Regulation of UDP-glucose:ceramide glucosyltransferase-1 by ceramide

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Abstract **We report that the expression of mRNA and the activity of UDP-glucose:ceramide (Cer) glucosyltransferase-1 (GlcT-1) of human hepatoma Huh7 and mouse melanoma B16 cells increases after treatment with bacterial sphingomyelinase or upon addition of short-chain Cer. Interestingly, however, GlcT-1 gene transcription was not increased by Cer when GlcT-1 cDNA was introduced with the CMV promoter in GlcT-1-deficient GM95 cells, suggesting that the normal promoter region of GlcT-1 gene is essential for the response. The conversion of C6-Cer to C6-GlcCer occurred much more rapidly in GlcT-1-overexpressing Huh7 cells than in mock transfectants. As a result, GlcT-1-overexpressing cells acquired a greater resistance to C6-Cer-mediated cell death.**

Key words: Ceramide; UDP-glucose:ceramide glucosyltransferase-1; Sphingolipid; Glycosphingolipid; Sphingomyelin; Sphingomyelinase

1. Introduction

Ceramide (Cer) is a common lipid backbone of glycosphingolipids (GSLs) and sphingomyelin. Recently, Cer and its metabolites, sphingosine and sphingosine-1-phosphate, have emerged as a new class of lipid biomodulator of cell functions [1–3]. The core structure of the majority of GSLs is glucosylceramide (GlcCer), which is synthesized on the cytoplasmic face of the Golgi apparatus by UDP-glucose (Glc):Cer glucosyltransferase-1 (GlcT-1). This enzyme is a typical type III glycosyltransferase and catalyzes the transfer of a Glc residue from UDP-Glc to Cer [4]. The balance of GSL synthesis and degradation is completely regulated within the cell but the mechanism that regulates GSL metabolism still remains to be elucidated.

Previousl,y we reported that genetic and pharmacological blockade of GlcT-1 resulted in intracellular accumulation of Cer [5]. In the present paper, we report that Cer regulates GlcT-1, thus controlling the content of Cer in human hepatoma Huh7 cells and mouse melanoma B16 cells.

2. Materials and methods

2.1. Materials

D-*Threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and *N*-hexanoylsphingosine (C6-Cer) were purchased from Matreya (USA) and thin-layer chromatography (TLC) plates (silica gel 60) from Merck (Germany). Sphingomyelinase (SMase) from *Bacillus cereus* was obtained from Funakoshi Co. (Japan). [^{14}C]Galactose (Gal) was purchased from Du Pont-New England Nuclear (USA), [α-^{32}P]dCTP (6000 Ci/mmol) was obtained from Amersham (USA) and [^{14}C]palmitic acid and [^{14}C]UDP-Glc were from American Radiolabeled Chemicals (USA). Human GlcT-1 cDNA was isolated by expression cloning as described in [6]. All other chemicals were of the highest grade available.

2.2. Cells

GlcT-1-deficient mutant cells, GM95 cells, were obtained from B16 melanoma cells after mutagenesis [7]. GlcT-1 revertant cells, CG1 cells, were generated from GM95 cells by introduction of GlcT-1 cDNA with the cytomegalovirus (CMV) promoter. Human hepatoma Huh7 cells were kindly donated by Dr. A. Komori, Faculty of Medicine, Kyushu University (Japan).

2.3. Cell culture and metabolic labeling

Cells were cultured at 37°C in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) in a humidified 95% air, 5% CO_2 incubator. For labeling experiments, cells were incubated in 200 μl of Dulbecco's MEM (DMEM) supplemented with 5% FBS containing 1 μCi of [^{14}C]Gal or 0.5 μCi of [^{14}C]palmitic acid for the times indicated.

2.4. Extraction and determination of radiolabeled sphingolipids from cells

Cells (1×10^5) were harvested by centrifugation at 2000 rpm for 5 min and washed twice with phosphate-buffered saline (PBS). Total lipids were extracted with 750 μl of *i*-propanol/hexane/water (55:35:10, v/v) with sonication for 20 min. After centrifugation at 13 000 rpm for 5 min, the supernatants obtained were dried under N_2 gas, dissolved in 25 μl of chloroform/methanol (2:1, v/v), and applied to TLC plates, which were developed with chloroform/methanol/0.02% $CaCl_2$ (5:4:1, v/v). Radiolabeled GSLs separated on TLC plates were quantified using a BAS1500 imaging analyzer (Fuji Film, Japan). Identification of C6-GlcCer was performed by the method described in [8].

2.5. Assay for GlcT-1

GlcT-1 activity shown in Fig. 2C was determined using a radiolabeled UDP-Glc as a donor according to the method of Basu et al. [9] with some modification. To prepare cell lysates, cells were washed with 1 ml of PBS and suspended in 50 μl of 10 mM Tris–HCl buffer, pH 7.5, containing 2 mM KCl and 5 mM $MgCl_2$. Standard incubation mixture (50 μl) contained 0.5% Triton X-100, 500 μM [^{14}C]UDP-Glc (0.02 μCi/reaction), 0.3 mM natural Cer (type III, Matrea, USA), and cell lysate (500 μg as protein) in 20 mM Tris–HCl buffer, pH 7.5. After incubation at 32°C for 2 h, 100 μl of chloroform/methanol (2:1, v/v) was added to terminate the reaction, and the lower layer was applied to TLC plates, which were then developed with chloroform/

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Abbreviations: Cer, ceramide; C6-Cer, *N*-hexanoylsphingosine; CMV, cytomegalovirus; Gal, galactose; GlcT-1, UDP-glucose:ceramide glucosyltransferase-1; Glc, glucose; GlcCer, glucosylceramide; GSL(s), glycosphingolipid(s); MEM, minimal essential medium; DMEM, Dulbecco's minimal essential medium; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; SMase, sphingomyelinase; TLC, thin-layer chromatography

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methanol/12 mM $MgCl_2$ (65:25:4, v/v). The [^{14}C]GlcCer produced was detected and quantified with an imaging analyzer. GlcT-1 activity shown in Fig. 3A was determined using fluorescent Cer as a donor by the method described in [5].

50 μg of C6-NBD-Cer was mixed with 500 μg of phosphatidylcholine and 10 μg of sulfatide in 1 ml of distilled water to form liposomes. To prepare cell lysates, cells (5×10^5) were washed with 1 ml of PBS and were suspended in 50 μl of 10 mM Tris–HCl buffer, pH 7.5. The incubation mixture contained 10 μl of liposomes containing C6-NBD-Cer, 500 μM UDP-Glc, 1 mM EDTA and cell lysate (50 μg as protein) in 50 μl of 16 mM Tris–HCl buffer, pH 7.5. After incubation at 32°C for 1 h, 100 μl of chloroform/methanol (2:1, v/v) was added to terminate the reaction, and the lower layer was applied to TLC plates, which were then developed with chloroform/methanol/12 mM $MgCl_2$ (65:25:4, v/v). The NBD-GlcCer produced was detected and quantified with a Shimadzu CS-9300 chromatoscanner (excitation 475 nm, emission 525 nm).

2.6. Cell viability assay

Cell viability was measured using a cell counting kit (Dojindo, Japan) according to the manufacturer's instructions. Cells were seeded on 24-well plates at a density of 5×10^4 cells/well for 12 h. After treatment with C6-Cer for an appropriate time, 10 μl of the cell counting reagent was added to the culture and the cells were incubated at 37°C for 30 min. Aliquots of culture were transferred into the wells of a 96-well plate and measured at 490 nm by a microplate reader (Bio-Rad, USA).

2.7. Northern blotting analysis

Aliquots of 30 μg of total RNA were subjected to electrophoresis on 1% agarose gels containing 18% formaldehyde, and transblotted onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech, UK). Nylon membranes were hybridized with ^{32}P-labeled GlcT-1 probe at 42°C for 24 h. A human β-actin cDNA (Wako Co., Japan) was used as an internal control.

2.8. Overexpression of GlcT-1

Human hepatoma Huh7 cells (1×10^5 cells/well) were seeded in a 6-well plate containing MEM in the presence of 10% FBS. After incubation at 37°C for 18 h in a CO_2 incubator, cells were transfected with 1 μg of PIRES-EGFP expression vector (Clontech, USA) alone or with vector containing GlcT-1 cDNA construct, and 6 μl of PLUS reagent plus 4 μl of LipofectAMINE reagent per well were added essentially according to the manufacturer's instructions. After 4 h, the medium was changed to 2 ml of MEM-α containing 10% FBS and incubated at 37°C for the times indicated.

3. Results and discussion

Previously, we reported that genetic or pharmacological dysfunction of GlcT-1 resulted in the accumulation of Cer in cells [5]. In this study, we examined the effects of Cer accumulation on the expression of GlcT-1 mRNA using variants of B16 mouse melanoma cells, GlcT-1-deficient mutant GM95 cells and GlcT-1 revertant CG1 cells, in which the cDNA encoding GlcT-1 was introduced into GM95 cells with the CMV promoter. The accumulation of Cer in CG1 cells was found to be much lower than that in GM95 cells but higher than that in B16 cells after treatment with bacterial SMase (Fig. 1A). In the presence of PDMP, a potent inhibitor of GlcT-1 [10], the accumulation of Cer in both CG1 and B16 cells increased markedly after SMase treatment. However, PDMP had no effect on the Cer content of GlcT-1-deficient GM95 cells after SMase treatment (Fig. 1A). The expression of GlcT-1 mRNA in B16 cells increased in proportion to the accumulation of the Cer in the cells (Fig. 1B, left). Interestingly, however, CG1 cells in which the promoter region of the GlcT-1 gene was replaced by the CMV promoter showed no increase in the expression of the introduced GlcT-1 mRNA (Fig. 1B, right), regardless of Cer accumulation (Fig. 1A). These results indicate that Cer regulates GlcT-1 partly at the transcriptional step of GlcT-1 synthesis, for which the normal promoter region of the GlcT-1 gene is essential.

It has been reported that addition of GlcCer stimulates the cell growth and DNA synthesis of hepatocytes [11], and that lipopolysaccharides markedly increase the mRNA level of hepatic GlcT-1 [12]. These observations appear to indicate a biological role of GlcT-1 in hepatocytes. In this study, we

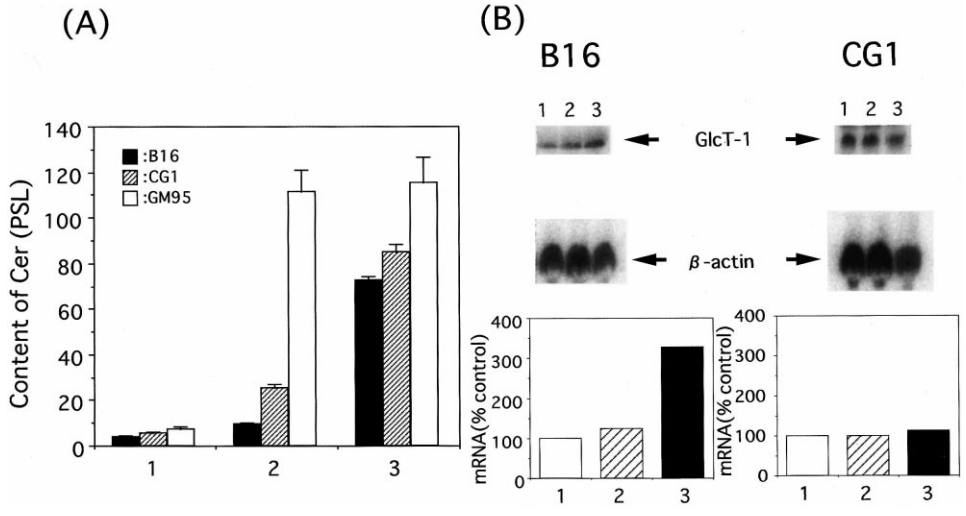

Fig. 1. The effects of Cer contents on the expression of GlcT-1 mRNA expression in B16 cells, GlcT-1-deficient GM95 cells and GlcT-1 revertant CG1 cells. (A) Effects of SMase and PDMP on Cer contents. Cells (1×10^5) were metabolically labeled with 0.5 μCi [^{14}C]palmitic acid for 24 h, washed with fresh DMEM and recultured in DMEM containing 20 mU of SMase from *B. cereus* for 12 h in the presence or absence of PDMP (20 μM). Lane 1, control without SMase; 2, treatment with SMase; 3, treatment with SMase in the presence of PDMP. PSL: radioactivity expressed as photo-stimulated luminescence/mm^2. (B) Effects of Cer contents on GlcT-1 mRNA expression in B16 cells and CG1 cells. B16 or CG1 cells (6×10^6) were treated with 500 mU of SMase from *B. cereus* for 12 h in 5 ml of MEM supplemented with 5% FBS. Total RNA was isolated from cells using a RNeasy Mini kit (Qiagen, Germany) and analyzed by the method described in Section 2. Lanes: 1, control experiment without SMase; 2, treatment with SMase; 3, treatment with SMase in the presence of PDMP. The values are the means of duplicate determinations.

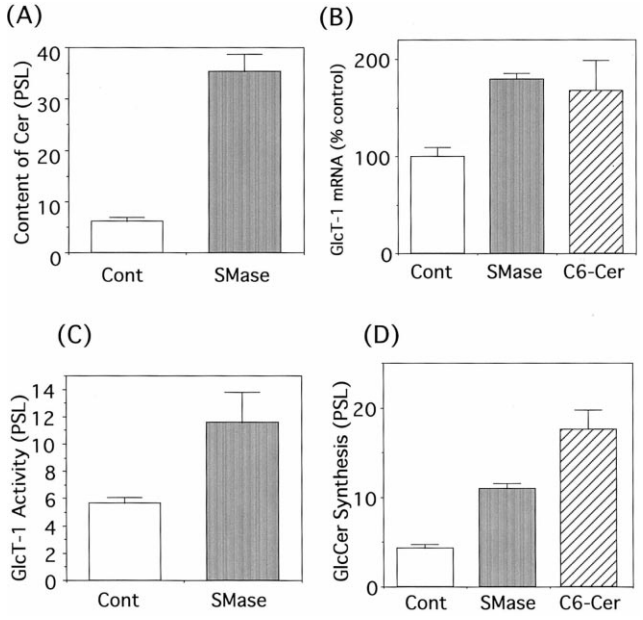

Fig. 2. Effects of Cer on GlcT-1 mRNA expression, GlcT-1 activity and GlcCer synthesis of human hepatoma Huh7 cells. (A) Cer contents. The experiment was conducted as shown in the legend of Fig. 1A. Cont, control experiment without SMase treatment. (B) Northern blotting analysis of GlcT-1 mRNA. The experiment was conducted as shown in the legend of Fig. 1B. (C) GlcT-1 activity. Huh7 cells (4×10^6) were treated with 500 mU of SMase from *B. cereus* in 5 ml of DMEM supplemented with 5% FBS for 12 h. GlcT-1 activity was measured using [^{14}C]UDP-Glc and a natural Cer (type III) as described in Section 2. (D) GlcCer synthesis. After treatment with 20 mU of SMase from *B. cereus* or 20 μM C6-Cer for 12 h, Huh7 cells were washed with fresh DMEM and metabolically labeled with 1 μCi of [^{14}C]Gal for 3 h in the same medium without SMase or C6-Cer. Total lipids were extracted and analyzed by TLC as described in Section 2. CMH produced was identified to be GlcCer, but not GalCer, by borate-impregnated TLC using chloroform/methanol/water (65:25:3.5, v/v) as described in [17]. The values in the figure are the means ± S.D. for triplicate determinations. PSL: radioactivity expressed as photo-stimulated luminescence/mm^2.

examined the effects of Cer on GlcT-1 mRNA expression, GlcT-1 activity and GlcCer synthesis in human hepatoma Huh7 cells to clarify whether or not GlcT-1 is regulated by Cer in liver cells. The content of Cer in the cells was found to be increased about 7-fold after treatment with a bacterial SMase (Fig. 2A). GlcT-1 mRNA expression (Fig. 2B), GlcT-1 activity (Fig. 2C) and GlcCer synthesis (Fig. 2D) were also increased concomitantly with Cer accumulation. Addition of C6-Cer to the culture of hepatoma cells also stimulated transcription of the GlcT-1 gene (Fig. 2B) and GlcCer synthesis (Fig. 2D). These results indicated that Cer regulates GSL synthesis in not only mouse melanoma cells but also human hepatoma cells via activation of GlcT-1, which occurs partly at the transcriptional step of GlcT-1 synthesis.

Huh7 cells were transfected with the expression vector alone (mock cells) or with vector containing a GlcT-1 cDNA construct (GlcT-1-overexpressing cells), and then the GlcT-1 activities of both cell lysates were examined using C6-NBD-Cer as an acceptor substrate. As expected, the GlcT-1 activity of lysates of GlcT-1-overexpressing cells was much higher than that in lysates of mock cells (Fig. 3A). When C6-Cer was added to cultures of GlcT-1-overexpressing cells and mock cells, the short-chain Cer was promptly glycosylated to metabolize C6-GlcCer. It should be noted, however, that the conversion ratio of C6-Cer to C6-GlcCer in GlcT-1-overexpressing cells was much higher than that in mock cells (Fig. 3B). Short-chain Cer is generally toxic to cells when added exogenously in excess [13]. In fact, cell viability was decreased by 20% when C6-Cer was added to the culture of Huh7 mock cells at a concentration of 40 μM, whereas short-chain Cer treatment did not affect the viability of the GlcT-1-overexpressing Huh7 cells at the same concentration (Fig. 3C). These results showed that Huh7 cells acquired insensitivity to the short-chain Cer when they overexpressed GlcT-1 possibly

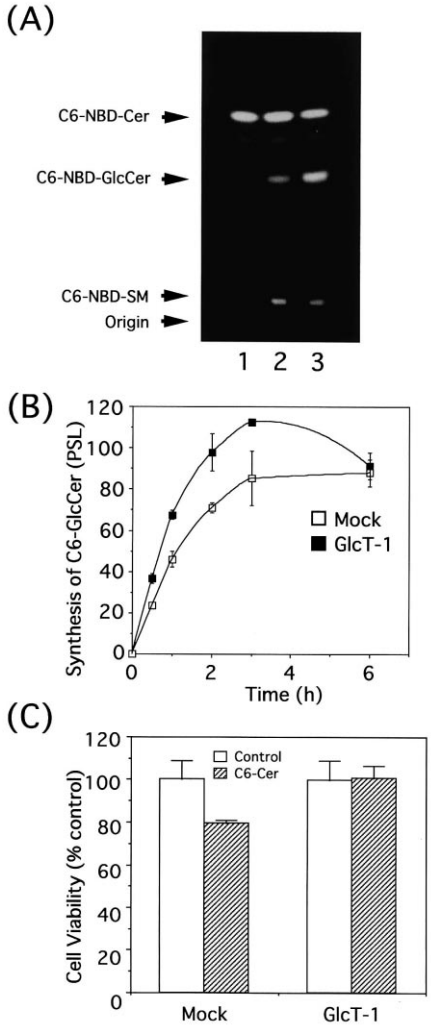

Fig. 3. Effects of C6-Cer on GlcT-1-overexpressing human hepatoma Huh7 cells. (A) TLC showing GlcT-1 activity of cell lysates using C6-NBD-Cer as an acceptor. Details are given in Section 2. 1, C6-NBD-Cer standard; 2, mock transfectants; 3, GlcT-1-overexpressing cells. (B) Generation of C6-GlcCer in GlcT-1-overexpressing cells or mock transfectants. GlcT-1-overexpressed cells or mock transfectants (1×10^5) were incubated at 37°C for 3 h in 200 μl of DMEM supplemented with 5% FBS containing 1 μCi [^{14}C]Gal and 20 μM C6-Cer. (C) Cell viability of GlcT-1-overexpressing cells or mock transfectants after treatment with C6-Cer. GlcT-1-overexpressing cells or mock transfectants (5×10^4) were incubated with 40 μM C6-Cer at 37°C for 24 h in 200 μl of MEM-α supplemented with 10% FBS. Cell viability was determined using a cell counting kit as described in Section 2. The values are the means ± S.D. for triplicate determinations.

due to reduction of the intracellular concentration of short-chain Cer. It was found that some of the C6-GlcCer produced by both normal and overexpressing cells was released into the culture medium (data not shown). It is interesting to note that cytotoxic resistance to tumor necrosis factor-α, which increases the intracellular Cer content, has been conferred in human breast cancer cells by introduction of the GlcT-1 gene [14].

The precise mechanism by which the activity of GlcT-1 is stimulated by Cer is still unknown. In this study, we found that the promoter region of GlcT-1 is indispensable for transcriptional activation of the GlcT-1 gene by Cer. Recently, Ichikawa et al. reported the structure of the GlcT-1 gene and revealed the presence of motifs for the binding sites of NF-κB, AhR and AP-2 in the promoter region of GlcT-1 [15]. We speculate that GlcT-1 could be stimulated by Cer partly through the activation of gene transcription by specific factor(s) such as NF-κB. It is interesting to note that tumor necrosis factor-α increased the intracellular level of Cer concomitantly with activation of NF-κB [16]. GlcT-1 could also be activated at the post-translational level, since activation of GlcCer synthesis by Cer was also observed in the presence of cycloheximide in B16 cells [5].

In summary, we conclude that Cer regulates GlcT-1 partly at the transcriptional step of GlcT-1 synthesis for which the promoter region of the GlcT-1 gene is essential, and that GlcT-1 regulates the content of Cer in cells especially when Cer is overproduced. However, further studies are needed to clarify the mechanism by which GlcT-1 is up-regulated by Cer.

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References

[1] Hannun, Y.A. (1996) Science 274, 1855–1859.
[2] Spiegel, S. and Merrill, A.H. (1996) FASEB J. 10, 1388–1397.
[3] Lee, M.-J., Van Brocklyn, J.R., Thangada, S., Liu, C.H., Hand, A.R., Menzeleev, R., Spiegel, S. and Hla, T. (1998) Science 279, 1552–1555.
[4] Ichikawa, S. and Hirabayashi, Y. (1998) Trends Cell Biol. 8, 198–202.
[5] Komori, H., Ichikawa, S., Hirabayashi, Y. and Ito, M. (1999) J. Biol. Chem. 274, 8981–8987.
[6] Ichikawa, S., Sakiyama, H., Suzuki, G., Hidari, K.I.-P.J. and Hirabayashi, Y. (1996) Proc. Natl. Acad. Sci. USA 93, 4638–4643.
[7] Ichikawa, S., Nakajo, N., Sakiyama, H. and Hirabayashi, Y. (1994) Proc. Natl. Acad. Sci. USA 91, 2703–2707.
[8] Komori, H. and Ito, M. (1995) FEBS Lett. 374, 299–302.
[9] Kaufman, S., Basu, B. and Roseman, S. (1973) J. Biol. Chem. 248, 1388–1394.
[10] Radin, N.S., Shayman, J.A. and Inokuchi, J. (1993) Adv. Lipid Res. 26, 183–213.
[11] Datt, S.C. and Radin, N.S. (1988) Lipids 23, 508–510.
[12] Memon, R.A., Holleran, W.M., Uchida, Y., Moser, A.H., Ichikawa, S., Hirabayashi, Y., Grunfeld, C. and Feingold, K.R. (1999) J. Biol. Chem. 274, 19707–19713.
[13] Obeid, L.M., Linardic, C.M., Karolac, L.A. and Hannun, Y.A. (1993) Science 259, 1769–1771.
[14] Liu, Y.-Y., Han, T.-Y., Giuliano, A.E., Ichikawa, S., Hirabayashi, Y. and Cabot, M.C. (1999) Exp. Cell Res. 252, 464–470.
[15] Ichikawa, S., Ozawa, K. and Hirabayashi, Y. (1998) Biochem. Biophys. Res. Commun. 253, 707–711.
[16] Van Antwerp, D.J., Martin, S.J., Verma, I.M. and Green, D.R. (1998) Trends Cell Biol. 8, 107–111.
[17] Hirabayashi, Y., Hamaoka, A., Matsumoto, M. and Nishimura, K. (1986) Lipids 21, 710–714.