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Clear differences in ceramide metabolism between glycosphingolipids and sphingomyelin in a human promyelocytic leukemia cell line HL-60 stimulated by a differentiation inducer

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Abstract Although the ceramide components of both glycosphingolipids (GSLs) and sphingomyelin (SM) in HL-60 cells were identical, the molecular species of the ceramides preferentially used in biosynthesis were quite different in GSLs and SM. When HL-60 cells were stimulated to differentiate into macrophage-like cells by phorbol ester after their sphingolipids had been metabolically labeled with L-[3-^{14}C]serine to saturation point, marked changes in the radioactivities of the ceramide residues were observed in GSLs, showing the activation of a biosynthetic pathway of ganglioside GM3. No significant changes were, however, observed in the ceramide residues of SM. These results indicate that it is necessary to consider the overall metabolism of ceramides, including their origin, when investigating the functions of ceramides in signal transduction systems.

Key words: Glycosphingolipid; Sphingomyelin; Ceramide; Differentiation (phorbol ester-induced); Two-dimensional thin-layer chromatography; HL-60 cell

1. Introduction

GSLs, especially certain gangliosides, have recently been reported to participate in essential biological processes such as cell–cell interactions, cell differentiation and signal transduction [1]. Sphingosine derivatives, including ceramides, which are considered as breakdown products of GSLs and SM, have also been demonstrated to modulate a variety of biological events following initial reports of their effects on protein kinase C [1,2]. Subsequent studies on the generation of sphingosine derivatives led to the hypothesis that SM might be degraded and generate ceramides in response to various cellular stimuli, and may thus be involved in a novel signal transduction system [3]. However, this hypothesis does not include any consideration of the possible role of GSLs, even though both GSLs and SM contain ceramide. To clarify the physiological significance of sphingolipids, it is important to study the overall cellular stimulus-induced metabolism of GSLs and SM. However, only a few attempts have so far been made to investigate the relationship between their metabolic pathways [4,5].

In the present study, we first established a simple method which enabled simultaneous analysis of GSLs and SM in the total lipid fraction of cultured cells by two-dimensional HPTLC. Early changes in sphingolipid metabolism, including GSLs and SM, during the macrophage-like differentiation of HL-60 cells [6,7] were then analyzed to estimate the physiological significance of sphingolipids in the differentiation process.

2. Materials and methods

2.1. Materials

L-[3-^{14}C]Serine was purchased from Amersham, UK. CMP-[^{3}H]NeuAc and [4-^{14}C]cholesterol were purchased from NEN, Boston, MA. TPA was obtained from Calbiochem, La Jolla, CA. PC, PE, PS, SM, PGly and CL were purchased from Avanti Polar Lipids, Alabaster, AL. PI, CH and triolein were obtained from serdary Research Laboratories, Ontario, Canada. CMH, CDH, PG and GM3 were purchased from Iatron, Tokyo, Japan. Cer and Sp were obtained from Sigma, St. Louis, MO; SpIP and HL-60 cells were kindly donated by Dr. Senitiroh Hakomori (University of Washington, Seattle) and Dr. S.J. Collins (University of Washington, Seattle), respectively. Mouse melanoma cell line B16 was obtained from American Type Culture Collection. All other reagents were of analytical or HPLC grade.

2.2. Cell culture

HL-60 cells were grown in RPMI 1640 containing 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. In the metabolic labeling study, the cells were cultured in the presence of 300 nCi/ml L-[3-^{14}C]serine. Monocytic differentiation was induced by TPA, and was evaluated morphologically and cytochemically as described previously [8,9]. B16 cells were cultured under the same conditions as those for HL-60 cells.

2.3. Extraction of total lipids

The cells were washed twice with phosphate-buffered saline. Then total lipid was extracted sequentially with a lower phase of isopropanol/n-hexane/water 55:25:20 (v/v/v), chloroform/methanol 1:2 (v/v) and chloroform/methanol 2:1 (v/v) [10]. In the radiolabeling study, [4-^{14}C]cholesterol was used as an internal standard.

2.4. Two-dimensional thin-layer chromatography

The total lipid extract from each cell preparation was spotted in the lower left-hand corner of a HPTLC plate (Merck #5641, 10 cm × 10 cm). The first chromatographic run was performed with chloroform/methanol/formic acid/water 65:25:8.9:1.1 (v/v/v/v) [11]. The second run was performed with chloroform/methanol/4.4 N ammonia 50:40:10 (v/v/v) in rotating 90° from the original direction. Then the

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Abbreviations: CDH, lactosylceramide; Cer, ceramide; CH, cholesterol; CL, cardiolipin; CMH, glucosylceramide; FAB-MS, fast atom bombardment mass spectrometry; GLC, gas-liquid chromatography; GM3, II^{3}NeuAc-lactosylceramide; GSL, glycosphingolipid; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; NeuAc, *N*-acetylneuraminic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, paraglboside; PGly, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; RP, reversed-phase; SM, sphingomyelin; Sp, sphingosine; SpIP, sphingosine-1-phosphate; TG, triacylglycerol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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third run was performed with diethylether in a direction opposite to that of the second run to separate ceramides from other neutral lipids. The plate was sprayed by H_2SO_4 and the spots were visualized by heating. Each spot was identified by comparison with authentic standards. The radioactivity of each spot was determined using a BAS 2000 imaging analyzer (Fuji Film, Tokyo, Japan).

2.5 Synthesis of [^{3}H-NeuAc]GM3

[^{3}H-NeuAc]GM3 was synthesized enzymatically as described previously [12], with a lysate of B16 cells as an enzyme source [13] and both CDH and CMP-[^{3}H]NeuAc as substrates.

2.6 Analysis of the molecular species of CMH, CDH and SM

After hydrolysis of the glycerolipids with methanolic sodium hydroxide [14] and dialysis against water overnight, the sphingolipids were recovered from a silica gel column using a stepwise gradient of methanol in chloroform [15]. They were then applied to a silica gel HPLC column (Iatrobeads 6RSP-8010, 0.46 × 25 cm, Iatron, Tokyo, Japan) with isopropanol/*n*-hexane/water 35:64:1 (v/v/v). CMH and CDH were eluted using a linear gradient of isopropanol/*n*-hexane/water 55:44:1 (v/v/v) [16]. SM was purified by sequential HPLC from the total lipid fraction. First, silica gel HPLC was performed in isopropanol/*n*-hexane/water 70:20:10 (v/v/v), and PC and SM were isocratically eluted and found to overlap [17]. Then SM was isolated on the same column using acetonitrile/methanol/water 70:20:10 (v/v/v) [18].

CMH and CDH were separated into molecular species using a RP-HPLC column (Senshu Pack, PEGASIL ODS, 0.46 × 15 cm) in methanol at 40°C [19]. SM was separated in methanol containing 0.1% (v/v) triethylamine. The molecular mass of the sphingolipids contained in each fraction was analyzed by negative- or positive-ion FAB-MS with a JEOL JMS-HX110 mass spectrometer with triethanolamine [20] as the matrix. Their fatty acid residues were analyzed by GLC as methyl ester derivatives [21] on a capillary column of cross-linked methylsilicone [22]. The correlation between the upper or lower spot and each molecular species was estimated by TLC.

3. Results

3.1. Separation of HL-60 cell total lipids by two-dimensional TLC

Fig. 1 shows the separation pattern of HL-60 cell total lipids by HPTLC. Phospholipids (PC, PE, PS, PI, SM), major neutral GSLs (CMH, CDH, PG), and GM3 were well separated as distinct spots. Most of the GSLs and SM showed doublet spots, each consisting of an upper spot and a lower one. Gangliosides with longer sugar chains were separated in the second run, although they were developed closely to each other. Major neutral lipids (CH, TG) were developed in the upper right-hand corner of the plate after the second run and separated in the third run as slightly large vague spots. When [^{3}H-NeuAc]GM3 was developed using the present solvent system, no detectable degradation of GM3 was observed. Intermediates of biosynthesis and/or breakdown products of GSLs and SM, such as ceramide, sphingosine and sphingosine-1-phosphate were not detected by chemical-level analysis of HL-60 total lipids.

3.2. Changes in sphingolipids of HL-60 cells during monocytic differentiation induced by TPA

HL-60 cells were cultured in the presence of L-[3–^{14}C]serine. The radioactivity incorporated into the sphingolipid fraction of the cultured cells was only 0.3% of the added count. The incorporation of the radioactivity into each sphingolipid almost reached a plateau after 4 days of culture. All GSLs incorporated a larger amount of radioactivity into the upper spot of the doublet. In SM, the reverse was the case.

HL-60 cells differentiated to macrophage-like cells morpho-

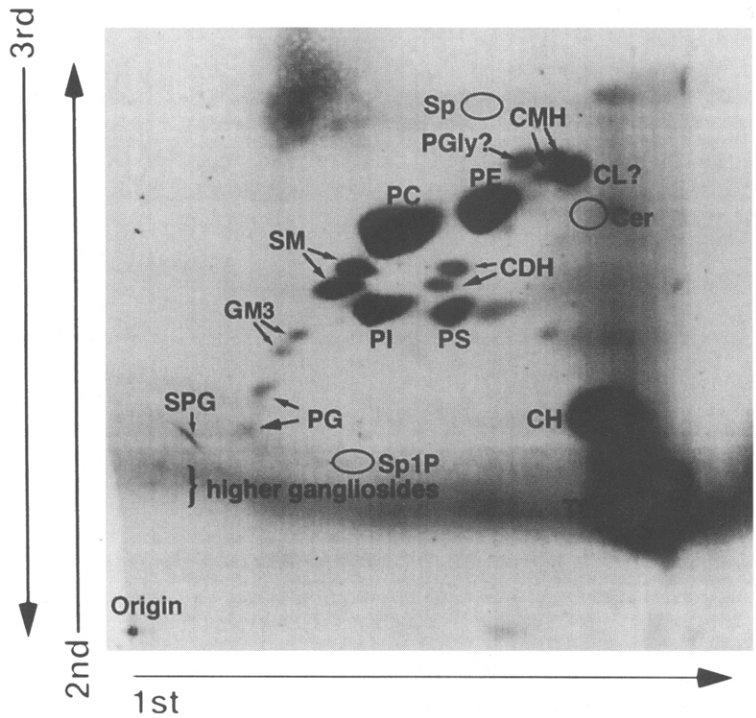

Fig. 1. Two-dimensional thin-layer chromatogram of total lipids from HL-60 cells. Total lipids were applied to the lower left-hand corner of a HPTLC plate. The first chromatographic run was performed with chloroform/methanol/formic acid/water 65:25:8.9:1.1 (v/v/v/v). Then the plate was developed with chloroform/methanol/4.4 N ammonia 50:40:10 (v/v/v) for the second dimension. The third run was performed with diethylether in the opposite direction to the second run. The plate was sprayed with H_2SO_4 and the spots was visualized by heating.

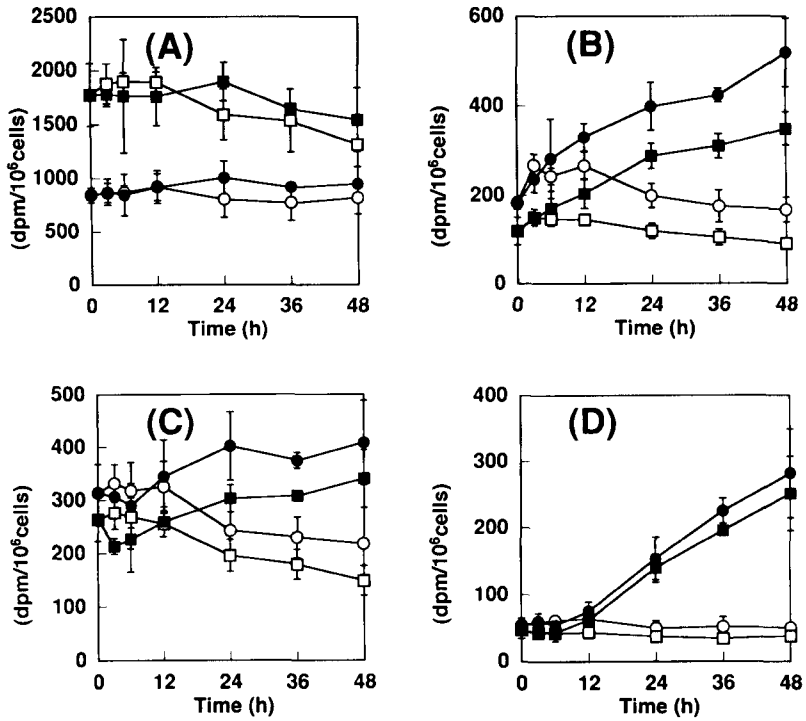

Fig. 2. Changes in radioactivities of sphingolipids in HL-60 cells induced to differentiate with 16 μM TPA. HL-60 cells metabolically labeled with L-[3–^{14}C]serine at equilibrium level were induced to differentiate to macrophage-like cells with TPA (●,■) or in a control culture (○,□). The radioactivity of each spot was analyzed by a BAS 2000 imaging analyzer. ○,●, upper spot; □,■, lower spot. A, SM; B, CMH; C, CDH; D, GM3.

logically and cytochemically with TPA in a dose-dependent manner, and the differentiation reached a plateau at 16 nM, as described previously [7]. No significant cell damage or toxicity was observed even at 1.6 μM TPA. When the cells were stimulated with 16 nM TPA, transient increases of CMH, CDH, GM3 [23] and SM [24] were observed simultaneously. On the other hand, when the cells were stimulated with 1.6 μM TPA, CMH, CDH and GM3 increased (Fig. 2). However, SM did not change significantly. The radioactivity of the lower spot of SM and the upper spots of GSLs maintained a larger count than their respective counterparts during these changes.

3.3 Molecular species of the HL-60 sphingolipids

CMH, CDH and SM were separated as three major peaks by RP-HPLC. The first peak of CMH gave a pseudo-molecular ion ($[M-H]^-$) at *m/z* 698 by negative-ion FAB-MS and was shown to contain C16:0 fatty acid by GLC, indicating that this peak contained sphingenine (d18:1) as the sphingoid base. The second peak of CMH was analyzed in the same way and found to be composed of two molecular species, d18:1-C22:0 (*m/z* 782) and d18:1-C24:1 (*m/z* 808). The third peak was d18:1-C16:0 (*m/z* 810). The first peak, corresponding to the lower spot of CMH, was developed on a TLC plate, as were the second and third peaks, upper spot (Table 1). The first, second and third peaks of CDH gave pseudo-molecular ions ($[M-H]^-$) at *m/z* 860, 944 plus 970 and 972 by negative-ion FAB-MS and were shown by GLC to contain C16:0, C22:0 plus C24:1 and C24:0 fatty acids, respectively. In the case of SM, three peaks gave pseudo-molecular ions ($[M+H]^+$) at *m/z* 703, 787 plus 813 and 815 by positive-ion FAB-MS and were shown by GLC to contain C16:0, C22:0 plus C24:1 and C24:0 fatty acids, respectively. Both CDH and SM were found to contain sphingenine (d18:1) as the sphingoid base. The behavior of the peaks of CDH or SM on TLC were analogous to those of CMH. These data indicated that the ceramides of the upper spots of CMH, CDH and SM were composed mainly of d18:1 sphingenine and C24:0, C22:0 and C24:1 fatty acids, whereas the lower ones were composed of d18:1 sphingenine and C16:0 fatty acid.

Table 1
Molecular species of CMH, CDH and SM in HL-60 cells.
The molecular species of each of the lipids were isolated by RP-HPLC as described in section 2. Each was analyzed by TLC, FAB-MS and GLC.

Sphingolipids	Peaks on HPLC	Spots on TLC	Molecular species of ceramides	*m/z*[a]
CMH	first	lower	d18:1-C16:0	698[b]
	second	upper	d18:1-C22:0	782[b]
			d18:1-C24:1	808[b]
CDH	third	upper	d18:1-C24:0	810[b]
	first	lower	d18:1-C16:0	860[b]
	second	upper	d18:1-C22:0	944[b]
			d18:1-C24:1	970[b]
SM	third	upper	d18:1-C24:0	972[b]
	first	lower	d18:1-C16:0	703[c]
	second	upper	d18:1-C22:0	787[c]
			d18:1-C24:1	813[c]
	third	upper	d18:1-C24:0	815[c]

[a]Pseudo-molecular ion.
[b]$[M-H]^-$ by negative-ion FAB-MS.
[c]$[M+H]^+$ by positive-ion FAB-MS.

4. Discussion

In the present study, we established a simple method for simultaneous analysis of GSLs and SM from the total lipid fraction of cultured cells by two-dimensional HPTLC. Although gangliosides contain acid-labile ketoside bonds [25], the use of an acidic solvent was necessary to separate major GSLs including gangliosides, phospholipids and neutral lipids. However, no detectable degradation of [^{3}H-NeuAc]GM3 was observed, indicating that the ketoside bonds of gangliosides were sufficiently stable during development.

Most of the GSLs and SM of HL-60 cells showed doublet spots. The molecular species analysis revealed that the ceramides of the upper spots of both GSLs and SM were composed mainly of d18:1 sphingenine and C24:0, C22:0 and C24:1 fatty acids whereas the lower ones were composed of d18:1 sphingenine and C16:0 fatty acid. The upper spot of each GSL contained higher radioactivity than the lower one. In contrast, SM contained higher radioactivity in the lower spot. These relationships were also maintained during the metabolic changes in sphingolipids during HL-60 cell differentiation induced by TPA. These results indicate that the molecular species of ceramides which are used preferentially for biosynthesis differ between GSLs and SM, although both GSLs and SM are composed of the same molecular species of ceramides. The biological activities of ceramide have been assessed by utilization of synthetic analogues such as *N*-acetyl-sphingosine (C2-ceramide) to overcome the problem of aqueous insolubility of the natural compound [26]. In addition, C2-ceramide has been considered to substitute for ceramide derived from SM. However, our results strongly suggest that further studies should be performed to determine which molecular species of ceramide possess biological activities, in order to elucidate the overall stimulus-induced sphingolipid metabolism through which signals appear to be flowing.

The radioactivities of both spots of GSL doublets changed in response to 1.6 μM TPA, although the radioactivities of the SM doublet did not change. These results suggest that not only ceramide pools but also their metabolic regulation of ceramide in GSLs differ from those of SM, contrary to other investigators who have speculated recently that the metabolic pathways of GSLs and SM interdependent [24] and that stimulation of ganglioside GM3 biosynthesis might be closely associated with macrophage-like cell differentiation of HL-60 cells, the 'sphingomyelin cycle' possibly being unnecessary for this event.

Most studies which have shown alterations of GSL profiles have focused only on the hydrophilic moiety. The present data support the consideration that further study should be performed taking into account not only the hydrophilic moiety but also the hydrophobic moiety in order to elucidate the precise mechanisms which regulate the metabolic pathways of GSLs and SM during cell differentiation. The present system should prove useful for such a study.

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