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ORIGINAL ARTICLE

Tumor specific cytotoxicity of glucosylceramide

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Abstract To develop a new taxon of anti-cancer agent with lower side effect, this study described a tumor selective cytotoxicity of glucosylceramide extracted from malt feed of beer brewing waste. Interpretation of ^{13}C- and ^{1}H-NMR spectra identified the chemical structure of major component of glucosylceramide as 1-*O*-β-D-glucopyranosyl-2(2′-hydroxyeicosanoylamino)-4,11-octadecadiene-1,3-diol. Selective cytotoxicity was studied with three pairs of normal and cancer cells: liver, skin and lung. The glucosylceramide selectively lowered the relative viability of cancer cells. Of the pairs, the selectivity was most pronounced with the liver cells, and, for this reason, further experiment was conducted with this pair of normal (CS-HC) and cancer cells (HepG2) to get more insight into the selective toxicity. The glucosylceramide significantly increased the cell population at G_2/M phase in HepG2 cells, and also increased the numbers of apoptotic (sub-G_0/G_1) cells, but to much lesser extent compared with the increase in G_2/M phase. Treatment of HepG2 cells with this agent selectively disrupted the mitochondrial membrane integrity without activation of caspase pathway to induce apoptosis. These findings suggested that the glucosylceramide specifically suppressed the growth of cancer cells by inhibiting cell renewal capacity rather than induction of apoptosis. The underlying mechanism for the selectivity remains to be answered in the forthcoming study.

Keywords Glucosylceramide · Tumor selective · Cytotoxicity · Plant · Ceramide

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Introduction

Glycosphingolipids are ubiquitous components of plant materials [1], and have been shown to display a variety of biological activities: anti-tumor activity [2–4]; anti-microbial activity [5, 6]. There is considerable structure variation among the sphingolipids with respects to the types of polar head groups and ceramide backbones. The biological activities, therefore, showed variation with their chemical structure: carbon-chain structure of sphingoid bases and/or compositions of amide-linked fatty acids. In the past decade, ceramide has also been shown to be of crucial significance in several cell functions including apoptosis, cell growth, senescence and cell cycle modulation. These sphingolipids are important membrane components of animal cells as well, and are emerging as signaling lipid in several biological pathways [7, 8].

Because of their biological activities, a number of studies have been devoted to isolate the sphingolipids from many natural raw materials [9–11]. Of the plant materials, rice bran or wheat germ have been used as a commercial source of glucosylceramide. Malt feed is the largest solid waste of beer production, and expected to contain bioactive lipids such as glucosylceramide because sphingolipids are widely distributed within the plant kingdom [1, 12]. From the economical point of view, utilization of the brewing waste as a source of bioactive agents attracted our attention. This study describes the isolation of glucosylceramide from brewing waste, and its selective cytotoxicity to tumor cells. The feature of tumor selectivity is very important for the development of new taxon of anti-cancer agent with low side effect. Special emphasis was therefore placed on the tumor specific toxicity of this compound, and the possible mechanisms for the cytotoxicity was discussed.

Materials and methods

Chemicals

Lipids standards were purchased from Nacarai Tesque (Kyoto, Japan) or Funakoshi Co. Ltd. (Tokyo, Japan). MitoCapture reagent was purchased from Biovision, Inc. (CA, USA). Other chemicals were all guaranteed grade, and obtained from domestic suppliers.

Preparation of crude ceramide

Malt feed was dried and extracted with ethanol at 60°C for 4 h. The extract was filtered through sheet filter (Advantec Type 26), and stored at −35°C for 2 days to precipitate lipid solute. The precipitate was washed with distilled water, and collected by centrifugation at 8,000×*g* for 5 min, and freeze-dried.

Purification of glucosylceramide

Two grams of crude ceramide in 4 ml of chloroform was mixed with 4 ml of 0.6 N NaOH in methanol, and methanolysed at 50°C for 30 min. To this methanolysate were added 2.6 ml of 1 N HCl and 1 ml of water to split the lysate into methanol/water and chloroform layers. The chloroform layer containing approximately 1 g of lipids was applied to silica gel (Wako gel, Wako Chemicals Co., Osaka, Japan) column (1.5 cm ID × 30 cm), and was eluted with chloroform/methanol/water (65/15/2, by vol.). Presence of ceramide in the eluent was checked by analysis on HPTLC plates (Merck, Dermstadt, Germany) developed with chloroform/methanol/water (65/15/2, by vol.). The ceramide fraction was concentrated to dryness, and taken up in about 500 μl of chloroform for further purification by HPLC equipped with an evaporative light scattering detector (ELSD-LT, Shimadzu Co., Kyoto, Japan). Aliquot of the ceramide fraction in 100 μl was injected into pre-packed HPLC silica gel column (Develosil 60–30, 8 mm ID × 250 mm), and eluted with chloroform/methanol (87/3, by vol.) The peak corresponding to glucosylceramide was collected and used for the identification of its chemical structure. Yield of purified glucosylceramide was usually 2% against crude ceramide as starting material.

Identification of chemical structure

^{13}C- and ^{1}H-NMR spectra were obtained at 125 MHz for ^{13}C by a Jeol α-500 spectrometer. The ^{13}C signals of the solvent (CD_3OD) were used as secondary references (49.0 from TMS). Aliquot of this sample was also subjected to acid hydrolysis for analysis of its fatty acid and sugar constituents. For the analysis of fatty acid component, purified ceramide was transmethylated with methanolic HCl at 50°C for 12 h. Methyl esters produced were analyzed by gas chromatography (GC) or GC-mass spectrometer (GC-MS). Chemical structures for fatty acids were identified by interpretation of mass spectra, and or by similarity comparison of mass spectra with those of database library (NIST 147, Shimadzu Co., Kyoto, Japan). For the analysis of sugar component, purified ceramide was dissolved in 1 ml of 0.1 M HCl at 90°C for 2 h. This reaction mixture was washed three times with ethyl acetate. The aqueous layer was neutralized with 0.1 M NaOH, and the sugar composition was analyzed by DXc-500 sugar analysis system [(Dionex Corporation, CA, USA), column; CarboPac PA 1 (4.0 i.d. × 250 mm), guard column; CarboPac PA1 GUARD, solvent; 10 mM NaOH, flow rate; 1.0 ml/min, detector; electro chemical detector ED-50].

Cells and tissue culture

The antitumor assay was performed using 16 different human normal or cancer cell lines. Of the cells, WI-38 (lung normal diploid fibroblast), WI-38 VA13 sub 2 RA (SV-40 transformed lung cells), OUMS-36 (normal human embryo fibroblast cell), OUMS-36T-2F (normal human embryo fibroblast cell), A549 (lung adenocarcinoma cell line), A431 (epidermoid carcinoma), TIG (human normal skin fibroblast), Raji (Burkit hyphoma), HepG2 (liver cancer cells) and KATOIII (stomach

cancer) were purchased from Japan cancer research source bank (JCRB, Ibaragi, Japan). Cells of Colo-201 (colon adenocarcinoma), Molt-4 (leukemia cell), MIA-PaCa2 (pancreatic cancer) and VMRC-LCP (lung squamous cell carcinoma) were from Health Science Resources Bank (Osaka, Japan). Cells mentioned above were cultured at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium, DMEM or EMEM medium supplemented with 10% fetal bovine serum.

SKBR-3 (breast cancer cells) and CS-HC (human primary hepatocytes) were purchased from Dainippon Pharmaceuticals Co. (Osaka, Japan). Culture medium for SKBR-3 was McCOY's 5A containing 10% FBS and CS-C medium kits from Dainippon Pharmaceuticals for CS-HC. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. The exponentially growing cells were used throughout the experiments.

Cell cytotoxicity assay

Glucosylceramide was dissolved in PBS containing 0.8% tween 80 as described previously [13]. Cell cytotoxicity titration curve was constructed with serial dilution of ceramide in a 96-well microplate. Cells seeded at density of 5,000 cells/well and pre-cultured in the medium for 24 h were treated with serially diluted ceramide for another 24 h, and the viable cell numbers were determined by MTS assay according to the manufacture's instruction (CellTiter® AQueous Non-Radioactive Cell Proliferation Assay, Promega Co., Madison, USA). Cell cytotoxicity was thus expressed as the relative viability against control cells treated only with the vehicle solutions.

Lipid analysis

Cells were pelleted by centrifugation at 1,500 rpm (800×g) for 5 min, and lipids were extracted with the method of Bligh-Dyer [14]. Lipids dissolved in chloroform were analyzed by HPLC system equipped with ELSD as described in the foregoing section. Column used was pre-packed silica gel column (Cosmosil 4.6 × 150 mm, nacalai tesque, Kyoto, Japan) and elution program was described previously [15]. Authentic samples used for the construction of standard curve were as follows: cholesterol ester (CE), cholesteryl oleate; triacylglycerol (TG), triolein; free fatty acid (FFA), stearic acid; phosphatidylethanolamine (PE); phosphatidylinositol (PI); phosphatidylserine (PS); phosphatidylcholine (PC) and glucocerebroside. Cell proteins were determined by the method of Lowry [16], and the lipid concentrations were expressed as mg/mg cell protein.

Quantitation of apoptotic cells

Cells (1×10^4 cells/dish) were pre-cultured in the medium for 24 h, and incubated further with 200 μM ceramide for 5 h. On completion of the incubation, cells were washed with PBS and re-suspended in 100 μl of binding buffer containing Anexin V (MBL, Nagoya, Japan) and incubated for 30 min. Cells were washed with binding buffer, and apoptotic cells stained with Anexin V were counted with a fluorescence microscope. Data were expressed as percentages of apoptotic cells in the total cells.

Mitochondrial membrane integrity

Cells cultured for 24 h on coverslips were incubated with 200 μM ceramide sample for 4 h, and loaded with MitoCapture (Biovision, USA) reagent for 2 h at 37°C under 5% CO_2 atmosphere. Cells were observed with a fluorescence microscope using band-pass filter. MitoCapture aggregates and fluoresces red in the mitochondria of healthy cells. However, in apoptotic cells, MitoCapture cannot accumulate in the mitochondria, it remains as monomers in the cytoplasm, and fluoresces green. Number of mitochondria disrupted cells were counted for four to six separate microscopic fields, and the percentages are shown.

Measurement of caspase activity

Cells were pre-cultured in the medium for 24 h, and were further incubated with 200 μM ceramide for 4 h. Cells were lysed in buffer containing 10 mM Tris–HCl (pH 7.5), 10 mM $Na_2H_2PO_4/Na_2HPO_4$, 130 mM NaCl, 1% Triton X-100 and 10 mM sodium pyrophosphate. The cell lysates (45 μl) were mixed with 5 μl of fluorogenic caspases-3 substrate (500 μM) DEVD-R110 (Roche Diagnostics, Mannheim, Germany) in 96-well micro titer plate according to the manufacturer's instruction. The plate was incubated at 37°C for 1 h, and then fluorescence was monitored with excitation and emission wavelengths at 485 and 535 nm, respectively.

Flowcytometry

HepG2 cells were treated with 150 μM glucosylceramide for 8, 16 and 24 h. Cells were harvested with trypsin-EDTA, followed by washing with PBS. Cells were fixed with 75% ethanol at –20°C overnight. Cell pellets were re-suspended in 800 μl of PBS, mixed with 100 μl of 1 mg/ml RNase and 900 μl of 0.4 mg/ml propidium iodide, and incubated at 37°C in the dark for 30 min.

The cell suspension was filtered through a 35 mM nylon filter. Data acquisition and analysis were performed by a FACSCalibur flowcytometer system (BD Biosciences). Cell cycle analysis was performed with CellQuest (BD Biosciences) software. Every measurement usually counted at least 10,000 events.

Result

Figure 1 shows the possible chemical structure of the major component comprising almost 60% of glucosylceramide from malt feed. The ^{13}C- and ^{1}H-NMR spectra listed in Table 1 were comparable to the previous data [3], and identified the chemical structure of glucosylceramide as shown in Fig. 1. However, some uncertainty exists in the chemical structure of sphingoid base. It is often difficult to localize the double bond position in the long aliphatic carbon chain only based on the interpretation of ^{13}C- and ^{1}H-NMR spectra, and the same difficulty was encountered in the present study. The sugar component was identified as glucose by HPLC analysis (data not shown). Amide linked fatty acids were identified as α-hydroxy fatty acids by GC-MS spectrometry. The largest fatty acid component was α-hydroxyeicosanoic acid comprising about 50% of total amid linked fatty acids (Table 2).

The ^{13}C-NMR spectra for other minor components in the preparation were almost similar to that mentioned above, suggesting that these were glucosylceramides with varied chain length and sphingoid bases. This glucosylceramide preparation was used in this experiment without further purification.

Figure 2 depicts the effect of glucosylceramides on the growth of normal and cancer cells. Of the cell lines, limited number of normal and cancer cells from the same tissue have been available. Thus, three pairs of normal and cancer cells were employed in this study: liver, skin and lung. As shown in Fig. 2, the glucosylceramide specifically inhibited the growth of cancer cells. This is especially manifested with the pairs of liver cells: CS-HC and HepG2 cells. In the case of skin and liver cells, higher concentration of glucosylceramide slightly lowered the viability of normal cells but to definitely lesser extent compared to the case of cancer cells.

A series of normal and cancer cell lines were used to evaluate the cytotoxicity of the glucosylceramide. Of the cancer cells, Molt-4 and Colo 201 cells were susceptible to this ceramide, while Raji, MI-Paca, VMRC-LCP, KATO-III and SKBR-3 cells were resistant (data not shown). In the case of normal cells, no toxicity was observed for OUMS, OUMS-36 and WI-38 VA13 sub 2 RA (data not shown). The cancer specific cytotoxicity was most pronounced with the pair of liver cells. Further experiments on selective cytotoxicity were therefore carried out with the pair of these cells.

In order to examine the incorporation of glucosylceramide into cellular lipids, the lipid concentrations were analyzed. However, no appreciable amount of glucosylceramide was detected in both CS-HC and HepG2 cells after 4 h of treatment (data not shown).

Figure 3 shows cell viability (A) and cell cycle profile (B) of HepG2 cells grown in the presence of glucosylceramide for 0, 8, 16 and 24 h. Cell viability decreased to 54% at 16 h, and was almost 20% of control at 24 h (Fig. 3a). The cell population of G_2/M phase increased from 25% at 0 h to 31% at 16 h, and to 46% at 24 h of treatment (Fig. 3b). At 16 h, there was a slight increase in the population of apoptotic cells (sub-G_0/G_1 phase) accounting for about 3% of total cells, which decreased to 0.4% at 24 h (Fig. 3b).

Figure 3 demonstrated the presence of apoptotic cell (sub-G_0/G_1) population in HepG2 cells grown in glucosylceramide. To confirm this apoptotic event, cells grown in glucosylceramide were stained with Anexin V, and the apoptotic cells were counted by use of fluorescence microscope. Glucosylceramide specifically increased the numbers of apoptotic cells only in HepG2 cells (Fig. 4). This was not the case of normal cell in which no effect was noted. Maximal induction of apoptosis was attained at 75 μM with HepG2 cells, and the percentages of apoptotic cell decreased with further increase in the glucosylceramide concentrations (Fig. 4).

Mitochondria play a central role in the induction of apoptosis [17]. To gain more insight into the mechanism of apoptosis induction by this glucosylceramide, mitochondrial membrane integrity was examined after 5 h of incubation with this agent. In order to study the membrane integrity, MitoCapture reagent

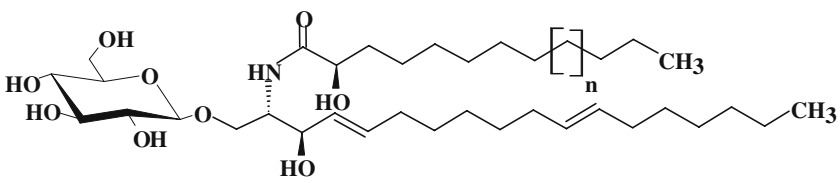

Fig. 1 Chemical structure of the major glucosylceramide extracted from malt feed of beer brewery waste

Table 1 ^{13}C and ^{1}H NMR spectral data of ceramides from malt feed

Position	C	H (*J* value)
1a	69.1	4.02
1b		3.79, dd (3.8, 10.0)
2	54.6	4.24, d (7.4)
3	71.6	4.08, m
4	130.8 or 130.7	5.35, m
5	130.8 or 130.7	5.35, m
6	32.9	1.24–1.24
7–9	31.0–30.1	1.28–1.40
10, 13	33.7	2.06–2.00
11	130.1 or 130.8	5.35, m
12	130.1 or 130.8	5.35, m
14–16	31.0–30.3	1.28, m
17	23.8	1.28, m
18	14.5	0.89, t
1′	177.1	–
2′	72.9	3.96, m
3′	35.9	1.24–1.24
4′	26.1	1.28, m
5′-n′-3	31.0–30.3	1.28, m
n′-2	32.9	1.28, m
n′-1	23.8	1.28, m
n′	14.5	0.89, t
Glc-1	104.7	4.27, d (7.9)
Glc-2	75.5	3.35, m
Glc-3	77.9	3.29, m
Glc-4	71.6	3.28, m
Glc-5	77.9	3.30, m
Glc-6a	62.6	3.66, m
Glc-6b		3.80, dd (10.5, 3.8)

Glc Glucose

Table 2 Compositions of α-hydroxy fatty acid

Chain length	Percentage
16:0	7.6 ± 0.4
18:0	15.5 ± 0.6
20:0	49.1 ± 0.7
22:0	10.5 ± 0.5
24:0	6.5 ± 0.2
24:1	10.3 ± 1.0

Data are mean ± SE of triplicate analyses

was used. In healthy cells, MitoCapture accumulate and aggregates in the mitochondria, and gives off a bright red fluorescence, while in apoptotic cells, this reagent cannot enter the mitochondria due to altered mitochondrial trans membrane potentials, and therefore remain predominantly in the cytosol in its monomeric form fluorescing green. Incubation of HepG2 cells with glucosylceramide significantly increased the number of mitochondria disrupted cells (Fig. 5). The loss of mitochondrial membrane integrity was observed only with HepG2 cells, but not with normal cells of CS-HC.

Disruption of mitochondrial membrane integrity releases pro-apoptotic factors, and activate the caspase cascade to induce apoptosis [17]. However, no increase in caspase 3 activity and caspase gene expressions were observed in this study, suggesting that glucosylceramide induced apoptosis via caspase independent pathway (data not shown).

Discussion

This study demonstrated the selective cytotoxicity of glucosylceramides for the first time. Several previous studies have demonstrated the anti-carcinogenic activity of dietary glucosylceramides [2–4]. However, no reference to its tumor selective cytotoxicity has been made. The tumor selectivity was manifested with all cell lines used in this study, and no cytotoxicity to normal cells was noted. This feature is especially beneficial to develop an anti-cancer agent of low side effect. Furthermore, addressing the mechanisms responsible for the selectivity may open up another possibility for molecular target in cancer chemotherapy.

Several glycolipids exert their biological activities by functioning as ligand to certain receptor proteins expressed on the cell membrane [18–20]. One postulate to explain the selectivity may be that the glucosylceramide specifically binds to the receptors expressed on the cancer cell membrane with the consequence of growth inhibition. However, it should be mentioned that the growth inhibition by glucosylceramide requires rather higher concentration (more than 100 μM) than that usually expected for ligands or agonists. The glucosylceramide used in this study is a mixture of ceramides with varied chain length (Table 2). In general, the biological activity of ceramide depends on the chemical structures of its sphingoid base and fatty acid constituent. Requirement for higher concentration in this study, therefore, may be explained in part by the lower concentration of active ceramide component in our preparation. Alternatively, it can be postulated that the higher concentration of glucosylceramide added to culture medium influenced the physicochemical property of cancer cell membrane. Membrane structure or microdomain of cancer cell may be susceptible to this agent compared with the normal cells, and this point should be studied to clarify the underlying mechanisms of the selective cytotoxicity.

Several lines of studies have demonstrated the anti-carcinogenic activities of exogenous synthetic or natural ceramides [5, 21–23]. The cellular targets of the ceramide are numerous, and include intracellular signaling pathway (Bax, Bad, Bcl-2, Akt/PKB, PI3K and PKC)

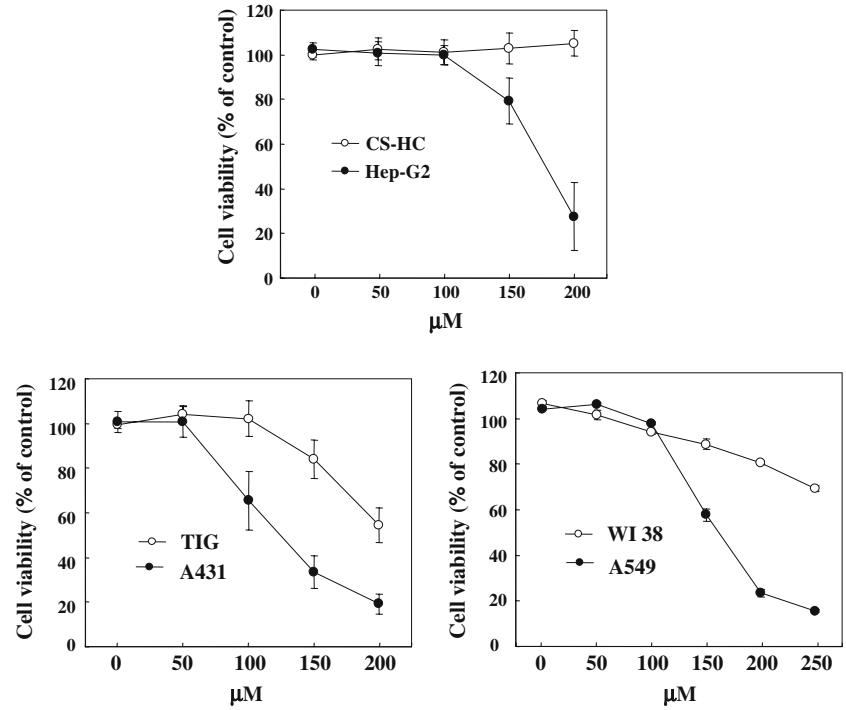

Fig. 2 Tumor selective cytotoxicity of glucosylceramide. *Open* and *closed circle* show the viabilities for normal and cancer cells, respectively. Cells used were originated from liver (CS-HC and HepG2), skin (TIG and A431) and lung (WI38 and A549). Cells seeded at density of 5,000 cells/well and pre-cultured in the medium for 24 h were treated with indicated concentrations of glucosylceramide for another 24 h, and the viable cell numbers were determined by MTS assay

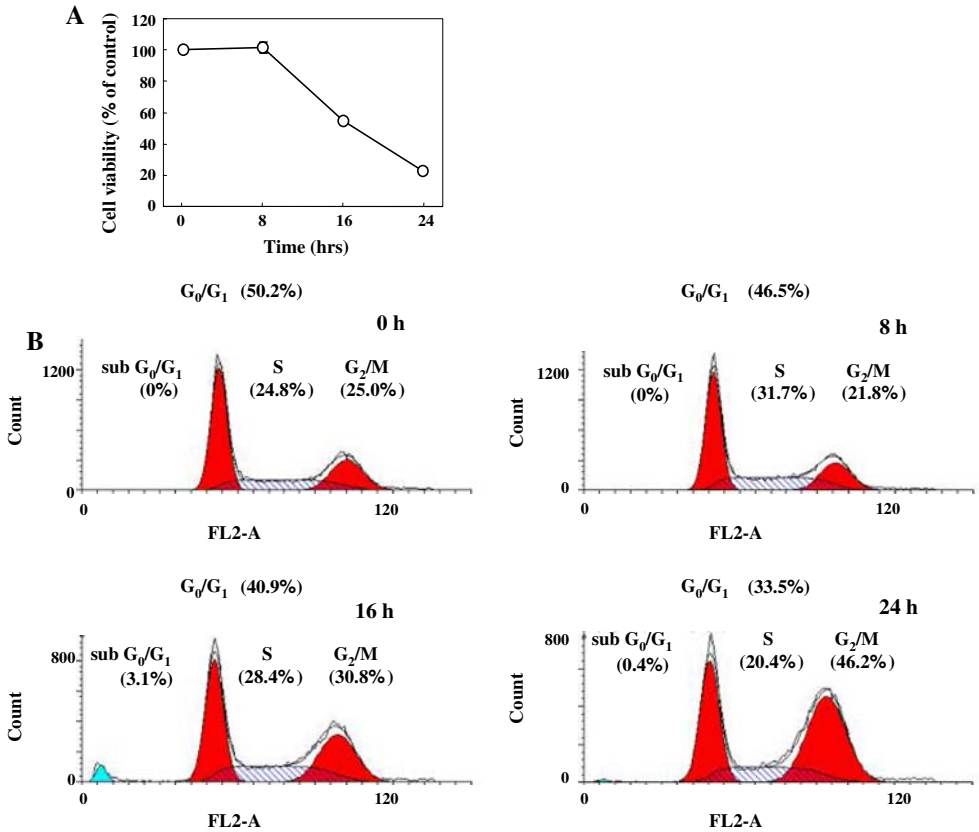

Fig. 3 Effect of glucosylceramide on the viability (**a**) and cell cycle (**b**) of HepG2 cells. Cells were grown with 150 μM of glucosylceramide for 0, 8, 16 and 24 h, and their viabilities and cell cycle profile were determined as described in the Materials and methods section

and cell cycle regulator (CIP/KIP family). Another mechanism by which ceramide may limit the self renewal capacity is ceramide-dependent inhibition of the gene expression of telomerase [24]. The proliferating cells protect themselves from these unfavorable effects of ceramide by converting ceramide to glucosylceramide or

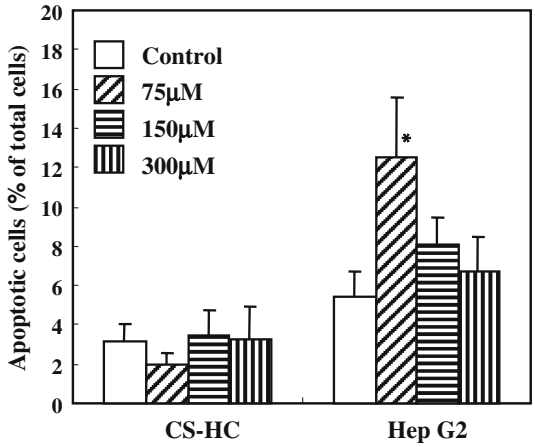

Fig. 4 Effect of glucosylceramide on the induction of apoptotic cell death. Cells were treated with 200 μM of glucosylceramide for 5 h, and the apoptotic cells were counted with a fluorescence microscope. *Asterisk* denotes the statistically significant difference from normal cells by Student's *t* test ($P < 0.05$)

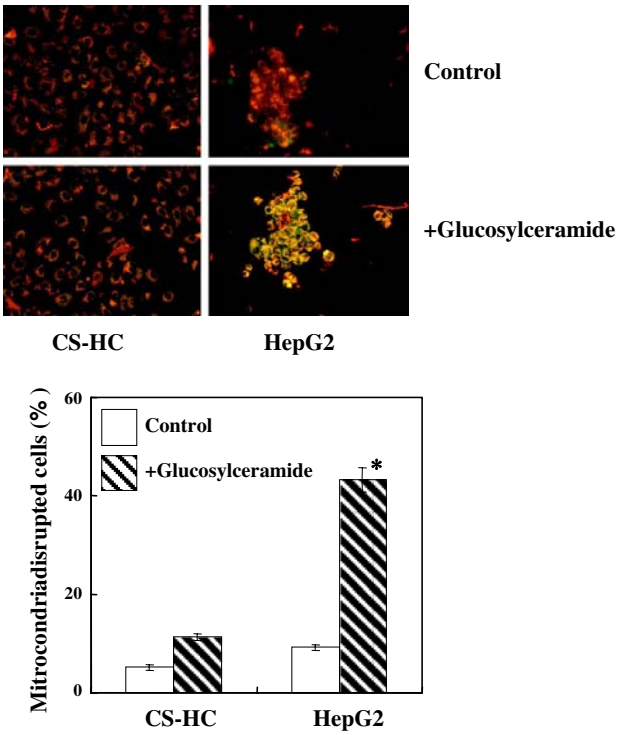

Fig. 5 Effect of glucosylceramide on the mitochondrial membrane integrity. Cells cultured for 24 h on coverslips were incubated with 200 μM glucosylceramide for 4 h, and loaded with MitoCapture (Biovision, USA) reagent for 20 min at 37°C under 5% CO_2 atmosphere. Cells were observed with a fluorescence microscope using band-pass filter. *Asterisk* denotes the statistically significant difference from normal cells by Student's *t* test ($P < 0.05$)

other glycosylceramide [7]. Thus our and previous study showing the cytotoxicity of glucosylceramide may impose another question to be answered. Natural long-chain ceramide is not water-soluble, and is considered to be difficult to insert from out side into a phospholipid bilayers [8]. In this context, it is noteworthy that the lipid analysis found no accumulation of this lipid in both normal and cancer cell suggesting that no significant incorporation of this lipid into cellular compartment. Analysis of fatty acid composition of cellular lipid classes (cholesterol ester, triacylglycerol, phosphatidylethanolamie and phosphatidylcholine) found trace of the glucosylceramide-derived α-hydroxy fatty acid in these lipid fractions (data not shown), also may support the view that glucosylceramide modulate the cell growth without entering the cells. It is thus unlikely that the activity of glucosylceramide synthase is involved in the susceptibility of cancer cells to exogenously added glucosylceramide. Glucosylceramide therefore may alter the cell proliferation by interfering with the signal transduction across cell membrane specifically in cancer cells. Another possibility to mention is that glucosylceramide modulated the structural role of membrane ceramide with consequences of microdomain function or lipid vesicular trafficking to mitochondria to alter its membrane permeability [8, 25].

Exposure of the cells to ceramide induced several modes of response depending on the cell types. Ceramide treatment induced non-apoptotic programmed death with a necrotic-like morphology in human glioma cells [26]. On the other hand, ceramide induced apoptosis via Bax and caspase-dependent pathway [27]. HepG2 cells grown in glucosylceramide showed immunocytochemical morphology of apoptosis as stained by Anexin V (Fig. 4). This apoptotic cells accounted for only 3% of total cell on flow cytometry (Fig. 3), and is probably due to disruption of mitochondrial membrane integrity. By association, cell cycle of HepG2 cells was arrested at G_2/M phase by glucosylceramide, and the population increased from 24% at 0 h to 46% at 24 h of treatment. Thus, the cytotoxicity of the glucosylceramide was mainly explained by cell cycle arrest rather than by induction of apoptosis. The viability of cancer cell decreased to about 23% after 24 h of treatment with glucosylceramide (Fig. 3), suggesting that most of these cells are not viable, and unable to re-enter the cell cycle after removal of this agent from the culture medium. However, the clonogenic cell survival assay should be conducted before coming to the definite conclusion.

Cell cycle arrest at G_2/M phase contrasted with the most prevailing observations of G_0/G_1 phase arrest induced by ceramides [28–30]. This suggested that the mechanism for the growth inhibition by glucosylceramide may differ from those for ceramide. Sphingoid

bases have been reported to arrest cell renewal at G_2/M phase [31]. Although, this study failed to detect any increase of exogenously added glucosylceramide in HepG2 cells, the extent to which this molecule entered the phospholipid bilayer or cell compartment still remain somewhat obscure. The occurrence of glucose moiety makes this molecule more water-soluble, and may increase the membrane permeability. Thus, it may be possible that small amount of ceramide permeate into cells, and its hydrolyzed sphingoid base affects the cell physiology.

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References

1. Sullards MC, Lynch DV, Merrill AH Jr, Adams J (2000) Structure determination of soybean and wheat glucosylceramides by tandem mass spectrometry. J Mass Spectrom 35:347–353
2. Schmelz EM, Sullards MC, Dillehay DL, Merrill AH Jr (1999) Colonic cell proliferation and aberrant crypt foci formation are inhibited by dairy glucosphingolipids in 1,2-demethylhydrazine-treated CF1 mice. J Nutr 130:522–527
3. Symolon H, Schmelz EM, Dillehay DL, Merrill AH Jr (2004) Dietary soy sphingolipids suppress tumorigenesis and gene expression in 1,2-dimethylhydrazine-treated CF1 mice and ApcMin/+ mice. J Nutr 134:1157–1161
4. Inamine M, Suzui M, Morioka T, Kinjo T, Kaneshiro T, Sugishita T, Okada T, Yoshimi N (2005) Inhibitory effect of dietary monoglucosylceramide 1-*O*-beta-glucosyl-*N*-2′-hydroxyarachidoyl-4,8-sphingadienine on two different categories of colon preneoplastic lesions induced by 1,2-dimethylhydrazine in F344 rats. Cancer Sci 96:876–881
5. Chen JH, Cui GY, Liu JY, Tan RX (2003) Pinelloside, an antimicrobial cerebroside from *Pinellia ternata*. Phytochemistry 64:903–906
6. Cateni F, Zilic J, Falsone G, Scialino G, Banfi E (2003) New cerebrosides from *Euphorbia peplis* L.: antimicrobial activity evaluation. Bioorg Med Chem Lett 13:4345–4350
7. Bieberich E (2004) Integration of glycosphingolipid metabolism and cell-fate decisions in cancer and stem cells: review and hypothesis. Glycoconj J 21:315–327
8. van Blitterswijk WJ, van der Luit AH, Veldman RJ, Verheij M, Borst J (2003) Ceramide: second messenger or modulator of membrane structure and dynamics? Biochem J 369:199–211
9. Naka T, Fujiwara N, Yano I, Maeda S, Doe M, Minamino M, Ikeda N, Kato Y, Watabe K, Kumazawa Y, Tomiyasu I, Kobayashi K (2003) Structural analysis of sphingophospholipids derived from *Sphingobacterium spiritivorum*, the type species of genus Sphingobacterium. Biochim Biophys Acta 1635:83–92
10. Cateni F, Zilic J, Falsone G, Hollan F, Frausin F, Scarcia V (2003) Preliminary biological assay on cerebroside mixture from *Euphorbia nicaeensis* All. Isolation and structure determination of five glucocerebrosides. Farmaco 58:809–817
11. Rupcic J, Maric V (2004) Cerebrosides of *Candida lipolytica* yeast. Appl Microbiol Biotechnol 64:416–420
12. Takakuwa N, Saito K, Ohnishi M, Oda Y (2005) Determination of glucosylceramide contents in crop tissues and by-products from their processing. Bioresour Technol 96:1089–1092
13. Wongtangtintharn S, Oku H, Iwasaki H, Inafuku M, Toda T, Yanagita T (2005) Incorporation of branched-chain fatty acid into cellular lipids and caspase-independent apoptosis in human breast cancer cell line, SKBR-3. Lipids Health Dis 4:29
14. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
15. Christie WW (1985) Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. J Lipid Res 26:507–512
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
17. Bras M, Queenan B, Susin SA (2005) Programmed cell death via mitochondria: different modes of dying. Biochemistry (Mosc) 70:231–239
18. Kobayashi E, Motoki K, Yamaguchi Y, Uchida T, Fukushima H, Koezuka Y (1996) Enhancing effects of alpha-, beta-monoglycosylceramides on natural killer cell activity. Bioorg Med Chem 4:615–619
19. Ishihara S, Nieda M, Kitayama J, Osada T, Yabe T, Kikuchi A, Koezuka Y, Porcelli SA, Tadokoro K, Nagawa H, Juji T (2000) Alpha-glycosylceramides enhance the antitumor cytotoxicity of hepatic lymphocytes obtained from cancer patients by activating CD3-CD56+ NK cells in vitro. J Immunol 165:1659–1664
20. Zhou D, Mattner J, Cantu C III, Schrantz N, Yin N, Gao Y, Sagiv Y, Hudspeth K, Wu YP, Yamashita T, Teneberg S, Wang D, Proia RL, Levery SB, Savage PB, Teyton L, Bendelac A (2004) Lysosomal glycosphingolipid recognition by NKT cells. Science 306:1786–1789
21. Maru M, Haraguchi M, Higashi H, Kato S, Kurimura T, Naiki M, Wakamiya N (1993) Anti-tumor activity of ceramides and glycosphingolipids in a murine tumor system. Int J Cancer 53:645–650
22. Schmelz EM, Merrill AH Jr (1998) Ceramides and ceramide metabolites in cell regulation: evidence for dietary sphingolipids as inhibitors of colon carcinogenesis. Nutrition 14:717–719
23. Sugiki H, Hozumi Y, Maeshima H, Katagata Y, Mitsuhashi Y, Kondo S (2000) C2-ceramide induces apoptosis in a human squamous cell carcinoma cell line. Br J Dermatol 143:1154–1163
24. Ogretmen B, Schady D, Usta J, Wood R, Kraveka JM, Luberto C, Birbes H, Hannun YA, Obeid LM (2001) Role of ceramide in mediating the inhibition of telomerase activity in A549 human lung adenocarcinoma cells. J Biol Chem 276:24901–24910
25. Morales A, Colell A, Mari M, Garcia-Ruiz C, Fernandez-Checa JC (2004) Glycosphingolipids and mitochondria: role in apoptosis and disease. Glycoconj J 20:579–588
26. Mochizuki T, Asai A, Saito N, Tanaka S, Katagiri H, Asano T, Nakane M, Tamura A, Kuchino Y, Kitanaka C, Kirino T (2002) Akt protein kinase inhibits non-apoptotic programmed cell death ilduced by ceramide. J Biol Chem 277:2790–2797
27. von Haefen C, Wieder T, Gillissen B, Starck L, Graupner V, Dorken B, Daniel PT (2002) Ceramide induces mitochondrial activation and apoptosis via a Bax-dependent pathway in human carcinoma cells. Oncogene 21:4009–4019
28. Kuroki J, Hirokawa M, Kitabayashi A, Lee M, Horiuchi T, Kawabata Y, Miura AB (1996) Cell-permeable ceramide

inhibits the growth of B lymphoma Raji cells lacking TNF-alpha-receptors by inducing G0/G1 arrest but not apoptosis: a new model for dissecting cell-cycle arrest and apoptosis. Leukemia 10:1950–1958
29. Connor CE, Burrows J, Hearps AC, Woods GM, Lowenthal RM, Ragg SJ (2001) Cell cycle arrest of hematopoietic cell lines after treatment with ceramide is commonly associated with retinoblastoma activation. Cytometry 43:164–169
30. Takai N, Ueda T, Kawano Y, Nishida M, Nasu K, Narahara H (2005) C2-ceramide exhibits antiproliferative activity and potently induces apoptosis in endometrial carcinoma. Oncol Rep 14:1287–1291
31. Ahn EH, Schroeder JJ (2002) Sphingoid bases and ceramide induce apoptosis in HT-29 and HCT-116 human colon cancer cells. Exp Biol Med (Maywood) 227:345–353