. In lysosomal fraction (Nos. 7 and 8 fractions in Fig. 4 a) it was 33, 51, and 46%, respectively. In order to get more information about the accumulating site of CS at the plasma membrane plus Golgi fraction, the first Percoll gradient fractions (4–7 fractions from top) of 24 h pulse was subjected to the second Percoll gradient and it was found that the radioactivity was cofractionated with plasma membrane marker (Fig. 4 b). In the longer pulse study (1, 3, and 7 d), the radioactivity of unhydrolyzed CS was in-

creased in plasma membrane plus Golgi fractions (34 to 51%) whereas that was decreased in lysosomal fractions (44 to 27%). This confirmed that unhydrolyzed CS was accumulated in not only lysosome but also plasma membrane.

^{14}C-CS metabolism in skin fibroblasts from patients with infantile GLD. Three cell lines from patients with infantile GLD were used in this study and the results obtained were very similar among those lines. Typical TLC pattern was shown in Fig. 5 a–c. In 1-h pulse and subsequent chase experiments, the radioactivity of ^{14}C-CS was decreased (from 60 to 25%), whereas that of GalCer was almost unchanged (from 26 to 28%). Distribution of GalCer was found to be in bimodal fashion, showing two peaks at No. 2 and No. 6 fractions at 2-h chase and the curve was getting plateau during 24-h chase (Fig. 5 d). It is suggested that unhydrolyzed GalCer was distributed to all subcellular fractions almost evenly during 24-h chase study. In order to determine a precise site of accumulation of GalCer, pulse studies for 1, 3, and 7 d were undertaken. As shown in Fig. 6, in proportion to the length of pulse period, the radioactivity of GalCer in No. 2 fraction (plasma membrane plus Golgi) was clearly increased and that of GalCer in Nos. 7 and 8 fractions (lysosome) was decreased. This result strongly indicated that an accumulating site of unhydrolyzed GalCer was plasma membrane rather than lysosome.

Discussion

Previous studies using radiolabeled CS were mainly concerned with diagnosis and characterization of the genetic heterogeneity among MLD (13, 18). In the present study, using subcellular fractionation method by Percoll gradient after pulse-chase study, we have examined the metabolism of CS and subcellular distribution of its metabolites in cultured skin fibroblasts from controls and patients with MLD and GLD.

^{14}C-CS dissolved in the medium with or without 10% FCS was equally taken up in a trypsin resistant way by cultured skin fibroblasts. However translocation of ^{14}C-CS to lysosome was strongly influenced by the presence of FCS. When FCS was present in medium, ^{14}C-CS taken up by control skin fibroblasts was translocated to lysosome and metabolized to GalCer and Cer within 1 h. Then, the radioactive phospholipids were
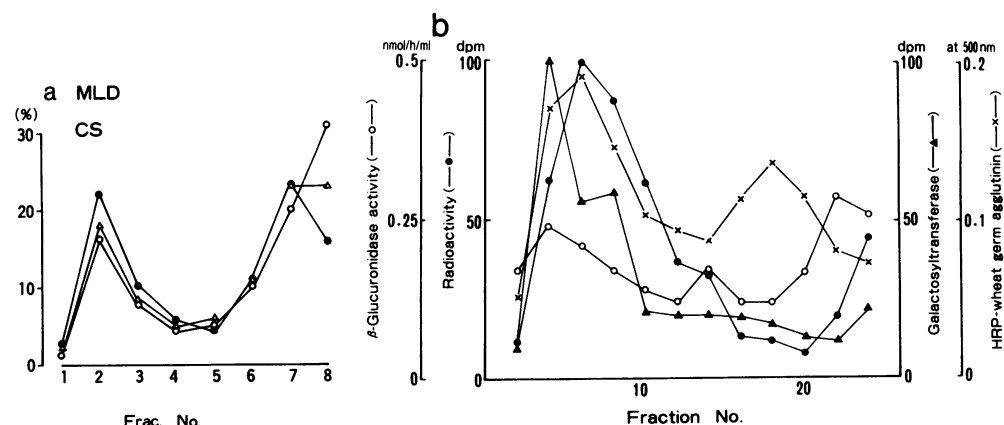

Figure 4. (a) The accumulation pattern of CS from the subcellular fractions in MLD skin fibroblasts after the pulse-chase study of ^{14}C-CS: cells were pulsed for 1 h with 40 nmol of ^{14}C-CS and chased for 2 h (●), 6 h (○), and 24 h (△). At each time point cell homogenates were subjected to the first Percoll gradient and lipids were extracted, separated and visualized as described in Methods. The amount of CS was calculated as percentage of total intracellular radioactivity.

(b) The second Percoll gradient profile of radioactivity, enzymic activity, and markers of subcellular fractions in MLD skin fibroblasts: MLD skin fibroblasts were pulsed for 24 h with 40 nmol of ^{14}C-CS and subjected to the first Percoll gradient and then the combined fractions from 4 through 7 in the gradient (plasma membrane plus Golgi fraction) was subjected to the second Percoll gradient as described in Methods. Fractions were collected from top and activities determined. Radioactivity (●); β-glucuronidase (○); galactosyltransferase (Golgi marker) (▲); HRP-wheat germ agglutinin (plasma membrane marker) (×).
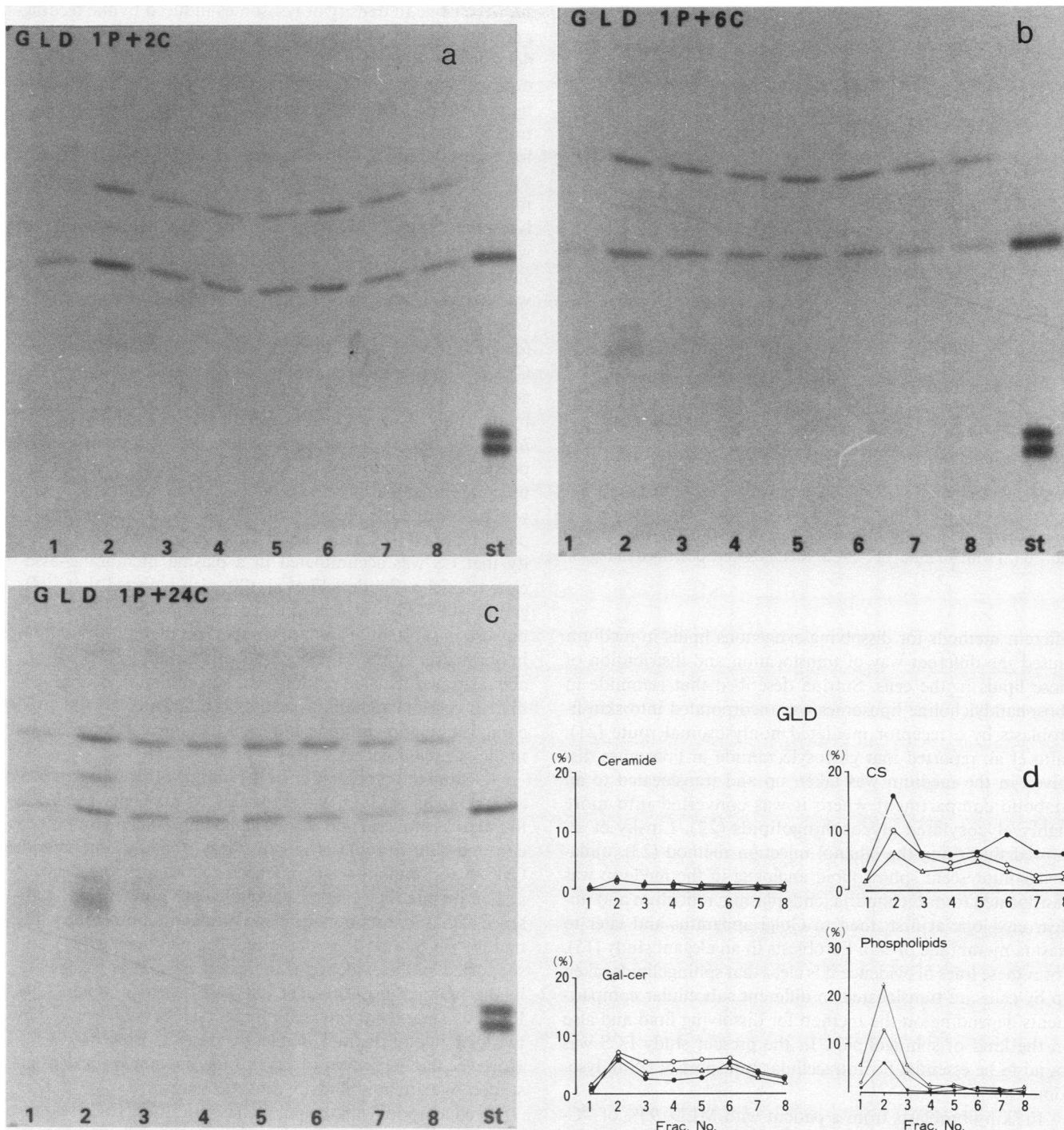
Figure 5. Autoradiogram (*a–c*) and distribution pattern of radioactivity (*d*) of major lipids extracted from the subcellular fractions in GLD skin fibroblasts after the pulse-chase study of ^{14}C-CS: GLD cells were pulsed for 1 h with 40 nmol of ^{14}C-CS and chased for 2 h (*a*, • in *d*), 6 h (*b*, ○ in *d*) and 24 h (*c*, △ in *d*). Methods were described in Fig. 3.

found to be increasing at plasma membrane but no accumulation of CS, GalCer, and Cer was found in lysosome (Fig. 2). These results indicate that CS was hydrolyzed rapidly in lysosomes. Its metabolites were translocated and radioactive fatty acids were reused for the synthesis of phospholipids found at plasma membrane (Figs. 1–3). Sphingosine, the counterpart of fatty acid, was probably reused to produce other lipids as reported in the study using [^{3}H]sphingosine-labeled GM$_2$ ganglioside (19, 20). Although we could not detect the sites of lipid

synthesis, we positively found reuse or salvage of lipid in the skin fibroblasts.

When FCS was absent in the medium, ^{14}C-CS taken up by the control fibroblasts remained unhydrolyzed mainly at plasma membrane as long as 24 h, being a great contrast to the finding that 76% of ^{14}C-CS metabolized with the complete medium. These data suggest that FCS plays an important role in the translocation of CS to lysosome and that CS is hydrolyzed only in lysosome. It has been often reported that the
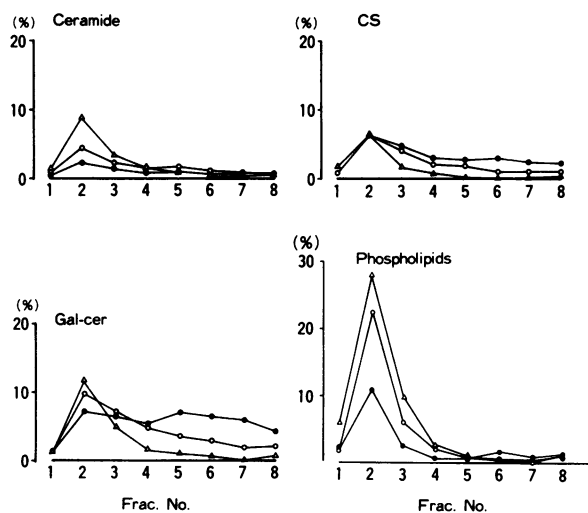
Figure 6. Distribution pattern of radioactivity of major lipids extracted from the subcellular fractions in GLD skin fibroblasts after the long-term pulse study: GLD cells were pulsed for 1 d (●), 3 d (○), and 7 d (△) with 40 nmol of ^{14}C-CS. Methods were described in Fig. 3.

different methods for dissolving exogenous lipids in medium caused the different way of translocation and distribution of those lipids in the cells. Sutrina described that ceramide in phosphatidylcholine liposomes was incorporated into skin fibroblasts by a receptor mediated nonlysosomal route (21). Saito et al. reported that glucosylceramide in liposomes dissolved in the medium was taken up and translocated to an anabolic compartment where it was converted into more highly glycosylated glycosphingolipids (22). Lipsky et al. showed that using the ethanol injection method (23), unilamellar fluorescent sphingolipid analogue in the medium was translocated to mitochondria, endoplasmic reticulum and nuclear envelope at first, then to Golgi apparatus and later to plasma membrane of skin fibroblasts in an elegant study (15). From these lines of evidence it is clear that sphingolipids taken up by cells are translocated to different subcellular compartments depending on the method for dissolving lipid and also on the kind of sphingolipids. In the present study FCS was found to be essential for intracellular transport of CS to lysosome.

In skin fibroblasts from a patient with MLD, 95% of ^{14}C-CS taken up remained unhydrolyzed during 24-h chase and the unhydrolyzed CS was accumulated in lysosome and plasma membrane (Fig. 4 a and b). In longer pulse study, radioactivity of CS was increased in plasma membrane plus Golgi and decreased in lysosome. In skin fibroblasts from patients with GLD, ~ 30% of ^{14}C-CS taken up remained unhydrolyzed during 24-h chase. This result was compatible with the observations that complete block in the degradation of GalCer was not found in skin fibroblasts from patients with GLD (13, 24). The unhydrolyzed GalCer (26 to 28%) was distributed to all subcellular fractions during 24-chase. In longer pulse period with ^{14}C-CS (1–7 d), radioactivity of GalCer was clearly increased in plasma membrane and decreased in lysosome (Fig. 6). These results could indicate (a) translocation of CS or GalCer from lysosomes during hydroly-

sis, (b) change in density of lysosomes induced by the feeding experiments, (c) two populations of lysosomes of different densities, or (d) the presence of a plasma membrane associated organelle capable of CS metabolism. To consider these possibilities, we performed the following experiments. When control fibroblasts were preincubated with leupeptin (20 μg/ml) for 3 d and then ^{14}C-CS was loaded with leupeptin for 1 d, CS was dominantly accumulated in lysosomal fraction, suggesting impaired lysosomal function and translocation of lipids from lysosome. When control fibroblasts were preincubated for 3 d with the complete medium plus monensin (20 μg/ml) and subsequently ^{14}C-CS was loaded with monensin for 1 d, CS was unhydrolyzed and located in plasma membrane plus Golgi fraction, suggesting impaired translocation to lysosome (data not shown). The second possibility is eliminated because CS was dominantly accumulated in lysosomal fraction by the addition of leupeptin. The third possibility is also eliminated by the study using the second Percoll gradient method (Fig. 4 b). By our studies, it seems reasonable that in spite of a slight possibility of rupture of lysosomal compartment during the preparation, unhydrolyzed CS or GalCer in plasma membrane was translocated from lysosome. However, according to the paper reported by Chang and Moudgil (25), there is a possibility that CS was accumulated in a plasma membrane–associated vesicles. Sonderfeld et al. (20) also reported that [^{3}H]-ganglioside GM_2 was accumulated not only in the lysosomal but also in substantial amounts in the light membrane fraction in fibroblasts from GM_2 gangliosidosis, variant B. It is reasonably assumed that the pathological accumulation of sphingolipid in plasma membrane causes significantly altered lipid composition of membrane and leads to neuronal dysfunctions in the sphingolipidoses.

Accumulation of GalCer in the kidney of twitcher mouse, animal model of GLD, was reported (26) and GalCer was also found unhydrolyzed and accumulated in plasma membrane of cultured skin fibroblasts in our study. On the other hand, GalCer accumulation has not been reported in the nervous system despite the genetic catabolic block. To explain the absence of GalCer accumulation and the devastation of the white matter, psychosine hypothesis has been presented (27) and supported by the evidence of increased amount of psychosine in the brain of GLD patient (28) and twitcher mouse (29). Recently an another explanation that GalCer is hydrolyzed by two genetically distinct β-galactosidases is presented (30). However the derivation of psychosine has not been demonstrated. We are now studying this mechanism.

In conclusion, with the pulse-chase study of ^{14}C-CS and the subsequent subcellular fractionation by Percoll gradient we have investigated the metabolism of CS and translocation of its metabolites in the fibroblasts from controls, MLD and GLD. Our results suggest that the added lipid was transported to lysosome, metabolized there rapidly and the resultant metabolites were reused efficiently as a constituent of the cell organelle. In disease state, unhydrolyzed metabolite was accumulated in not only lysosomes but also nonlysosomal compartment like plasma membrane. This translocation of unhydrolyzed lipid from lysosome in lipidoses may result in an imbalance of the sphingolipid pattern on the cell surface. Then, these changes, when occurred in neurons, may lead to significant neurological disturbances at the early stage before massive storage in lysosomes mechanically impair the neuron itself.

Acknowledgments

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