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Rapid internalization of exogenous ganglioside GM3 and its metabolism to ceramide in human myelogenous leukemia HL-60 cells compared with control ganglioside GM1

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Abstract Incorporation and metabolism of exogenous GM3 in human myelogenous leukemia HL-60 cells were analyzed using ^{3}H-labeled GM3 ([^{3}H]GM3). [^{3}H]GM3 was rapidly internalized into the cells (trypsin-resistant fraction) 8 times more than the control, ^{3}H-labeled GM1 ([^{3}H]GM1). In addition, not only incorporation but also metabolism of [^{3}H]GM3 was more rapid than [^{3}H]GM1 in HL-60 cells. Moreover, one of the metabolites was found to co-migrate with ceramide in thin-layer chromatography analysis and ceramide formation from exogenous GM3 is more rapid than that from exogenous GM1. These results suggested that there would be some preferential mechanism to produce ceramide from differentiation-inducible GM3 in HL-60 cells rather than from non-inducing GM1.

Key words: Ganglioside; Ceramide; Differentiation; HL-60 cell; Monocytic lineage

1. Introduction

Ceramide, sphingosine, and their derivatives have been suggested to serve as metabolic second messengers, and especially ceramide was reported to induce differentiation and apoptosis in various cells [3]. The ceramide level is thought to be regulated by several metabolic pathways: de novo synthetic enzymes, sphingomyelinase, choline phosphotransferase, sphingosine *N*-acyltransferase, ceramidase, ceramide glucosyltrans-ferase, and glycolipid degrading enzymes including glucosylceramidase. Formation of bioactive ceramide has been intensively studied and suggested to be from sphingomyeline through the action of neutral sphingomyelinase [4]. In particular cell lines, however, the other pathways have been suggested to play some critical roles on the ceramide formation [5,6]. It would be of interest to analyze whether ceramide from the other pathways, including glycolipid metabolism, plays some important roles on cellular differentiation and apoptosis [1].

On the other hand, particular ganglioside molecules were suggested to play important roles on the regulation of differentiation in human leukemia cell lines [7–9]. In addition, particular glycosyltransferases were reported to play an important role on the accumulation of such gangliosides in the cell surface membrane [9–12]. Further, regulation of glycosyltransferase expression by antisense oligodeoxynucleotides was reported to result in monocytic differentiation in HL-60 cells [13]. Moreover for monocytic differentiation by GM3, we have reported the characteristic incorporation of [^{3}H]GM3 in HL-60 cells [14].

For the metabolism of exogenous gangliosides, extensive studies have been accomplished mainly by the groups of Tettamanti [15,16], and it was reported that free sphingosine and ceramide are formed from exogenous ganglioside GM1 [17]. In the present study, we have investigated internalization and metabolism of the incorporated [^{3}H]GM3 in HL-60 cells comparing with control ganglioside [^{3}H]GM1 to know whether there is any difference on the formation of ceramide between differentiation inducing ganglioside GM3 and non-inducing GM1. Here we show the results that GM3 was more rapidly internalized and metabolized than GM1, and that GM3 was more expeditiously metabolized to ceramide than GM1.

2. Materials and methods

2.1. Materials

Ganglioside GM3 was isolated from dog erythrocytes and GM1 from normal human brain. GM3 and GM1 were tritiated by catalytic reduction of the sphingosine double bond using [^{3}H]$NaBH_4$ (New England Nuclear, Boston, MA) [18]. Tritiated GM3 ([^{3}H]GM3) and GM1 ([^{3}H]GM1) were freed from radioactive impurities using silica gel chromatography, and the final purity of [^{3}H]GM3 (248 Ci/mol) and [^{3}H]GM1 (118 Ci/mol) was at least 98%. Radioactivity was determined by using a liquid scintillation counter. The amount of gangliosides in the tritiated preparations was estimated by HPTLC (Silica Gel 60, Merck Co., Darmstadt, Germany) using a resorcinol-HCl spray followed by heating at 105°C. Human myelogenous leukemia HL-60 cells were maintained, prepared in the synthetic medium, and treated with [^{3}H]GM3 or [^{3}H]GM1 as mentioned earlier [14]. All other reagents were of the highest grade available.

2.2. Extraction and analyses of incorporated [^{3}H]gangliosides

The cells were incubated with up to 50 μM [^{3}H]GM3 or [^{3}H]GM1 at 4 or 37°C for various lengths of time. After incubation, the cells were washed twice with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} using centrifugation at 1000×*g*, and the supernatants were combined (medium fraction). The cells were then treated with 2 ml of trypsin solution for 10 min at 37°C and washed twice with PBS. The radioactivities that appeared in the supernatants were combined (trypsin-sensitive fraction) and measured. Both the medium and trypsin-sensitive fractions were applied to reverse-phase silica C18 cartridges, respectively, and lipid fractions were recovered and evaporated. On the other hand, the lipid fractions were prepared from the washed cells as described (trypsin-resistant fraction) [19]. The radioactivities

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Abbreviations: LacCer, lactosylceramide; GM3, II^{3}NeuAc-LacCer; GM2, II^{3}NeuAc-GgOse$_3$Cer; GM1, II^{3}NeuAc-GgOse$_4$Cer; Cer, ceramide; HPTLC, high-performance thin-layer chromatography; PBS, phosphate-buffered saline

[1] Glycosphingolipids are designated according to the recommendation of the Nomenclature Committee of the IUPAC [1]. Gangliosides are designated as described [2]. Structures corresponding to each designation are as shown in Abbreviations.

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of each fraction were determined in a scintillation counter. The remaining extracts were applied on HPTLC plates and developed with chloroform/methanol/0.22% $CaCl_2$ (65:35:8; v/v/v).

2.3. Analyses of [^{3}H]ceramide

Radioactivities scraped from the area co-migrated with authentic ceramide on HPTLC plate were extracted by chloroform/methanol/water (60:30:4, v/v/v) and evaporated. The residue was hydrolyzed in methanolic HCl [20] and analyzed the mobility on HPTLC plate comparing with bovine brain dihydrosphingosine standards (Sigma, St. Louis, MO). The amount of [^{3}H]ceramide converted from the incorporated [^{3}H]GM3 was estimated by counting radioactivities in the scraped fractions from the corresponding area on HPTLC.

2.4. Analysis of sphingosine

Sphingosine of GM3 from dog erythrocytes was analyzed by gas chromatography-mass spectrometry using the method described elsewhere [20] with slight modification; used column in gas chromatography was DB-5 fused silica capillary column (J&W Scientific, Folsom, CA) and the apparatus was JEOL SX-102A mass spectrometer equipped with a DA-7000 datalizer.

3. Results

3.1. Internalization of [^{3}H]GM3 into HL-60 cells

Incorporation of exogenous [^{3}H]gangliosides in HL-60 cells was firstly analyzed. Since the incorporation was shown to be linear up to 50 μM and up to 30 min [14], we chose 15–20 min incubation with 25 μM [^{3}H]gangliosides as a typical labeling condition. When the cells were incubated with 25 μM [^{3}H]gangliosides at 37°C for 15 min, the total incorporated amount of [^{3}H]GM3 was 3.24 ± 0.48 nmol/10^8 cells ($n = 6$), while 1.02 ± 0.32 nmol [^{3}H]GM1 ($n = 6$) was incorporated into 10^8 cells. In addition, the internalized fractions were discriminated from the cell surface-binding fractions by trypsinizing the cells after incubation with [^{3}H]gangliosides. As shown in Fig. 1, [^{3}H]GM3 was preferentially internalized in the cells over [^{3}H]GM1; [^{3}H]GM1 was found in the trypsin-sensitive (cell surface-binding) fraction more than in the trypsin-resistant (internalized) fraction. By contrast, [^{3}H]GM3 was found in the internalized fraction more than in the cell surface-binding fraction. Comparing two [^{3}H]gangliosides in each fraction, the amount of [^{3}H]GM3 in the trypsin-sensitive fraction was only about a half of [^{3}H]GM1. However, [^{3}H]GM3 was found in the trypsin-resistant fraction 8 times more than [^{3}H]GM1.

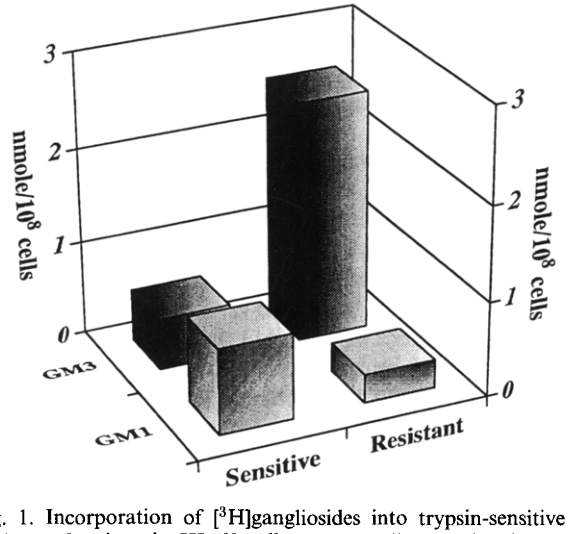

Fig. 1. Incorporation of [^{3}H]gangliosides into trypsin-sensitive and -resistant fractions in HL-60 cells. HL-60 cells were incubated with 25 μM of [^{3}H]gangliosides for 15 min at 37°C. Cells were harvested and divided into trypsin-sensitive and -resistant fractions. Values are means of the results of three separate experiments and presented as nmol/10^8 cells.

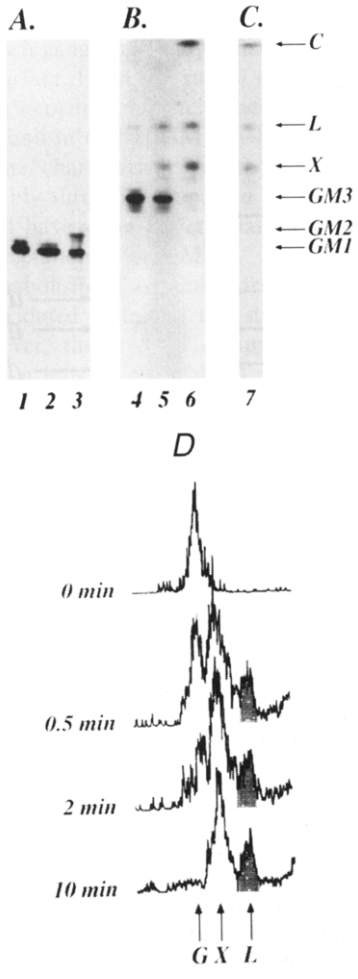

Fig. 2. Autoradiography and radiochromatoscanning of [^{3}H]gangliosides incorporated into HL-60 cells. A-C: After incubation for 20 min with [^{3}H]GM1 (A), with [^{3}H]GM3 (B) at 25 μM, and with [^{3}H]GM3 at 25 μM in the presence of 50 μM cold authentic GM3 (C), lipid fractions were extracted, and analyzed by HPTLC followed by autoradiography. Lanes 1 and 4: medium fractions. Lanes 2 and 5: trypsin-sensitive fractions. Lanes 3, 6 and 7: Trypsin-resistant fractions. Arrows C, L, and X represent the positions of ceramide, LacCer, and a compound X, respectively. Other arrows represent the positions of authentic GM3, GM2, and GM1 as indicated, respectively. D: Time course of exogenous [^{3}H]GM3 metabolism in HL-60 cells. The cells were pulsed with 25 μM of [^{3}H]GM3 at 37°C for the time indicated, spun down, washed twice with PBS, and trypsinized. The cells were kept at 4°C during spinning down and washing process except for the period of trypsinization. Trypsin-resistant fractions were analyzed by HPTLC followed by radiochromatoscanning. G: the position of GM3; X, compound X; L and hatched area, LacCer.

3.2. Rapid metabolism of incorporated [^{3}H]GM3 in HL-60 cells

Lipids were prepared from the medium, trypsin-sensitive and resistant fractions of HL-60 cells treated with 25 μM [^{3}H]gangliosides at 37°C for 20 min, analyzed by HPTLC plate, and detected by autoradiography. Fig. 2A,B demonstrate the results comparing [^{3}H]GM1 and [^{3}H]GM3, respec-

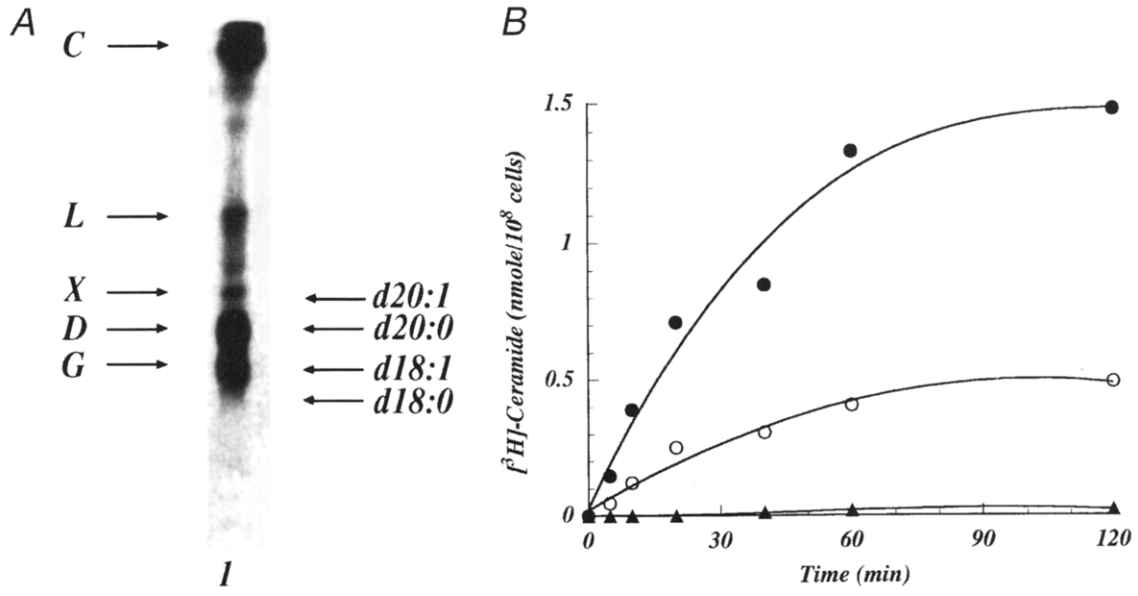

Fig. 3. Further metabolism of incorporated [^{3}H]GM3 in HL-60 cells. A: Autoradiogram of trypsin-resistant fraction from [^{3}H]GM3-labeled HL-60 cells. The cells were incubated with 25 μM [^{3}H]GM3 for 1.5 h at 37°C. Lipid was extracted from trypsin-resistant fraction and analyzed by HPTLC followed by autoradiography. Lane 1, trypsin-resistant lipid fraction from HL-60 cells. Arrows indicate each component's position of sphingosine and dihydrosphingosine standards; d20:1, d20:0, d18:1, and d18:0. Arrows C, L, X, D, and G indicate the positions of ceramide, LacCer, a compound X, a band corresponding to d20:0 dihydrosphingosine, and GM3, respectively. B: Time course of [^{3}H]ceramide formation from the incorporated [^{3}H]GM3 in HL-60 cells. The cells were pulsed with 25 μM [^{3}H]GM3 in the presence (○) or absence (●) of 50 μM cold authentic GM3 or with 25 μM [^{3}H]GM1 (▲) at 37°C for the time indicated, spun down at 4°C, and washed twice with PBS. Trypsin-resistant fractions were analyzed by HPTLC followed by scraping the area corresponding to ceramide. The data represent one set of determinations from two replicate experiments.

tively. As shown in Fig. 2A, [^{3}H]GM1 was found as the main band in all three fractions. While a band corresponding to GM2 was not found in the medium and trypsin-sensitive fractions, the band was detected obviously in the trypsin-resistant fraction. However, [^{3}H]GM1 did not seem to be further metabolized to GM3 nor LacCer at the point. By contrast, [^{3}H]GM3 was metabolized in a very different manner (Fig. 2B). While [^{3}H]GM3 remained as the main band in the medium and trypsin-sensitive fraction, a band corresponding to LacCer (arrow L) appeared in the trypsin-sensitive fraction. In addition, a band that migrated between LacCer and GM3 was also observed in the trypsin-sensitive fraction (arrow X). Moreover, a band corresponding to GM3 almost disappeared in the trypsin-resistant fraction, while bands L and X were detected as notable major ones. Since the [^{3}H]GM3 used was structurally different from unlabeled GM3 at the sphingosine double bond, we conducted another experiment to discover whether the metabolism of [^{3}H]GM3 was blocked by the cold authentic GM3 that was not hydrogenized at double bond of sphingosine. As shown in Fig. 2C, bands L and X became faint compared with lane 6 of Fig. 2B. This suggested that [^{3}H]GM3 and unlabeled GM3 behaved in the same manner as for their metabolism after incorporation in HL-60 cells.

Table 1
[^{3}H]Ceramide production at prolonged time intervals

Time	[^{3}H]Ceramide production (nmol/10^8 cells)
Day 0	0
Day 1	1.85
Day 2	2.10
Day 4	1.94
Day 6	2.07

Cells were labeled and processed as described in the legend to Fig. 3B. The data represent one set of determinations from two independent experiments.

Subsequently, the time course of [^{3}H]GM3 metabolism in HL-60 cells was investigated. We pulsed the cells with 25 μM [^{3}H]GM3 at 37°C for various periods of time and then kept the cells at 4°C during the spinning down and washing processes except for the period of trypsinization. At 4°C, we observed that incorporation of [^{3}H]GM3 in the cells was only one-fifth that at 37°C. As shown in Fig. 2D, the peak corresponding to GM3 disappeared in a time-dependent manner and [^{3}H]GM3 was metabolized in the cells to the peaks corresponding to compound X and LacCer. Strikingly, compound X and LacCer appeared at the 0.5 min point. However, the areas of peaks X and L did not seem to have any significant difference between the points from 0.5 to 10 min.

3.3. *Generation of ceramide from the incorporated [^{3}H]GM3 in HL-60 cells*

In Fig. 2B, not only bands X and L but also a band corresponding to ceramide (arrow C) was observed in lane 6. We could further detect band C when the cells were cultured with 25 μM [^{3}H]GM3 at 37°C for 1.5 h as shown in Fig. 3A. A more complex pattern of [^{3}H]GM3 metabolites was observed at 1.5 h than at 20 min. A band corresponding to GM3 (G) was clearly detected as well as bands X and L. In addition, a band corresponding to the area around d20:0 dihydrosphingosine (Fig. 3A, arrow D) was also found in the same intensity as band G, while there was no notable band corresponding to d18:0 dihydrosphingosine. However, at present, band D was not thought to be d20:0 dihydrosphingosine. Since long-chain bases of GM3 that was converted to [^{3}H]GM3 and used in this study were revealed as d18:1 and d20:1 sphingosines at a ratio of about 8:2 by gas chromatogra-

phy-mass spectrometry analysis (data not shown), a more intense band corresponding to d18:0 dihydrosphingosine would have appeared if band D was d20:0 dihydrosphingosine. By contrast, band C was considered to be ceramide, as radioactivities scraped from the area of band C co-migrated further with d18:0 and d20:0 sphingosine molecules after acid hydrolysis treatment (data not shown). Furthermore, the time course of [^{3}H]ceramide formation from the incorporated [^{3}H]GM3 was determined in the trypsin-resistant fractions of HL-60 cells. As shown in Fig. 3B, ceramide is generated from the internalized [^{3}H]GM3 in a time-dependent manner until 40–60 min. After these points, [^{3}H]ceramide formation was saturated. On the other hand, production of [^{3}H]ceramide from incorporated [^{3}H]GM1 was minimum compared with [^{3}H]GM3 treatment (Fig. 3B). Not only in short-term intervals but also in prolonged time periods, [^{3}H]ceramide was generated and its level was maintained (Table 1). Moreover, the formation of [^{3}H]ceramide was reduced by co-incubation with cold authentic GM3 (Fig. 2C, Fig. 3B). This suggested that [^{3}H]ceramide production in the cells was not specific to the saturation of the sphingosine double bond in GM3.

4. Discussion

Compared with [^{3}H]GM1, HL-60 cells were shown to uptake and internalize preferentially [^{3}H]GM3 in the present study. In addition, incorporated [^{3}H]GM3 was rapidly metabolized on the cell surface as well as inside the cells. This conversion from GM3 to LacCer on the cell surface would be mediated by cell-surface or extracellular neuraminidase [21]. However, we observed not only LacCer but also band X (Fig. 2). Although we do not have any rigid structural information about the band at present, an acetylated or intramolecular lactonized form of GM3 would be one possibility [22]. Further elucidation of an involvement of such an intermediate in the cell surface GM3 metabolism would be required. As demonstrated in Fig. 2, [^{3}H]GM3 was rapidly catabolized and was not detected after 30 min incubation. On the other hand, a band corresponding to GM3 was clearly observed after 1.5 h incubation. We interpreted these findings as follows; metabolic process would continue during 10 min trypsinization treatment at 37°C when [^{3}H]GM3 incorporation was not yet saturated. Once the incorporation was saturated, further processing of [^{3}H]GM3 would not be observed significantly during the trypsinization period.

For internalization and metabolism from the incorporated gangliosides, HL-60 cells seemed to have some preference for GM3 rather than GM1. It has been reported that GM1 and GM2 were metabolized in some cell systems to asialo-derivatives, LacCer, and ceramide, and that the metabolites were detected within 10–60 min, although the amounts were not so quite notable [15–17]. In the present study, however, [^{3}H]GM3 was shown to be metabolized remarkably to LacCer and ceramide in 0.5–20 min, while [^{3}H]GM1 was not significantly metabolized to ceramide up to 2 h. The ability of trypsin to release [^{3}H]GM1 but not [^{3}H]GM3 suggests that [^{3}H]GM1 is merely associating with the cell surface. The preference for GM3 rather than GM1 by HL-60 cells in the significant metabolism may be initially caused by the difference of each ganglioside in the association with the cell surface. As both have the same charge in the molecules, it is not thought that ionic interaction mechanism of gangliosides with the cell surface is the main reason for the difference. Rather, hydrophobicity of each ganglioside may influence on the association with the cell surface. However, such a difference in hydrophobicity may not explain completely the significant internalization and metabolism of [^{3}H]GM3 in HL-60 cells. Taken together with the characteristic metabolism of [^{3}H]GM3 to LacCer, probably through compound X, it is suggested that the cells would have some preferential mechanism for incorporation and metabolism of GM3.

Further metabolism of ceramide from [^{3}H]GM3 has not been fully elucidated including the structure of band D at present. However, the level of ceramide was shown to be maintained for a long time (Table 1). During monocytic differentiation by 12-*O*-tetradecanoylphorbol 13-acetate in HL-60 cells, the ceramide level was reported to increase significantly after 18–24 h together with an increased level of sphingomyelin [23] and GM3 [10]. Together with these findings, the present results may suggest that the supply of ceramide would increase from the accumulated GM3 during monocytoid differentiation; the level of ceramide would be elevated not only from sphingomyelin cycle but also from catabolism of glycolipids. Although the pools of ceramide from sphingomyelin cycle and glycolipid catabolism may be different, it would be of interest to elucidate whether the ceramide from glycolipid metabolism is involved in monocytic differentiation in HL-60 cells.

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