Conversion of short-chain ceramides to short-chain ceramide GM3 in B16 melanoma cells

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Abstract **We report that short-chain ceramide (Cer), C_2- and C_6-Cer, were immediately glycosylated and finally converted to short-chain Cer GM3 in B16 melanoma cells. By addition of either C_2- or C_6-Cer to a cell culture of B16 melanoma in the presence of [^{14}C]Gal, the radiolabeled precursor, was incorporated into each of two novel glycosphingolipids (GSLs) within 30 min along with synthesis of normal GSLs. These novel GSLs were identified as C_2-, C_6-Cer cerebrosides and C_2-, C_6-Cer GM3, respectively. In comparison with C_2-Cer, C_6-Cer was found to be much more efficiently converted to the GSLs, whereas no glycosylated sphingosine was detectable when it was added in place of short-chain Cer.**

Key words: Short-chain ceramide; C_2-ceramide; C_6-ceramide; Endoglycoceramidase; Glycosphingolipid; Ganglioside

1. Introduction

Ceramide (Cer), the common lipid backbone of sphingolipids, has been proved to act as an intracellular second messenger and cell regulatory molecule in numerous studies using short-chain synthetic Cer with improved cell-permeability, instead of natural Cer. For example, C_2- and C_6-Cer have been shown to induce cell differentiation [1] and programmed cell death [2], to mediate EGF receptor phosphorylation [3] and endocytosis [4], and to inhibit neutrophil superoxide formation and calcium influx [5]. However, there have been very few reports about the intracellular metabolism of these synthetic short-chain Cer. Lipsky and Pagano studied sphingolipid metabolism in cultured fibroblasts employing a fluorescent Cer analogue, C_6-NBD-Cer [6]. They reported that the C_6-NBD-Cer was converted to NBD-labeled sphingomyelin and NBD-labeled glucosylceramide (GlcCer), but never to more complex glycosphingolipids (GSLs). When [^{3}H]C_2-Cer was added to a culture of HL-60 cells, the labeled C_2-Cer was incorporated promptly into the cells but not metabolized to sphingosine (Sph), and substantially all Cer were detected as the intact forms even after prolonged incubation at 37°C [1]. It was indicated that only a small percentage of the label (2.8%) was converted to sphingomyelin and not to GSLs [1]. In contrast to these reports, we found that C_2- and C_6-Cer were immediately glycosylated and finally converted to short-chain Cer GM3 in B16 melanoma cells when the Cer was added in the presence of [^{14}C]Gal. This paper describes the identification of novel GSLs with short-chain Cer which were synthesized from corresponding short-chain Cer as primers and the kinetics of their intracellular formation.

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Abbreviations: Cer, ceramide; GSL(s), glycosphingolipid(s); C_2-Cer, *N*-acetylsphingosine; C_6-Cer, *N*-hexanoylsphingosine; NBD, nitrobenzo-2-oxa-1,3-diazole; Sph, sphingosine; GalCer, galactosylceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; Gal, galactose; EGCase, endoglycoceramidase; TLC, thin-layer chromatography; PBS, phosphate buffered saline; FCS, fetal calf serum.

2. Materials and methods

2.1. Materials

Sph, C_2-Cer and C_6-Cer were purchased from Matreya, USA [^{14}C]Gal was obtained from DuPont-New England Nuclear, USA and TLC plates (silica gel 60) were from Merck, Germany. β-Galactosidase (from jack bean) and β-glucosidase (from sweet almond) were from Seikagaku Co., Japan, and Funakoshi Co., Japan, respectively. D-Galactonic acid γ-lactone, psychosine and Triton X-100 were from Sigma, USA. D-Glucono-γ-lactone, decane and C_2-Cer GM3 were from Wako Co., Japan. Endoglycoceramidase (EGCase) was prepared from the culture fluid of *Rhodococcus* sp. M-777 [7–9] or purchased from Takara Shuzo Co., Japan. Sphingolipid ceramide *N*-deacylase was prepared from the culture fluid of *Pseudomonas* sp. TK-4 [10].

2.2. Cell culture and metabolic labeling

B16 melanoma cells were grown in MEM medium supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified 95% air/5% CO_2 incubator. For the labeling experiments, 1×10^5 cells were seeded in 24-well microplates and precultured for 16 h to allow the cells to attach to the plate. After preincubation, the medium was replaced with 200 μl of fresh MEM medium supplemented with 5% FCS containing 1 μCi [^{14}C]Gal and incubated for the time indicated.

2.3. Addition of synthetic short-chain Cer and Sph

Sph, C_2-, or C_6-Cer was first dissolved in ethanol/decane (98:2 v/v). The solution was added to the medium in a tube and mixed well with a Vortex mixer. The final concentration of the ethanol–decane solution in the medium was 0.5% [11].

2.4. Extraction of GSLs and analysis by TLC

B16 cells were harvested by centrifugation (800 rpm × 10 min) and washed with PBS. The cells were suspended in 750 μl of *i*-propanol/hexane/water (55:35:10 v/v), subjected to sonication for 20 min [12] and centrifuged at 13,000 rpm for 5 min. The supernatants obtained were dried, dissolved in 20 μl of chloroform/methanol (2:1 v/v) and applied to a TLC plate, which was then developed with solvent A (chloroform/methanol/0.2% KCl (5:4:1 v/v)) or solvent B (chloroform/methanol/0.2% CaCl2 (60:40:9 v/v)). Each radioactive GSL separated on the TLC plate was analyzed and quantified by a BAS1000 image analyzer (Fuji Film, Japan).

2.5. Preparation of standard short-chain Cer GSLs

C_2-Cer cerebroside was prepared from psychosine according to the method described previously [13]. C_6-Cer GM3 and C_6-Cer cerebroside were prepared from lyso-GM3 and psychosine, respectively, by the method as shown in ref. [14]. Lyso-GM3 was prepared from GM3 by digestion with sphingolipid ceramide *N*-deacylase [10].

2.6. Identification of short-chain Cer GSLs from B16 cells using a specific glycosidase

GSLs with short-chain Cer were scraped from the TLC plate and suspended in 1 ml of chloroform/methanol (2:1 v/v), followed by sonication for 1 h. After centrifugation at 12,000 rpm for 5 min, the

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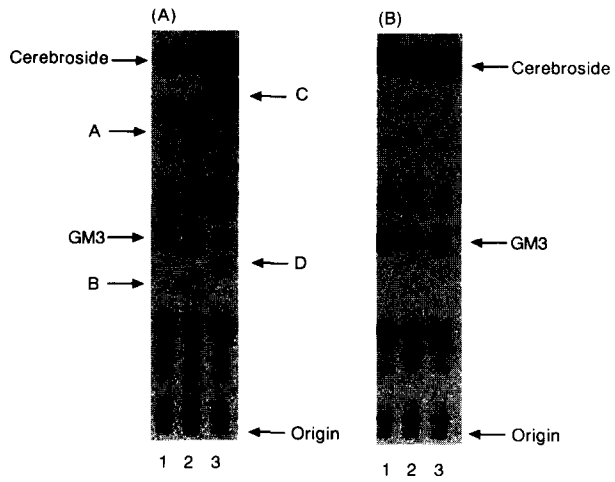

Fig. 1. TLC analysis of short-chain Cer GSLs in B16 cells. (A) Conversion of exogenously added short-chain Cer to short-chain GSLs. Lane 1 = control; lane 2 = C_2-Cer; lane 3 = C_6-Cer. (B) Effect of Sph on de novo GSL synthesis. Lane 1 = control; lane 2 = Sph 5 μM; lane 3 = Sph 10 μM. Details are given in the text.

supernatants were dried and dissolved in 20 μl of 10 mM acetate buffer, pH 6.0, containing 0.2% Triton X-100. For identification of GM3 with short-chain Cer, 10 μl of EGCase (5 mU) was added and incubated at 37°C overnight. For cerebrosides with short-chain Cer, 10 μl of either β-galactosidase (200 mU) or β-glucosidase (200 mU) was added instead of EGCase. Glycosidase inhibitor was included in the reaction at a concentration of 5 mM; D-galactonic acid γ-lactone was used for the β-glucosidase reaction to inhibit any contaminating β-galactosidase and D-glucono-γ-lactone to inhibit any β-glucosidase. After incubation at 37°C overnight, the reaction mixture was dried, dissolved in 20 μl of 50% methanol and applied to a TLC plate, which was then developed with solvent A. Each radioactive GSL separeted on the TLC plate was analyzed by a BAS1000 image analyzer (Fuji Film, Japan).

3. Results

3.1. Identification of novel GSLs with short-chain Cer

When B16 melanoma cells were cultivated in MEM medium containing C_2- or C_6-Cer at a concentration of 5 μM in the presence of 1 μCi [^{14}C]Gal for 2 h, two spots derived from each short-chain Cer were detected along with normal GM3 and cerebrosides by the image analyzer (Fig. 1A; A,B in lane 2; C,D in lane 3). In order to identify these novel radioactive spots, each of them was scraped from the TLC plate separately and extracted with chloroform/methanol (2:1 v/v). The R_f of spot B on TLC was identical to that of standard C_2-Cer GM3 using two different solvent systems (R_f 0.36 in solvent A; R_f 0.21 in solvent B), and that of spot D was standard C_6-Cer GM3 (R_f 0.41 in solvent A; R_f 0.26 in solvent B). Furthermore, an endoglycoceramidase (EGCase), which cleaves the glycosidic linkage between oligosaccharide and Cer of various GSLs [7–9], was used to confirm the identification of the novel GSLs forming spots B and D. As shown in Fig. 2A, radioactive oligosaccharides were released from the novel GSLs as well as normal GM3 by the action of EGCase, and all of these oligosaccharides had the same R_f as that of sialyllactose. These oligosaccharides were further hydrolyzed by neuraminidase from *Arthrobacter ureafaciens* to produce the radioactive spots corresponding to lactose (data not shown). These results indicated that the sugar chains of the novel GSLs forming spots B and D were both NeuAc-lactose. It was thus concluded that spots B and D were GM3 having C_2- and C_6-Cer, respectively.

To identify the radioactive spots A and C, β-glucosidase and

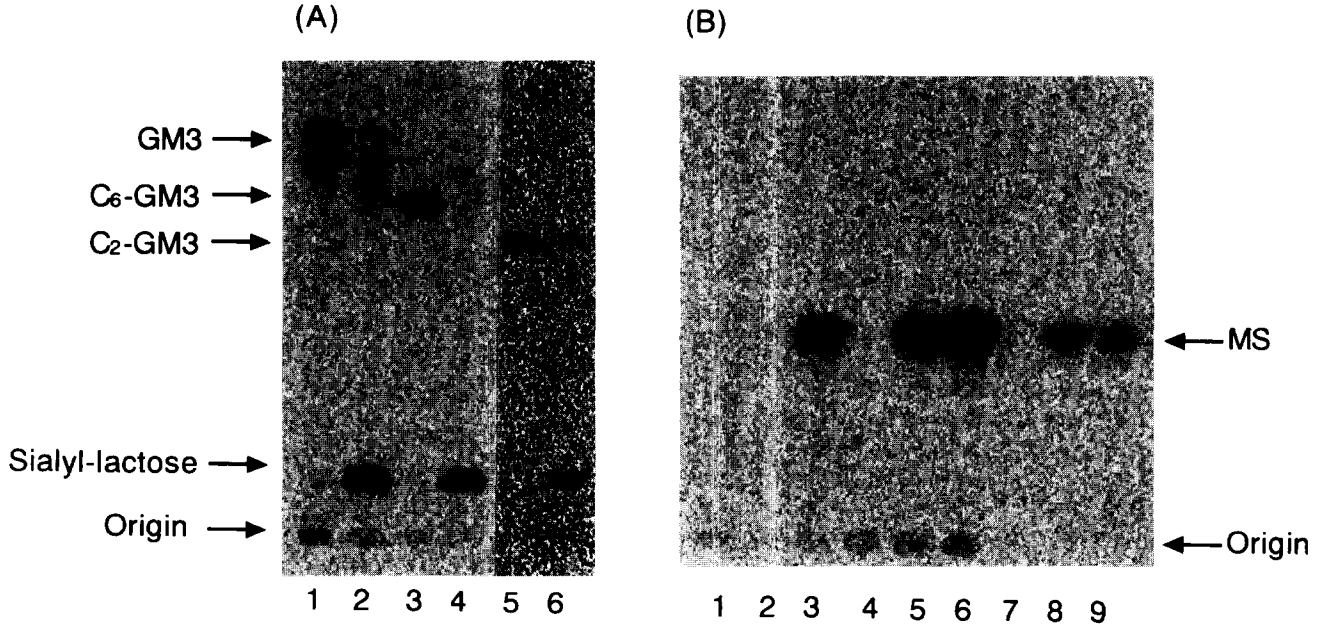

Fig. 2. Identification of short-chain Cer GSLs using specific glycosidases. (A) Identification of short-chain Cer GM3 by EGCase. Lane 1 = GM3; lane 2 = GM3 + EGCase; lane 3 = GSL isolated from spot D; lane 4 = GSL isolated from spot D + EGCase; lane 5 = GSL isolated from spot B; lane 6 = GSL isolated from spot B + EGCase. (B) Identification of short-chain Cer cerebrosides by exoglycosidase. Lane 1 = cerebroside; lane 2 = cerebroside + β-glucosidase; lane 3 = cerebroside + β-galactosidase; lane 4 = GSL isolated from spot C; lane 5 = GSL isolated from spot C + β-glucosidase; lane 6 = GSL isolated from spot C + β-galactosidase; lane 7 = GSL isolated from spot A; lane 8 = GSL isolated from spot A + β-glucosidase; lane 9 = GSL isolated from spot A + β-galactosidase. MS = monosaccharide. Details are given in the text.

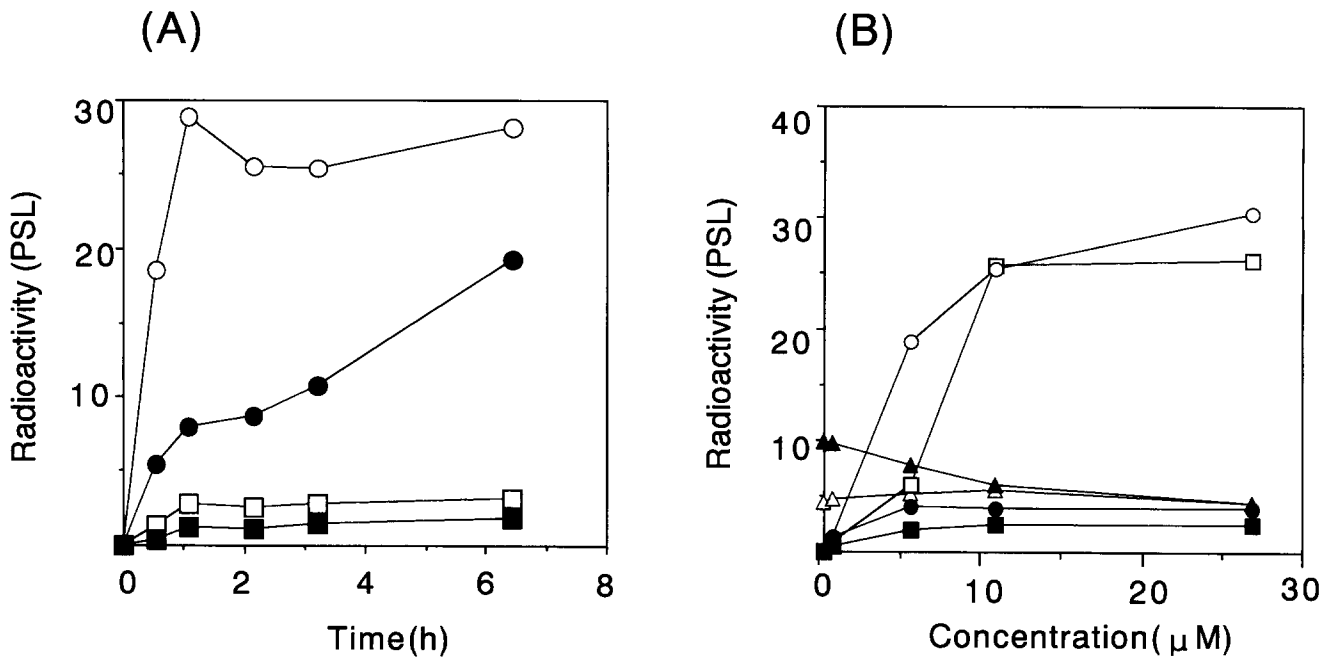

Fig. 3. Kinetics for intracellular formation of short-chain Cer GSLs in B16 cells. (A) Time course for de novo synthesis of short-chain Cer GSLs. The results are averages obtained from two different experiments. □ = C_2-Cer cerebroside; ■ = C_2-Cer GM3; ○ = C_6-Cer cerebroside; ● = C_6-Cer GM3. (B) Effects of concentration of short-chain Cer on de novo GSL synthesis. The results are averages obtained from two different experiments. △ = cerebroside; ▲ = GM3; □ = C2-Cer cerebroside; ○ = C6-Cer cerebroside; ■ = C2-Cer GM3; ● = C6-Cer GM3. PSL = photo-stimulated luminescence. Details are given in the text.

β-galactosidase were used with specific inhibitors as described in section 2. As shown in Fig. 2B, GSLs forming spots A and C were hydrolyzed by both β-glucosidase and β-galactosidase to produce monosaccharides (lanes 8,9 and 5,6), whereas normal cerebroside of B16 cells was hydrolyzed by β-galactosidase (lane 3) but was completely resistant to β-glucosidase (lane 2). Furthermore, R_f of spot A on TLC was shown to be identical to that of standard C_2-Cer cerebroside (R_f 0.66 in solvent A; R_f 0.58 in solvent B), and that of spot C was standard C_6-Cer cerebroside (R_f 0.74 in solvent A, R_f 0.66 in solvent B) using two different solvent systems. These results indicated that the normal cerebroside detected in this experiment was GalCer, and GSLs forming spots A and C were a mixture of GlcCer and GalCer with respective short-chain Cer. This result may suggest that short-chain Cer GlcCer was converted more slowly to short-chain Cer LacCer in comparison with the normal GlcCer, and thus short-chain Cer GlcCer could be detected transiently on TLC. It should be noted that in B16 cells no extension of sugar chains from GalCer could be occurred and therefore GalCer was apparently enriched. No novel GSLs were apparent on TLC when sphingosine (Sph) was added to a culture of B16 cells at a concentration of either 5 or 10 μM instead of short-chain Cer (Fig. 1B, lanes 2,3). Interestingly, however, de novo synthesis of cerebrosides and GM3 was distinctly increased when Sph was added. As reported previously [1], we also detected the C_2-Cer sphingomyelin when C_2-Cer was added to cultured B16 melanoma cells in the presence of [^{14}C]choline instead of [^{14}C]Gal (date not shown).

3.2. Kinetics for intracellular formation of short-chain Cer GSLs

In order to investigate the time course of the de novo synthesis of short-chain Cer GSLs, B16 melanoma cells were grown at 37°C for the indicated time in MEM medium containing 5 μM short-chain Cer in the presence of 1 μCi [^{14}C]Gal. C_2-, C_6-Cer cerebrosides and C_2-, C_6-Cer GM3 appeared within 30 min and increased gradually in a time-dependent manner (Fig. 3A). The de novo synthesis of all short-chain Cer GSLs reached a plateau within 2 h except for C_6-Cer GM3, which increased continuously even up to 6 h in the same manner as normal GM3. In comparison with C_2-Cer, C_6-Cer was found to be much more efficiently converted to short-chain Cer GSLs; de novo synthesis of C_6-Cer GM3 and C_6-Cer cerebroside at 3 h after addition of short-chain Cer was about 6.8-fold and 4.1-fold, respectively, in comparison to that of the C_2-Cer counterparts (means of 5 independent experiments).

To clarify the effects of concentration of short-chain Cer on de novo synthesis of GSLs, B16 melanoma cells were cultivated in MEM medium containing increasing amounts of synthetic Cer in the presence of 1 μCi [^{14}C]Gal for 2 h. The de novo synthesis of short-chain Cer GM3 increased gradually in a dose-dependent manner up to 25 μM, while that of short-chain Cer cerebrosides increased rapidly from the concentration of 5 μM (Fig. 3B). At a concentration of 10 μM, the synthesis of short-chain cerebrosides greatly exceeded that of normal cerebrosides. It was noteworthy that the de novo synthesis of normal GSL was little affected by the addition of short-chain Cer.

4. Discussion

Several lines of evidence have indicated that short-chain Cer, which could mimic natural Cer, evoke various physiological effects in different types of cell [1–5]. The most important finding of this study was that short-chain Cer could be converted to short-chain gangliosides GM3 in intact cells. This may indi-

cate that the physiological effects of short-chain Cer reported so far might be attributable in part to the formation of short-chain Cer gangliosides. It should be clarified whether short-chain Cer GM3 can be converted to more complex gangliosides using other cell lines, since the B16 melanoma cells used in this study do not possess a GM2-synthetic enzyme, and the final product of ganglioside is GM3.

It has been reported that a fluorescent Cer analogue C_6-NBD-Cer is quickly incorporated into the plasma membrane at 2°C and transported to the Golgi apparatus at 37°C, where C_6-NBD-Cer is converted to C_6-NBD-Cer sphingomyelin or C_6-NBD-Cer GlcCer but not to more complex GSLs [6]. These data are therefore in conflict with our observation that C_6-Cer were quickly converted to C_6-Cer GM3. One is forced to conclude that the incomplete extension of sugar chains observed in [6] is due to the attachment of a fluorescent molecule (NBD) to a Cer, and not to the length of the fatty acid chains. However, *N*-acyl chain or at least an *N*-acetyl group bound to the Sph moiety would be necessary for intracellular glycosylation, since this study showed that Sph could not be directly glycosylated in B16 (Fig. 1B). It is also noteworthy that a longer acyl chain ($C_6 > C_2$) in a Cer molecule led to better conversion to GM3 (Figs. 1A,3A).

It has been reported that exogenously added short-chain Cer have negative effects on cell activities, such as cell growth arrest [1] and programmed cell death [2]. In this study, a rapid increase of short-chain Cer cerebrosides was observed upon addition of short-chain Cer at 5–25 μM, although the synthesis of short-chain Cer GM3 was relatively slow at the same concentration (Fig. 3B). This may indicate the presence of a novel detoxification mechanism in B16 cells, i.e. in order to reduce the intracellular concentration of short-chain Cer, they might be quickly glycosylated and thus transported to the plasma membrane. This is reminiscent of a report indicating that exogenously added psychosine (GalSph) was quickly converted to GalCer in neuroblastoma cells to exclude the lyso-forms [15].

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