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ORIGINAL ARTICLE

Tumor specific cytotoxicity of β-glucosylceramide: structure–cytotoxicity relationship and anti-tumor activity in vivo

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Abstract This study describes the structure–cytotoxicity relationship of β-glucosylceramide (β-GluCer) and its anti-tumor activity in vivo. Unglycosylated ceramide had no selective cytotoxicity which demonstrated that the sugar moiety plays a critical role for the expression of selective cytotoxicity by β-GluCer. β-Galactosylceramide also showed tumor specific cytotoxicity suggesting that the chemical structure of sugar group is not a factor for the selective toxicity. Similarly, unglycosylated ceramides of short acyl chain also selectively inhibited the growth of cancer cells. These findings in concert point to the importance of the hydrophilicity of the ceramide molecule rather than the chemical structure for the cyto-selectivity. Treatment of the cells with β-GluCer increased the concentration of reactive oxygen species leading to cell cycle arrest and necrosis. Intraperitoneal administration of β-GluCer significantly suppressed the growth of tumor implanted to the back of mice. β-GluCer also induced antitumor immunity via the activation of NKT cells in vivo, and decreased the tumor metastasis of lymphoma cells. The present study thus demonstrated the antitumor activity of β-GluCer in vivo, and discussed the mechanisms responsible for the growth inhibition.

Keywords Glucosylceramide · Tumor selective · Cytotoxicity · Structure–activity relationship · Antitumor immunity

Introduction

Sphingolipids are members of membrane lipids and distributed widely in the animal and plant kingdom [1, 2]. It has been proposed that ceramide, a lipid molecule of sphingoid base backbone, plays important role in various aspects of cancer cell biology including apoptosis, proliferation, migration and senescence [3, 4]. Our previous results showed that the β-glucosylceramide (β-GluCer) isolated from malt feed selectively inhibited the growth of cancer cells [5]. The treatment of cells with this ceramide selectively disrupted the mitochondrial membrane integrity without activating the caspase-cascade to induce apoptosis, and alternatively caused cell cycle arrest in G2/M phase in HepG2 cells [5]. The present study extended our previous work and studied the structure–cytotoxicity relationship of β-GluCer and antitumor activity in vivo. Furthermore, glycosylceramides such as α-galactosylceramide (α-GalCer) and β-GluCer have been implicated in immunobiology [6]. It has been reported that α- and β-monoglycosylceramides have enhanced the activity of natural killer (NK) cells and inhibited the tumor growth [7]. Thus, the present study also shed the light on the induction of antitumor immunity by this β-GluCer.

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Materials and methods

Chemicals and antibodies

Lipids standards were purchased from Nacarai Tesque (Kyoto, Japan) or Funakoshi Co. Ltd (Tokyo, Japan). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA), a molecular probe for reactive oxygen species (ROS), was from Invitrogen (Carlsbad, CA, USA). Synthetic ceramides were supplied by Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA) and Larodan Fine Chemicals (Malmo, Sweden). Antibodies for flow cytometry were in fluoresceinisothiocyanate (FITC)- or phycoerythrin (PE)-form, and purchased from Pharmigen (San Diego, 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 β-GluCer

Two grams of crude ceramide in 4 ml of chloroform was mixed with 4 ml of 0.6 N NaOH in methanol, and methanolyzed 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 β-GluCer was collected and stored at −80°C. Yield of purified β-GluCer was usually 2% against crude ceramide as starting material. The chemical structure of β-GluCer is shown in Fig. 1.

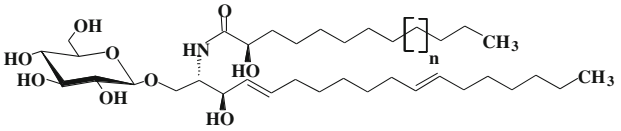

Fig. 1 Chemical structure of the major β-GluCer extracted from malt feed

Cells and tissue culture

The antitumor assay was performed using two pairs of human normal or cancer cell lines. Of the cells, WI-38 (lung normal diploid fibroblast), A549 (lung adenocarcinoma cell line) and HepG2 (liver cancer cells) were from Health Science Resources Bank (Osaka, Japan). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium or E-MEM medium supplemented with 10% fetal bovine serum.

CS-HC (human primary hepatocytes) was purchased from Dainippon Pharmaceuticals Co. (Osaka, Japan) and cultured in CS-C medium kits according to manufacturer's instruction. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Cells in exponential growth phase were used throughout the experiments.

Cell cytotoxicity assay

β-GluCer was dissolved in PBS containing 0.8% tween 80 as described previously [5]. 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.

Reactive oxygen species

Concentration of the ROS was measured by flow cytometry. Cells pre-cultured for 24 h in 60-mm Petri dishes were incubated with the test substances (β-GluCers, hydrogen peroxide and ascorbic acid) for 24 h. Cells were harvested with ACCTASE™ (Innovative Cell Technologies, Inc., San Diego, CA) and washed with PBS. Cell pellet was re-suspended in 1 ml of 20-µM H_2DCFDA, and incubated for 30 min at 37°C in 5% CO_2 atmosphere. The cell suspension was filtered through a 35-mM nylon filter. Data acquisition and analysis were performed by a FACSCalibur flow cytometer system (BD Biosciences). Every measurement usually counted at least 25,000

events. Fluorescence intensity distribution was recorded on a 4-decade logarithmic scale, and mean fluorescent intensities were recorded for each treatment.

Quantitation of apoptotic or necrotic cells

Cells (1×10^4 cells/dish) were pre-cultured in the medium for 24 h, and incubated further with 75-μM ceramide for 5 h. Cells were washed with PBS and re-suspended in 100 μl of binding buffer containing 1/100 volume of Annexin V solution (MBL, Nagoya, Japan) and placed on ice for 30 min, followed by incubation with 10-μM propidium iodide (PI) for 5 min. Cells were washed with binding buffer, and apoptotic cells stained with Annexin V and necrotic cells stained with PI were counted by using a fluorescence microscope. Data were expressed as percentages of apoptotic or necrotic cells in the total cells.

Cell cycle analysis

Cells treated with 100-μM glucosylceramide for 8, 16 and 24 h were harvested with ACCTASE™ (Innovative Cell Technologies, Inc., San Diego, CA) and washed with PBS. Cells were fixed with 75% ethanol at −4°C overnight. Cell pellets after centrifugation were re-suspended in 1 ml of PBS containing 0.1 mg/ml RNase and 10-μM propidium iodide, and incubated at 37°C in a humidified atmosphere of 5% CO_2 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.

Mice and diet

C3H/HeJJcl mice for tumor growth inhibition assay and C57BL/6 mice for tumor metastasis and survival test were purchased from Japan SLC inc. (Shizuoka, Japan). All animals were strictly maintained and ethically approved by the rules and regulations of the Animal Welfare Center, University of the Ryukyus, Okinawa, Japan, in specific pathogen-free condition with laminal airflow and humidity. All animals were kept in cages at room temperature (25°C), and ambient lightening was automatically controlled to provide 12-h light and dark cycles. Mice were allowed free access to commercial chow (CE2, Clea Japan Inc. Tokyo, Japan) and water throughout the experiment.

Tumor growth inhibition in mice

Single-cell suspensions of MH134 cells were prepared, and diluted in PBS. MH134 cells were of C3H/He background. A volume of 50 μl cell suspensions (approximately 10^4 tumor cells per animal) was injected subcutaneously into the back of male C3H/HeJJcl (5 weeks old). After confirmation of successful implantation (approximately 7–14 days after inoculation), β-GluCer (0.4 mg in 50 μl PBS containing 5% ethanol) was administered intraperitoneally everyday for a week. A volume of 50 μl vehicle solution (PBS containing 5% ethanol) was given as control at each administration time point. The tumor size was measured daily, and was calculated using the formula: $V = \text{length} \times \text{width} \times \text{width} \times 3.14\ (\pi)/6$ as described previously [8, 9]. Growth of tumor was expressed as fold-increase in the size against that at the starting point.

Phenotypic characterization of liver mononuclear cells

Mice were administered the β-GluCer twice 3 days before and on the day of tumor inoculation. The phenotypes of liver mononuclear cells (MNCs) were characterized by immunofluorescence staining and flow cytometry as previously described [10]. The liver was pressed through 200-gauge stainless steel mesh and suspended in Eagle's MEM medium supplemented with 5-mM HEPES and 2% FCS. Cells were washed once with the same medium, pelleted by centrifugation, and re-suspended in 35% Percoll solution containing 100 U/ml heparin and centrifuged at 2,000 rpm for 15 min. The pellet was re-suspended in red blood cell (RBC) lysis solution (155 mM NH4Cl, 10 mM KHCO3, 1 mM EDTA, 170 mM Tris, pH7.3) and then washed twice with the medium. The liver MNCs were stained with FITC-labeled anti-CD3 and PE labeled anti-NK1.1 antibodies. In order to detect apoptosis, the cells were further stained with FITC-labeled Annexin V or anti-CD69 antibody. Data acquisition and analysis were performed by a FACSCalibur flow cytometer system (BD Biosciences).

Tumor metastasis and survival test

EL4 (T-cell lymphoma) cells (5×10^5 cells/mouse) were injected intravenously (i.v.) into mice. This cell line is of C57BL/6 background. Mice were administered β-GluCer twice as described above. At day 14 after tumor inoculation, their livers were harvested and the number of tumors visible at the liver surface was counted. For the survival test, the inoculums size was 10^5 cells per mouse, and the survival rate was followed up to day 40 after inoculation.

Results

Our previous work demonstrated the tumor selective cytotoxicity of β-GluCer isolated from beer malt. The β-GluCer selectively lowered the viability of the liver, skin and lung

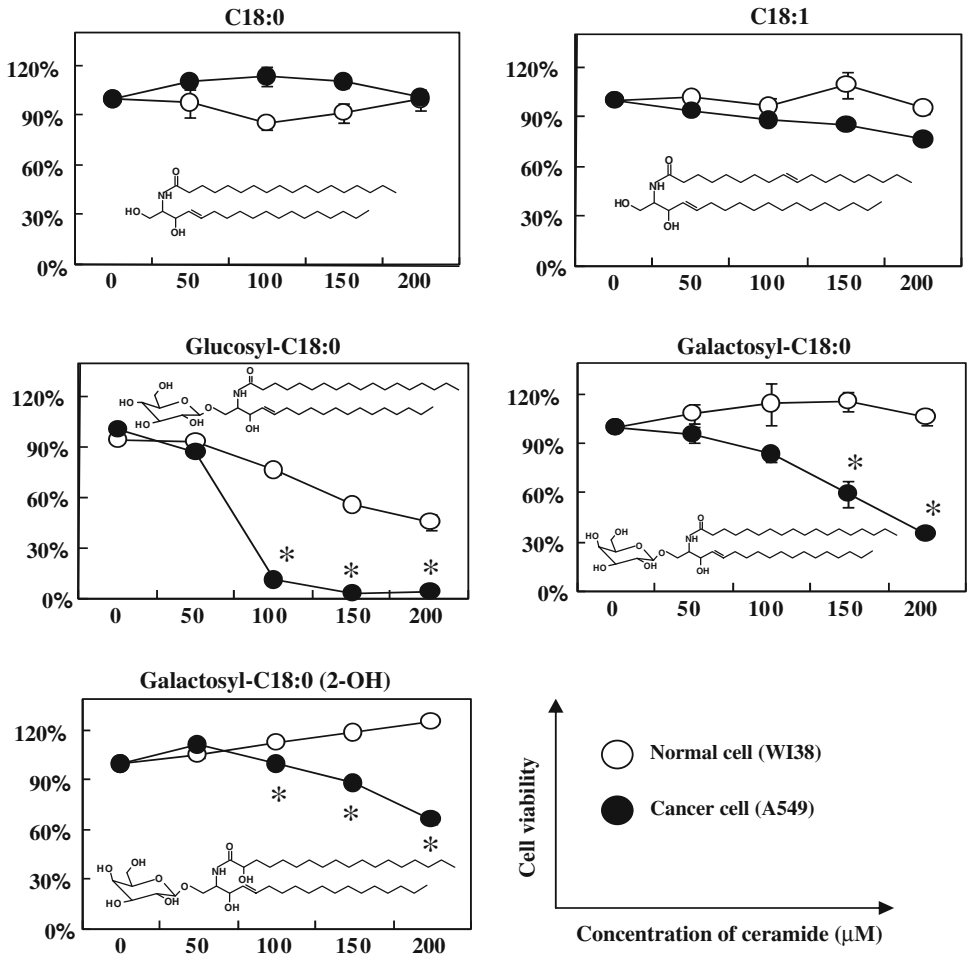

Fig. 2 Effect of various C18 ceramides on the viability of normal (WI38) and cancer cells (A549). Sphingoid base for these ceramides was sphingosine. Cells seeded at density of 5,000 cells/well and pre-cultured in the medium for 24 h were treated with indicated concentrations of β-GluCer for another 24 h, and the viable cell numbers were determined by MTS assay. Data are mean ± SE of triplicate analyses. *Asterisk* denotes statistically significant difference between normal and cancer cells by Student's *t* test ($P < 0.01$)

cancer cells compared to their normal counterparts. The ceramide treatment specifically disrupted the mitochondrial membrane integrity and caused cell cycle arrest in G2/M phase in HepG2 cells. However, the underlying mechanism for the selective cytotoxicity has been remained unanswered. The biological activity of ceramides shows variation with the chemical structure of sphingoid base and amide-linked fatty acids. It is therefore necessary to clarify the structure–antitumor activity relationship of β-GluCer to get more insight into the mechanism for the tumor selectivity.

To ascertain whether the sugar group plays a role for the selective toxicity, the authors first studied the effect of C18:0 ceramides with or without sugar moiety on the antitumor activity (Fig. 2). Sphingoid base of these ceramides was sphingosine. As shown in Fig. 2, unglycosylated form of ceramides with saturated or unsaturated fatty acid showed no cytotoxicity to cancer cells. β-Galactosylceramide (β-GalCer), as well as β-GluCers, was selectively toxic to cancer cells. Presence of α-hydroxy group in the fatty acyl chain had no effect on the selective cytotoxicity of the galactosylceramide (Fig. 2).

Chain length of amide-linked fatty acid is another factor to influence the biological activity of ceramides. Therefore, next study examined the effect of synthetic ceramides with varying length of acyl chains on the selective cytotoxicity. Ceramides of chain length shorter than C8 showed selective cytotoxicity as were the cases for β-GluCer and β-GalCer (Fig. 3). The selectivity, however, appeared to be somewhat attenuated with the ceramides of the shortest chain length C2 (Fig. 3).

Our previous study demonstrated that the β-GluCers specifically disrupted the mitochondrial membrane potential and caused the cell cycle arrest in G2/M phase [5]. It has been shown that the ceramide induced growth arrest or apoptosis by altering the cellular redox status [11]. It is therefore possible that the induction of selective cytotoxicity by β-GluCer associates with the changes in the cellular redox status as was the previous case. Thus, next study examined the effect of β-GluCer treatment on the level of ROS in the cancer cells (Fig. 4). ROS level was measured by a molecular probe H_2DCFDA, which enters the cell and reacts with ROS to produce fluorescent compound. Mean fluorescence intensity (MFI) counts shown in Fig. 4 therefore represent the ROS level in the cells. Treatment of the cells with β-GluCer elevated the cellular ROS to almost comparable level to that of the hydrogen peroxide treated

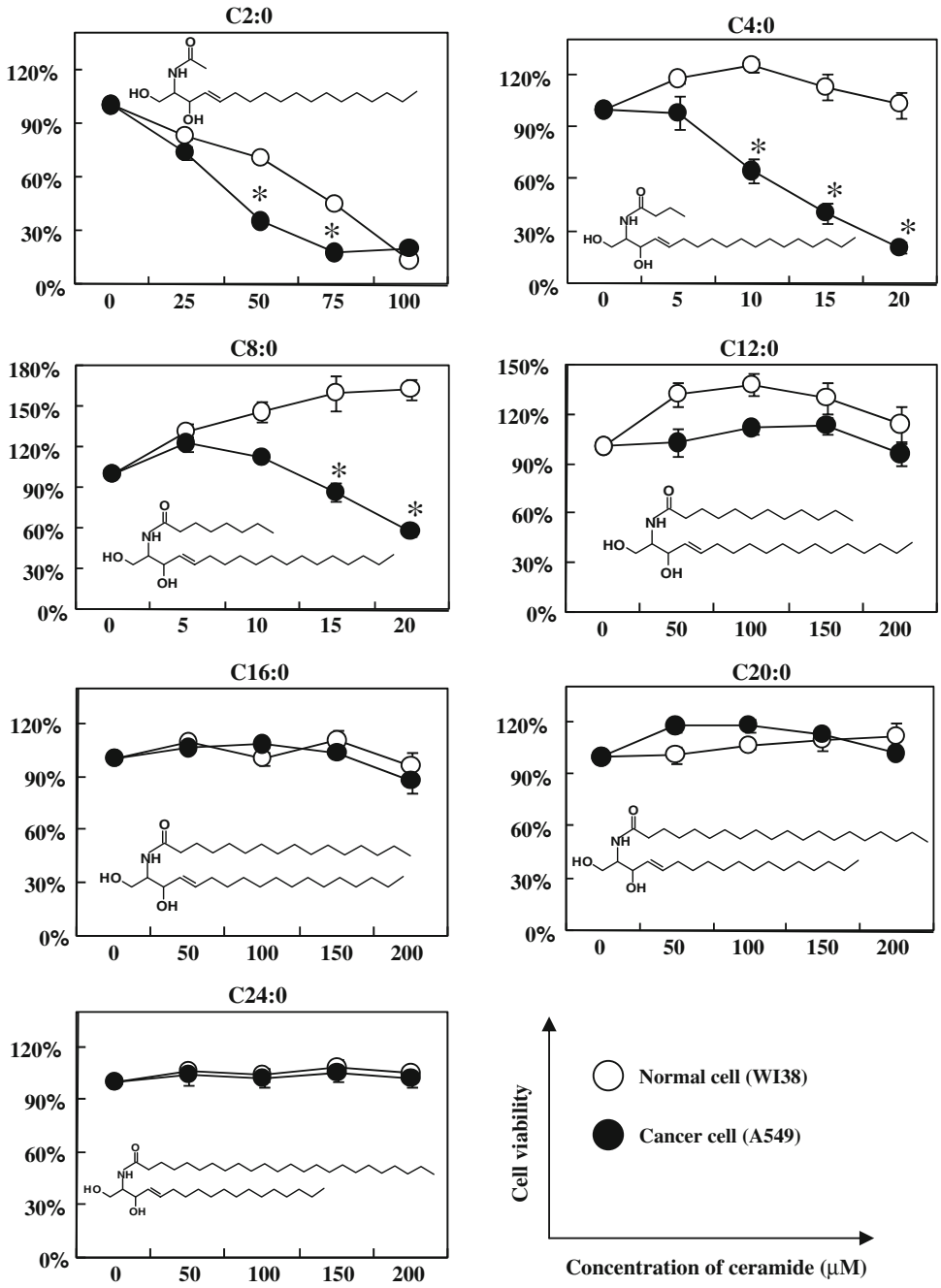

Fig. 3 Effect of ceramides of various chain length on the viability of normal (WI38) and cancer cells (A549). Cells seeded at density of 5,000 cells/well and pre-cultured in the medium for 24 h were treated with indicated concentrations of β-GluCer for another 24 h, and the viable cell numbers were determined by MTS assay. Data are mean ± SE of triplicate analyses. *Asterisk* denote statistically significant difference between normal and cancer cells by Student's *t* test ($P < 0.01$)

cells (Fig. 4). This increment in the cellular ROS by β-GluCer was circumvented by the presence of ascorbic acid (Fig. 4). To further confirm the involvement of ROS in the selective cytotoxicity of β-GluCer, cells were treated with this agent in the presence or absence of reducing agent ascorbic acid to scavenge the ROS. Supplementation of the medium with the reducing agent completely prevented the cells from the cytotoxicity of β-GluCer (Fig. 5).

An increased level of ROS and subsequent ATP depletion triggered necrosis in lymphoid cells [12]. Our previous study demonstrated a mitochondrial membrane disruption and cell cycle arrest by the β-GluCer treatment [5]. Fluorescence microscopic observation in this study revealed that necrotic cell death predominated over the apoptosis after treatment of the cell with the β-GluCer (Fig. 6). Consistent with our previous study, β-GluCer increased the cell population in G_2/M phase of A549 cells (Fig. 7).

A large body of in vitro study has been conducted to demonstrate the cytotoxicity of ceramides. However, it is more important for practical use to demonstrate the tumor growth inhibition in vivo. Next experiment therefore studied the effect of β-GluCer on the in vivo growth of tumor

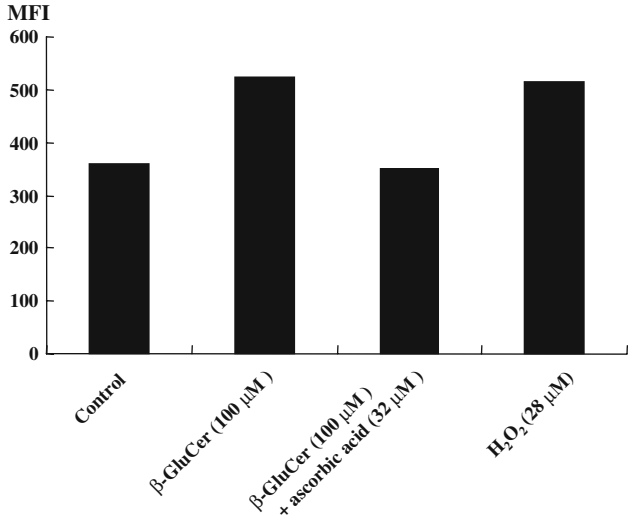

Fig. 4 Effect of β-GluCer on the cellular concentration of ROS. Cells (A549) cultured for 24 h were incubated with test substances (β-GluCer, ascorbic acid and hydrogen peroxide) for 24 h. Control is the vehicle buffer alone. ROS level was measured by a molecular probe H_2DCFDA as described in the method section

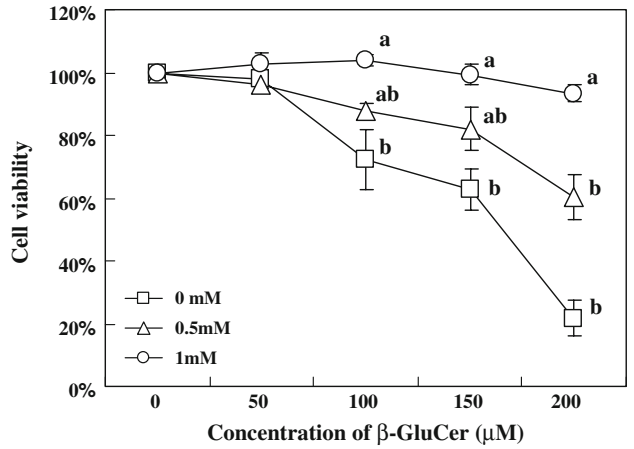

Fig. 5 Effect of ascorbic acid on the cytotoxicity of β-GluCer. Cells (A549) were treated with the β-GluCer in the presence of indicated concentration of ascorbic acid. Cell viability was determined by MTS method as described in the legend to Fig. 2 or 3. *a, b* There are no statistically significant difference between the values sharing the same letter. Criterion for the statistical significance was $P < 0.05$ by analysis of variance and following inspection of the difference between pairs of means by least significant difference method [40]

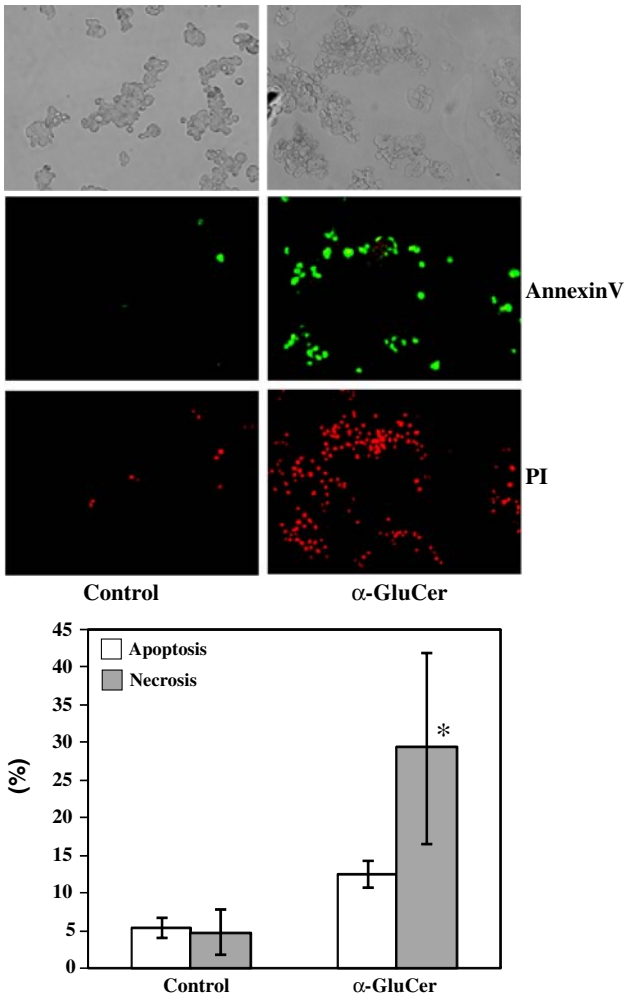

Fig. 6 Effect of β-GluCer on induction of apoptosis and necrosis. HepG2 cells were treated with 75-μM ceramide for 5 h and stained with Annexin V and propidum iodide (PI) as described in the method section. Apoptotic cells stained with *Annexin V* and necrotic cells stained with *PI* were, respectively, counted with a fluorescence microscope. Data are mean ± SE of triplicate analyses. *Asterisk* denotes statistically significant difference from control by Student's *t* test ($P < 0.05$)

cells. Our final goal is to develop a new anticancer therapeutics of low side-effect, and the selectivity of the β-GluCer was most pronounced with the liver cells. For this reason, liver cancer cells were chosen as carcinoma model, and implanted to the back of mice (C3H/HeJJcl). MH134 cells, liver cancer cells, were of background of C3H/He, and were found to be most susceptible to the β-GluCer in vitro among the liver cell lines compatible with the host mice (data not shown). The cancer cells were grown to approximately 7 mm in a diameter, and β-GluCer (0.4 mg/mouse) or vehicle solution was intraperitoneally administered daily. Relative growth rate of tumor cells was significantly lowered by the administration of β-GluCer (Fig. 8). The differences in the relative growth rate between β-GluCer-treated and control mice were statistically significant on day 3. This statistical significance was attenuated on day 5 ($P = 0.09$) due to a large fluctuation in the growth rate. Daily consecutive administration of β-GluCer had no effect on the body weight and serum levels of GOT and GPT (Table 1).

Several lines of studies have demonstrated that glycosylsphingolipid suppresses the growth of cancer cells by stimulation of immune system [6, 13, 14]. For example, α-galactosylceramide (α-GalCer) has been shown to activate NKT cells. Administration of α-GalCer leads to rapid

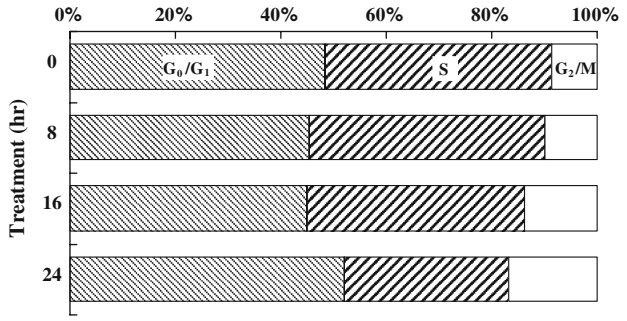

Fig. 7 Effect of β-GluCer on the cell cycle. A549 cells were treated with 100-μM glucosylceramide for 8, 16 and 24 h, and stained with 10-μM propidium iodide for flowcytometry as described in the method section. Cell cycle analysis was performed with CellQuest (BD Biosciences) software. Every measurement usually counted at least 10,000 events

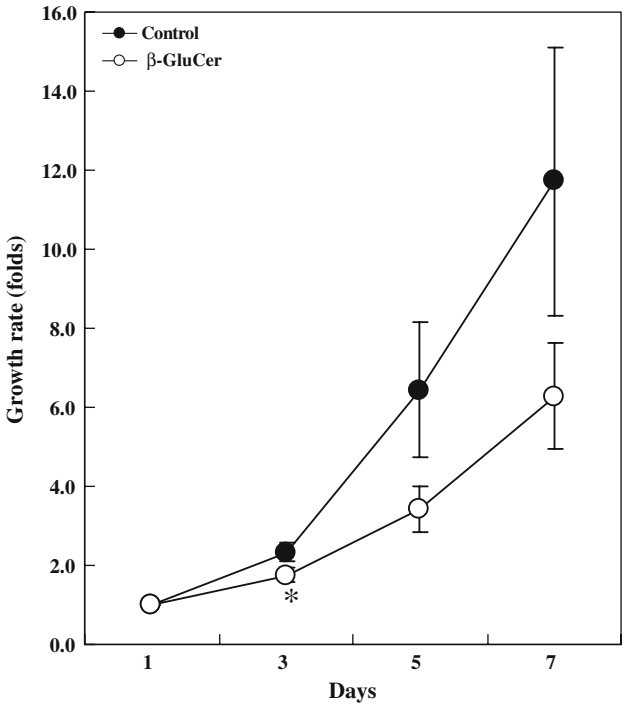

Fig. 8 Effect of β-GluCer on the growth of cancer cells in vivo. MH134 hepatoma cells were implanted to the back of C3H/HeJJcl mice. After confirmation of successful implantation, animals were divided into two groups: control and ceramide treatment group. β-GluCer (0.4 mg/mouse) was intraperitoneally administered everyday. Data are fold-increase in the tumor size against that at the starting point, and expressed as mean ± SE of 16–17 mice. *Asterisk* denote statistically significant difference between normal and cancer cells by Student's *t* test ($P < 0.05$)

production of both IFN-γ and IL-4 by NKT cells, with secondary activation of innate and adoptive immune responses [15, 16]. Furthermore, synthetic β-GluCer has been shown to activate NKT cells resulting in a beneficial clinical effect on tumor bearing mice: increase in survival rate with decreased tumor volume by intraperitoneal administration

Table 1 Effect of β-GluCer on body weight, serum GPT and GOT levels

	Treatment	
	Control	β-GluCer
Body weight (g)	23.1 ± 0.3	22.7 ± 0.3
GPT (IU/L)	10.8 ± 0.5	10.6 ± 1.0
GOT (IU/L)	90.8 ± 7.8	80.1 ± 8.3

Data are mean ± SE of 16–17 mice

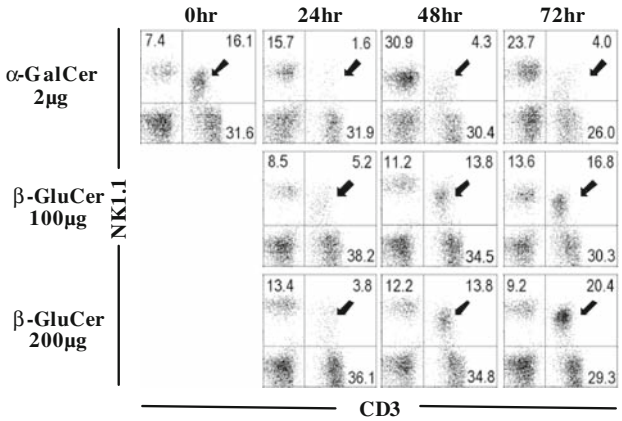

Fig. 9 Phenotype characterization of liver mononuclear cells (MNCs) in mice treated with β-GluCer or α-GalCer. Mice were administered the β-GluCer twice 3 days before and on the day of tumor inoculation. The phenotypes of liver MNCs were characterized by immunofluorescence staining and flow cytometry as previously described [10]. *X*- and *Y*-axis, respectively, shows the fluorescence intensities of FITC-labeled anti-CD3 and PE labeled anti-NK1.1 bound to the liver lymphocyte. Cells shown by *arrow* represents NKT cells stained by both anti-CD3 and anti-NK1.1 antibodies

[17]. These observations suggest that our β-GluCer preparation from malt feed also may activate the immune system.

Figure 9 shows the result of the flowcytometric analysis of liver lymphocytes stained with fluorescent monoclonal antibodies for CD3 (*X*-axis) and NK1.1 (*Y*-axis). Based on the fluorescence intensities on *X*-axis and *Y*-axis, cell population ($CD3^+$ $NK1.1^+$) shown by arrow represents NKT cells. α-GalCer decreased the proportion of NKT cells 24 h after intraperitoneal administration, reflecting an activation of NKT cells by this compound and following apoptotic cell death [18]. Although treatment of mice with 20 μg/mouse of our β-GluCer specimen had no effect on the proportion of NKT cells (data not shown), an increased dose of 100 or 200 μg/mouse apparently reduced the proportion as was the case for α-GalCer. This reduction appeared to be returned to the normal control level at 48 or 72 h after administration. In pathological conditions, NKT cells are activated and play a role in the defense against microbes and tumor cells [19–21]. Inflammation thus induced often causes liver injury. In this context, it is noteworthy that

Table 2 Effect of β-GluCer and α-GalCer on serum IFN-γ and GPT levels

	Treatment		
	Control	β-GluCer	α-GalCer
GPT (IU/L)	10.6 ± 2.0	19.2 ± 4.2	182.7 ± 18.3[a]
IFN-γ	41.2 ± 7.9	45.8 ± 16.0	2628.3 ± 48.5[a]

Data are mean $\pm$ SE of 5 mice

[a] Significantly different from control by Student's t test at $P < 0.01$

α-GalCer, being antitumor agent, was found to be problematic in that it induces liver injury at the same time [18, 22–24]. In order to test whether or not our specimen induces liver damage, serum levels of GPT and IFN-γ were measured (Table 2). Consistent with the previous observation, α-GalCer significantly increased the serum GPT more than tenfold, and IFN-γ to more than 60-fold over the level of control. In contrast, no increase in these parameters was noted for mice treated with β-GluCer.

Figure 9 suggested that the NKT cell in the liver underwent apoptotic cell death upon activation by the β-GluCer. To confirm this observation, the liver NKT cells (R1 fraction) were further stained with Annexin V to detect the cells in an early stage of apoptosis (Fig. 10a). With the non-treated control mice, there was a peak of non-apoptotic NKT cells giving off weak fluorescence (Fig. 10a, 0 h). Treatment of mice with α-GalCer decreased this peak area of weak fluorescence, and conversely intensified the peak of strong fluorescence representing the apoptotic cells labeled with Annexin V after 24 h of the treatment. Similarly, treatment of mice with β-GluCer increased the proportion of NKT cells in apoptosis to the same extent as the case for α-GalCer (Fig. 10a). The proportion of NKT cells in apoptosis returned to the initial normal level after 48 or 72 h of treatment in accordance with the restoration of NKT cell population to the normal level. Both α-GalCer and β-GluCer induced no apoptosis for conventional T cells (R2 fraction) of the mouse liver (Fig. 10a lower panels). Figure 10b shows the flowcytometric profile of NKT cells double-stained with PI and Annexin V (upper panel). The double-stain profile also showed NKT cells to undergo apoptosis not necrosis after β-GluCer treatment. The expression of CD69 in NKT cells was increased by the administration of β-GluCer to the same extent as observed for α-GalCer, indicating the activation of these cells (Fig. 10b, lower panel) by this agent.

Figures 11 and 12, respectively, show the effect of β-GluCer on tumor metastasis and survival rate of mice intravenously inoculated with the T-cell lymphoma EL4.

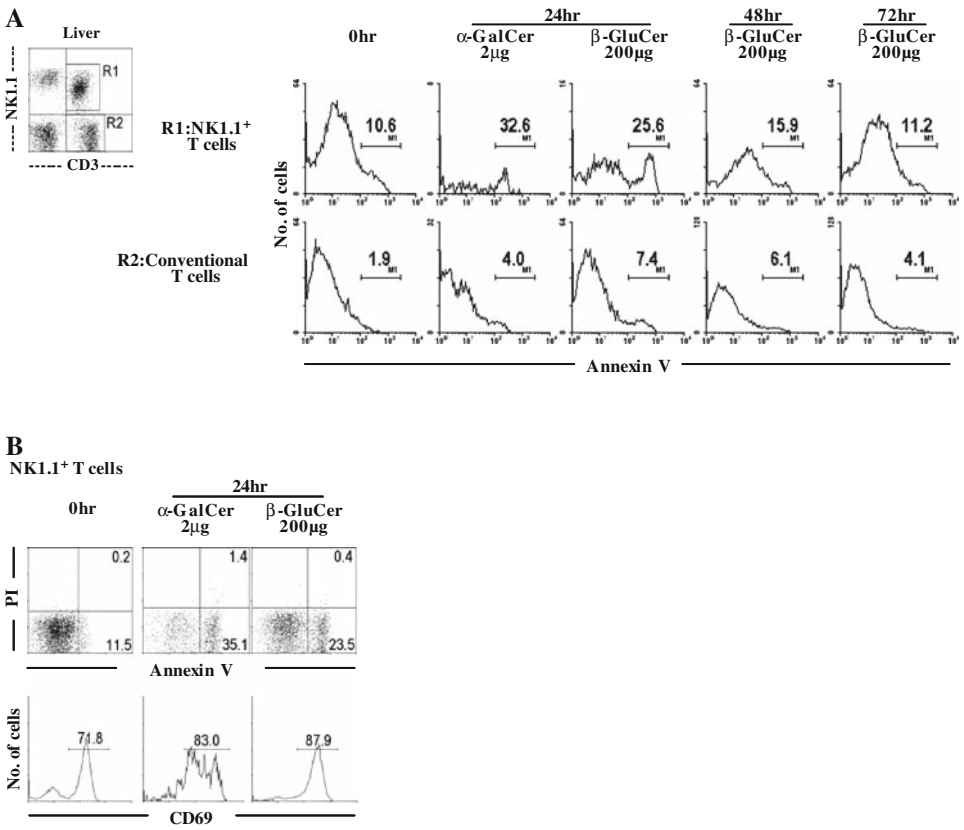

Fig. 10 Detection of apoptosis and necrosis of NKT cells. Cells stained with FITC-labeled anti-CD3 and PE labeled anti-NK1.1 were further reacted with Annexin V to detect the early stage of apoptosis (**a**). An analysis gate for NKT cells was established by CD3/NK1.1 expression, and gated lymphocyte events of 8,000 were acquired at least (**a**). To confirm NKT cells to undergo apoptosis not necrosis, uptake of PI and expression of CD69 following β-GluCer administration were studied in a separate experiment (**b**)

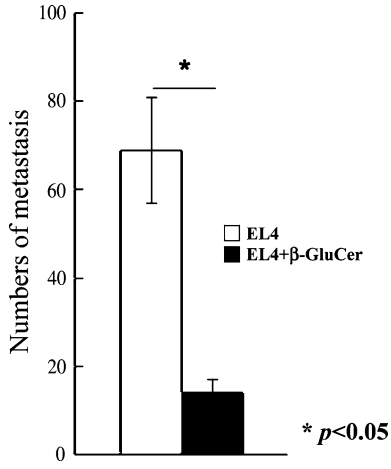

Fig. 11 Effect of β-GluCer on the degree of tumor metastasis. EL4 (T-cell lymphoma) cells (5×10^5 cells/mouse) were injected intravenously (i.v.) into mice, and mice were administered β-GluCer twice 3 days before and on the day of inoculation. At day 14 after tumor inoculation, the livers were harvested and the number of tumors visible at the liver surface was counted. Data are mean ± SE of 6 mice. *Asterisk* denotes the statistically significant difference by Student's *t* test ($P < 0.05$) between control and glucosylceramide treated group

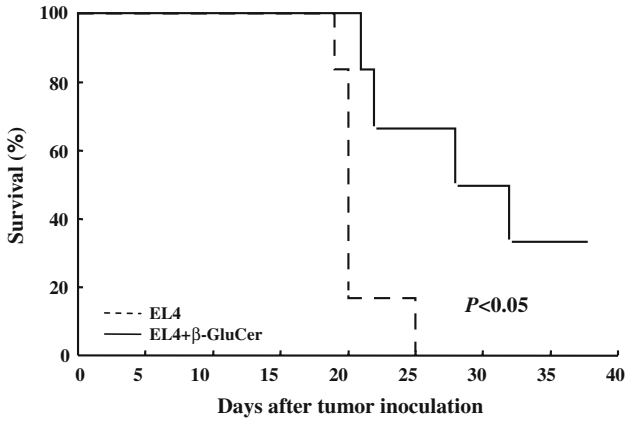

Fig. 12 Effect of β-GluCer on the survival rate after lymphoma inoculation. EL4 (T-cell lymphoma) cells (10^5 cells/mouse) were injected intravenously (i.v.) into mice, and mice were administered β-GluCer twice 3 days before and on the day of inoculation. The survival rate was followed up to day 40 after inoculation. *Asterisk* denotes the statistically significant difference by Kaplan–Meier test

Glucosylceramide significantly reduced the degree of metastasis and the survival rate compared to the control. Body weight loss and increase in the liver weight due to tumor metastasis was ameliorated by the β-GluCer treatment (data not shown). Unglycosylated C8 ceramide exhibited no inhibition of tumor metastasis pointing the importance of glucose residue for the induction of antitumor immunity in vivo (data not shown).

Discussion

We studied the structure–tumor specific cytotoxicity relationship of ceramides in this study. Unglycosylated form of ceramide had no selective cytotoxicity. Therefore, it was found that the occurrence of glycosyl group was critical for the expression of the tumor selective cytotoxicity. However, it was also found that the expression of the tumor selective cytotoxicity was independent of the chemical structure of sugar residue. As shown in Fig. 2, β-GalCer as well as β-GluCer selectively inhibited the growth of cancer cells. Furthermore, presence of hydroxyl group in the fatty acyl chain had little effect on the selective cytotoxicity.

The biological activities of ceramide vary with the chemical structure: carbon-chain structure of sphingoid base and/or composition of amide-linked fatty acids. It has been reported that glycosphingolipids exert their biological activities by interacting with the receptor proteins on the cell membrane, with consequences of the modulation of the signal transduction [6, 25, 26]. Given that β-GluCer binds to a certain receptor, there should be a structural requirement for a ligand. Both β-GalCer and β-GluCer inhibited the growth of cancer cells, suggesting that the selective cytotoxicity was independent of the chemical structure of sugar moiety. Furthermore, occurrence of hydroxyl group in the fatty acyl chain did nod influence the selective toxicity (Fig. 1). Taking these observations into consideration, it is unlikely that β-GluCer modulates the cell proliferation or cell death related events by receptor mediated mechanisms.

Natural long-chain ceramide is not water soluble, and is considered to be difficult to insert from outside into a phospholipid bilayers. In this regard, glycosylation of ceramides, either with glucose or galactose, may increase the water solubility of these molecules. Therefore, it is likely that the hydrophilicity of the molecule apart from the chemical structure appeared to be a factor for the expression of selective cytotoxicity.

The hydrophilicity of ceramide varies with the chain length of the amide-linked fatty acids. The water solubility of ceramide molecule increases with decrease in the chain length of fatty acyl chain. As shown in Fig. 3, ceramides of chain length shorter than C8 selectively inhibited the growth of cancer cells as was the case for glucosylceramide. This observation therefore may support the view that increased hydrophilicity due to glucosyl group may be a factor for the selective cytotoxicity of this compound. Ceramides with chain length shorter than C6 are permeable into cell. However, the permeability may not be the sole factor for the selective cytotoxicity because the difference in the susceptibility between normal and cancer cells appeared to be attenuated with the most water soluble, and hence, cell-permeable C2 ceramide (Fig. 3).

A number of extracellular stimuli, such as UV-irradiation, heat-stress, or chemotherapeutic agents increase the cellular level of long-chain endogenous-ceramides via the activation of de novo synthesis and/or sphingomyelinase [27, 28]. Ceramide then mediates anti-proliferative pathway or promote cell proliferation via the action of its catabolite sphingosine-1-phosphate (S1P) [29–31]. Thus, the effect of ceramide on cell proliferation was found to be bidirectional: endogeneous ceramide itself inhibit the cell growth, while increased level of its catabolite S1P promotes the cell proliferation. Furthermore, it is likely that the endogenous ceramide with different fatty acid chain length might have distinctive biological activities [27]. Likewise, our β-GluCer specimen may enter the cell and activate the pathway to induce the growth arrest. It is thus plausible that the cell membrane of cancer cells compared to normal cells may be more permeable to these rather hydrophilic ceramides. The difference in the membrane permeability therefore may explain in part the difference in the susceptibility to β-GluCer.

Several lines of recent studies demonstrated that formation of ROS and ATP depletion linked to the cell death mediated by cell-permeable ceramides [12]. Ceramide induced an increase in cellular ROS formation, which was blocked by treatment with antioxidant or scavenger [12]. Our present results largely agree with above results. Supplementation of the medium with antioxidant, ascorbic acid, completely blocked the formation of ROS, and hence, prevented the cell from death (Fig. 5).

Our previous study demonstrated that β-GluCer specifically disrupted the mitochondrial membrane integrity, and arrested the cell cycle in G2/M phase, leading to necrotic cell death. Necrosis has been considered to be the passive consequences of massive cell damage, and hence, accidental, uncontrolled and harmful by nature. However, recent studies suggest that this simplest view may need to be revised [32, 33]. Several recent reports indicate that cell death with necrotic feature may occur as a consequence of increased endogenous ceramide level [34, 35]. It therefore came to a notion that the necrotic cell death induced by ceramide has been identified as a regulated process and defined accordingly as necrosis-like programmed cell death distinguishable from the accidental necrosis, a passive process [32]. Induction of necrotic cell death by β-GluCer therefore may never lessen its therapeutic importance over the prevailing anticancer agent that induces non-harmful orchestrated sequence of apoptosis.

Glycosphingolipids have been shown to induce antitumor immune responses in various cancer models both in vitro and in vivo [6, 7, 36, 37]. In pathological conditions, NKT cells play an important role in the defense against tumor growth [19–21]. NKT cells regulate Th1 and Th2 immune responses by producing various immunoregulatory cytokines such as IL-4, IFN-γ and TNF-α [38]. β-GluCer in this experiment decreased the degree of metastasis after intravenous injection of T-cell lymphoma EL4, as was the case for α-GalCer. Intraperitoneal administration of β-GluCer induced apoptosis of NKT cells suggesting that the tumor cytotoxicity of this compound is mediated via the activation of this T-cell subset. The administration of α-GalCer leads to rapid production of both IFN-γ and IL-4 by NKT cells [15, 16]. In contrast, β-GluCer in our experiment induced no changes in serum level of IFN-γ (Table 2). This finding suggests that the antitumor mechanism for our β-GluCer differs from that proposed for α-GalCer. It has been demonstrated that IFN-γ produced by the NKT cells increase both innate antitumor cytotoxicity of NK cells, and adoptive antitumor response of $CD8^+$ Tcells [18]. In this regard, it has also been proposed that the direct effecter of α-GalCer-induced antitumor immunity is $CD3^-$ $CD56^+$ NK cells in human liver [25]. The phenotype of human hepatic lymphocyte was different from that of mice in terms of NKT cells [39]. It is therefore important to test the effectiveness of our β-GluCer preparation with human models in vivo for the clinical use.

Recent study by Zigmond et al. [17] also demonstrated a beneficial clinical effect of β-GluCer using murine hepatocellular carcinoma model. The β-GluCer modulated the intrahepatic and peripheral distribution of CD4 and CD8 lymphocytes as well as NKT cells [17]. Furthermore, they observed increased level of serum IFN-γ and elevated splenic level of STAT1 and STAT4 protein that play an important role in the regulation of IFN-γ and IL12 production at a transcriptional level. Their β-GluCer preparation therefore appeared to modulate the immune system by a largely similar mechanism proposed for α-GalCer. However, activation of NKT cells by our β-GluCer preparation was not associated with the enhanced production of IFN-γ (Table 2). This disagreement between our and their results may be explained in part by the difference in the chemical structure of ceramides: composition of amide-linked fatty acids and carbon skeleton of sphingoid base.

Activation of NKT lymphocytes by α-GalCer can lead to significant liver damage [18, 23]. The α-GalCer dependent antitumor cytotoxicity is mainly mediated by NK cells [25]. It has been suggested that activation of both NKT and NK cells may play a role in the onset of liver injury [18]. The elicitation of liver injury appeared to be a critical deficit in the application of α-GalCer for therapeutic use. Our β-GluCer specimen induced no changes in serum levels of GPT, GOT (Tables 1, 2) and morphology of the liver tissue with the daily consecutive administration. This feature may be potentially beneficial for the development of cancer chemotherapeutics of low side-effect.

The present study demonstrated the beneficial clinical effect of β-GluCer extracted from malt feed for the first

time. As shown in Figs. 8 and 11, our specimen suppressed the growth of the liver cancer implant as well as the tumor metastasis of lymphoma cells. These growth inhibitions can be attributable to the direct cytotoxicity of the β-GluCer or otherwise to the induction of antitumor immunity by the same compound. However, it is definitely difficult to determine to what extent these two mechanisms contributed to the growth inhibition in vivo by the β-GluCer. It also could be possible that these two mechanisms functioned synergistically to inhibit the growth of cancer cells. Twofold of antitumor specific cytotoxicity may have further advantage for the cancer chemotherapy.

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