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Inhibition of Sphingolipid Biosynthesis in Cultured Cells Enhances the Oligosaccharide Production from Exogenous Artifical Substrate

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Abstract: The effect of fumonisin B_1 which is an inhibitor of ceramide synthesis on saccharide elongation of dodecyl β-lactoside (Lac-C12), which is the analogue of lactosyl ceramide (LacCer), in mouse melanoma B16 cells and African green-monkey kidney (Vero) cells was examined. By adding fumonisin B_1 in the culture medium used for glycosylation of Lac-C12, mouse melanoma B16 cells synthesized GM3-type analogue twice much as the absence of fumonisin B_1, while the amount of endogenous GM3 remarkably decreased. The addition of fumonisin B_1 to the culture medium of Vero cells also elicited 50% augmentation in the amount of glycosylated Lac-C12. For the enhanced production of oligosaccharide using cell function, fumonisin B_1 acts effectively on cells which produce abundant endogenous glycolipid. The addition of fumonisin B_1 resulted in an increase in production of glycosylated Lac-C12 with decreasing natural ceramide synthesis.

Key words: Fumonisin B_1, saccharide primer, oligosaccharide, glycolipid

INTRODUCTION

The research on oligosaccharides had developed slowly owing to their complicated structures and the difficulty in predicting their functions. In recent years, research on oligosaccharide has been developed and its application to industrial materials has been attempted[1-5]. The functional roles of oligosaccharides on the cell surface have been extensively investigated. It has become clear that oligosaccharides are essential in many biological processes such as cell-cell interaction, proliferation, differentiation, cell-substrate interaction, carcinogenesis, bacteria or virus infection, immune response and inflammation[6].

Commonly, oligosaccharides are obtained from natural organic resources such as calf brain, human cataractous lenses and whole plant, etc[7-9]. However, the organic resources for oligosaccharides production have limited supply. To fill the demand for oligosaccharides, chemical[10,11] and enzymatic synthetic methods[12-15] have been extensively developed. In the early 1990's the novel saccharide primer method for obtaining oligosaccharides utilizing cell function was developed involving a combination of chemical synthetic method and enzymatic synthetic method. The most important advantage of the saccharide primer method[16-18] is that various oligosaccharides can be obtained without complicated processes such as preparation of glycosyltransferase and multi-step chemical reactions. The principle of the saccharide primer method is as follows. The culture medium is supplemented with saccharide primer such as dodecyl β-lactoside (Lac-C12) and used for incubation of cells. Consequently, the primer is taken into the cell and glycosylated by cellular glycosyltransferases and the glycosylated products are secreted into the medium.

Except for galactosylceramide, all kinds of glycosphingolipids are synthesized through lactosylceramide in mammals. Synthesis of lactosylceramide has been described to occur on the cytosolic face of the Golgi. Lactosylceramide is generated by galactosylation of glucosylceramide which, in turn, is synthesized from ceramide and UDP-Glucose on the cytosolic surface on the Golgi apparatus[19]. It is generally accepted that the saccharide primer is glycosylated by the same enzyme as in the synthesis of natural glycosphingolipids[20].

Fumonisin B_1, an inhibitor of ceramide synthesis, has been used to inhibit N-acylation of sphinganine[21,25]. It is also known that fumonisins block ceramide biosynthesis by inhibiting the

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conversion of sphinganine to dihydroceramides[21]. Therefore, the effect of sphingolipid synthesis inhibition by fumonisin B_1 on the glycosylation of saccharide primer in cells was tested. The effects of fumonisin B_1 on glycosylation of saccharide primer (Lac-C12) in mouse melanoma B16 cells and African green-monkey kidney (Vero) cells were investigated.

MATERIALS AND METHODS

Materials: Mouse melanoma B16 cells and African green-monkey kidney (Vero) cells were obtained from Riken Cell Bank (Tsukuba, Japan). High performance thin-layer chromatography (HPTLC) plate were from Merck (Darmstadt, Germany). D-MEM and Ham's F12 (1:1) (DMEM/F12), antibioyic-antimycotic (100×), Insulin-Transferrin-Selenium-X Supplement (100×) and trypsin/EDTA (10×) were purchased from Invitrogen (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from SAFC Biosciences (Lyon, France). Sep-Pak C18 columns were from Waters (Milford, USA). All organic solvents used for extraction of lipids and fumonisin B_1 were purchased from Nacalai Tesque (Kyoto, Japan).

Preparation of saccharide primer: Saccharide primer, *n*-dodecyl β-lactoside (Lac-C12), was prepared as described previously[18,26]. A mixture of *n*-dodecanol (2.39 g, 12.8 mmol) and lactose octaacetate (2.08 g, 2.98 mmol) in dichloroethane (100 mL) and molecular sieves 4A were stirred at 50°C for 1.5 h. Then, $BF_3 \bullet Et_2O$ (2.25 mL, 17.9 mmol) was added and the mixture was stirred at 50°C. After 3 h, the mixture was diluted with chloroform at room temperature and the molecular sieves were filtered off. The obtained chloroform solution was neutralized with aq. $NaHCO_3$, washed with H_2O, dried with anhydrous Na_2SO_4 and concentrated. Purification was performed by column chromatography (silica gel 60, 70-230 mesh, Merck, Darmstadt, Germany; hexane/AcOEt 2:1, v/v).

Cell culture: Mouse melanoma B16 cells and African green-monkey kidney (Vero) cells were cultured in DMEM/F12 supplemented with 10% FBS and 1% antibiotic-antimycotic and detached through application of 0.25% trypsin/EDTA, passaged every 3-4 d and maintained in humidified atmosphere of 5% CO_2 at 37°C.

Oligosaccharides synthesis by cells: 2×10^6 B16 cells or Vero cells were seeded into 100-mm culture dishes containing 7 mL of medium and incubated for 48 h. Then, the cells were washed with TI-DF (D-MEM/F12 containing 1% Insulin-Transferrin-Selenium-X Supplement and 1% antibiotic-antimycotic) and incubated with 50 μM of n-dodecyl β-lactoside (the primer was dissolve in DMSO at an initial concentration of 50 mM) at 37°C for 48 h. Prior to the incubation of cells with primer, it was essential to remove FBS from the growth medium by washing with TI-DF, since serum has previously been observed to interfere with ganglioside accumulation by the cells[27]. After incubation, culture media were collected and cells were washed with PBS (-). Collected glycolipid analogues from the culture media and PBS (-) were purified using a Sep-Pak C18 column. After elution from the column, the solution was evaporated and dissolved in 50 μL of chloroform/methanol (1:2, v/v) for analysis by HPTLC.

Extraction of intracellular lipids: After washing the dishes used for oligosaccharide synthesis with PBS (-), cells were detached from the culture dishes with 0.25% EDTA in PBS (-) and harvested by centrifugation at 1,000 rpm for 10 min. The cell pellet was suspended in 1 mL of chloroform/methanol (1:2, v/v). After sonication for 30 min and centrifugation at 15,000 rpm for 30 min, the extracts were transferred to a new tube. The remaining lipids from the cell pellet were extracted again with 1 mL of chloroform/2-propanol/water (7:11:2, v/v). The first and the second extracts were pooled. After evaporation, the resulting pellet was dissolved in 50 μL of chloroform/methanol (1:2, v/v) and analyzed by HPTLC.

Effect of fumonisin B_1 on cellular glycosylation: When seeding cells into culture dishes and incubating with 50 μM of n-dodecyl β-lactoside, culture medium was supplemented with fumonisin B_1 at a final concentration of 0.1, 1, 5, 25 or 50 μM. Then, the cells were treated according to the same method as described above.

HPTLC analysis: Glycolipids from the cell homogenate and culture medium fraction were analyzed by HPTLC with chloroform/methanol/0.25% aqueous KCl (5:4:1, v/v) as developing solvent. The HPTLC plates were sprayed with resorcinol reagent and heated at 120°C to detect the separated glycolipids[28,29]. Densitogram was obtained from HPTLC by using Scion Image Software (Scion Corporation, http://www.scioncorp.com) to quantify glycolipids.

Inverted microscopy: Cell images were obtained by using a Zeiss Axiovert 40 CFL inverted microscope (Carl Zeiss, Oberkochen, Germany) with a Power Shot A620 camera (Canon, Tokyo, Japan), driven by Axio Vision 4.5 digital image software (Carl Zeiss, Oberkochen, Germany).

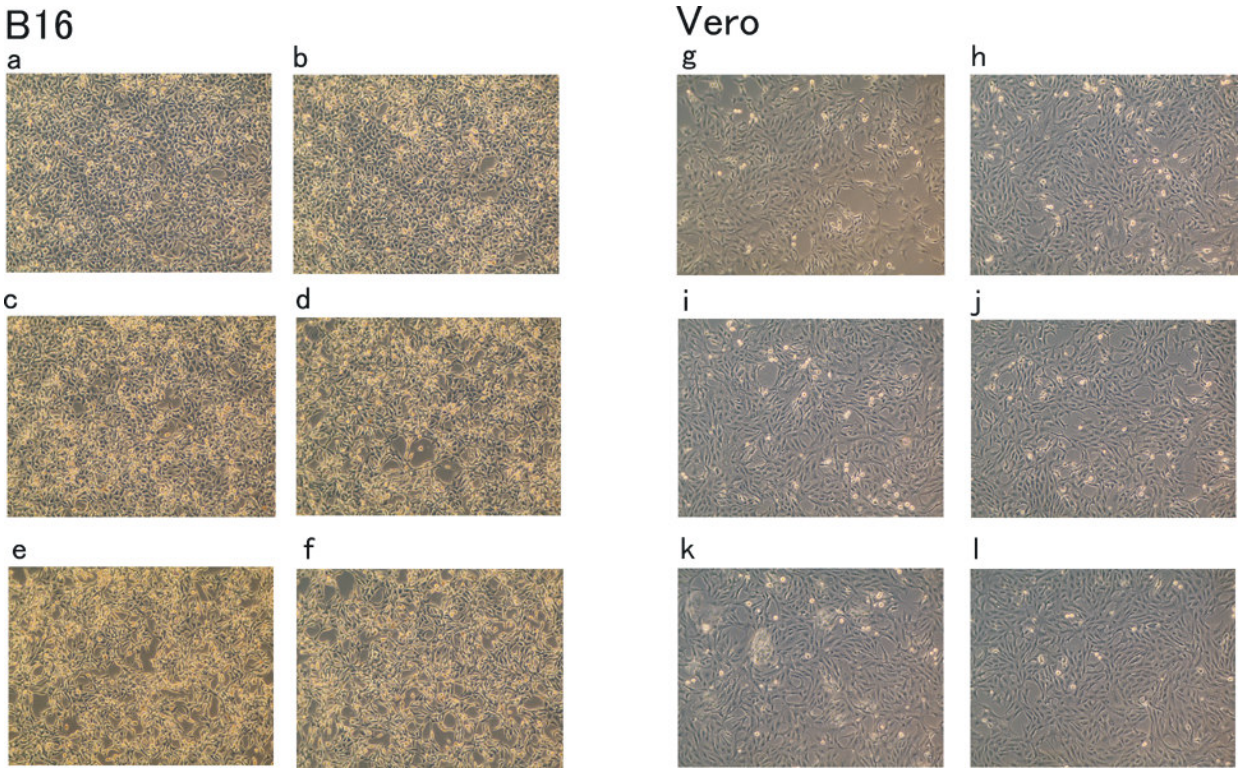

Fig. 1: Effect of fumonisin B_1 on proliferation of mouse melanoma B16 cells (a-f) and African green-monkey kidney (Vero) cells (g-l). Culture medium supplemented with fumonisin B_1 at a final concentration of 0 (a, g), 0.1 μM (b, h), 1 μM (c, i), 5 μM (d, j), 25 μM (e, k) and 50 μM (f, l).

RESULTS AND DISCUSSION

After incubation of mouse melanoma B16 cells and African green-monkey kidney (Vero) cells in culture medium containing 0.1, 1, 5, 25 or 50 μM of fumonisin B1 (preculture), the cells were used for oligosaccharide production via glycosylation of saccharide primer. On inverted microscopy observation, B16 cell grew well with low concentration of fumonisin B_1 (Fig. 1a-c), while the proliferation was inhibited with higher concentrations than 5 μM (Fig. 1d-f). Vero cells grew well with every concentration of fumonisin B_1 (Fig. 1g-l). The culture medium was replaced by TI-DF containing 50 μM of n-dodecyl β-lactoside with the same concentration of fumonisin B1 as used for preculture and the cells were incubated for 2 days. The glycosylated products and endogenous glycolipids in the culture medium and in the cells were extracted and analyzed by HPTLC, respectively. When mouse melanoma B16 cells were incubated in the presence of *n*-dodecyl β-lactoside, GM3-type oligosaccharide (NeuAc-Gal-Glc-C12) was accumulated in the culture medium as previously reported[17,18] (Fig. 2a). On the other hand, GM3-type, Gb3-type (Gal-Gal-Glc-C12) and a small amount of Gb4-type oligosaccharides (GalNAc-Gal-Gal-Glc-C12) were obtained when African green-monkey kidney (Vero) cells were incubated in the presence of n-dodecyl β-lactoside (Fig. 2a).

HPTLC results obtained from the culture of mouse melanoma B16 cells showed that the amount of GM3-type oligosaccharide accumulated in the culture medium increased with increasing the concentration of fumonisin B_1, while the amount of endogenous GM3 remarkably decreased. Fig. 2b shows the amount of GM3-type oligosaccharide that is glycosylated primer and endogenous GM3. The amounts of GM3-type oligosaccharide obtained from the culture with 25 μM

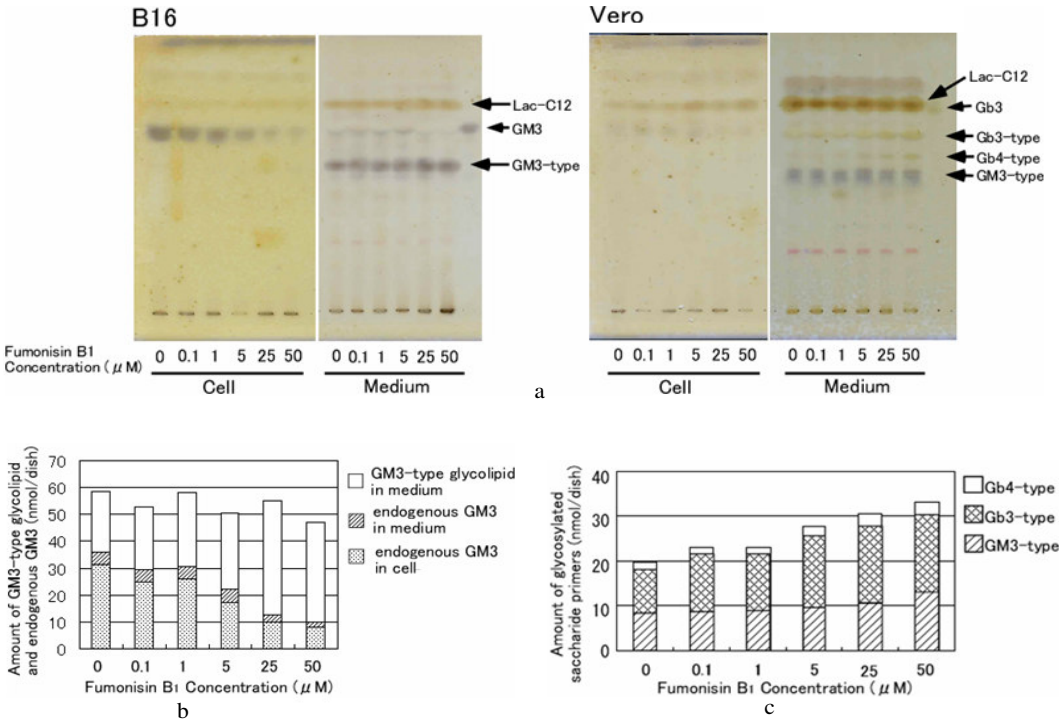

Fig. 2: Effect of fumonisin B_1 on glycolipid production in mouse melanoma B16 cells and African green-monkey kidney (Vero) cells. (a) HPTLC results of glycolipids extracted from the cell and culture medium fractions. (b) The amounts of GM3-type glycolipid and endogenous GM3 in culture medium and cells were determined after incubation. (c) The amount of glycosylated saccharide primers in medium were determined after incubation

or without fumonisin B_1 were 22.6 and 42.6 nmol/dish, respectively. The efficiency of glycosylation in cells incubated with fumonisin B_1 for 2 days increased to about two times as compared with the incubation without fumonisin B_1. It should be noted that the total amounts of GM3-type oligosaccharide and endogenous GM3 extracted from culture were little affected by the addition by the addition of fumonisin B_1.

On the other hand, African green-monkey kidney (Vero) cells could produce neutral Gb3-type and Gb4-type oligosaccharide in addition to acidic GM3-type oligosaccharide. These oligosaccharides were quantified (Fig. 2c). The amount of GM3-type oligosaccharide obtained by the culture with 50 μM or without fumonisin B_1 were 8.4 and 13.0 nmol/dish, respectively. The increasing rate brought by supplementing fumonisin B_1 to the African green-monkey kidney (Vero) cells culture was only 155%, which is smaller as compared with that from mouse melanoma B16 cells culture (188%). On the other hand, the amount of Gb3-type oligosaccharide obtained by the culture with 50 μM or without fumonisin B_1 were 9.87 and 17.2 nmol/dish, respectively. The amount of Gb4-type oligosaccharide obtained by the culture with 50 μM or without fumonisin B_1 were 1.46 and 2.95 nmol/dish, respectively.

When fumonisin B_1 was not supplemented to the culture medium during preculture but only during incubation for glycosylation reaction, the amount of oligosaccharide produced by glycosylation of the saccharide primer only slightly increased (data not shown). Consequently, fumonisin B_1 should be added during preculture of cells.

These results allowed us to conclude that fumonisin B_1 stimulated oligosaccharide production from saccharide primer that mimics lactosylceramide (LacCer), but inhibited the production of endogenous glycolipids. The n-dodecyl β-lactoside can be incorporated by cells in culture and can be utilized for glycolipid synthesis. Most of glycosphingolipids in mammals are synthesized through LacCer intermediate. The production of glycosylated primers owing to the inhibition of ceramide synthesis indicated that relatively large amount of n-dodecyl β-lactoside was preferentially glycosylated by cellular glycosyltransferases over small amount of LacCer

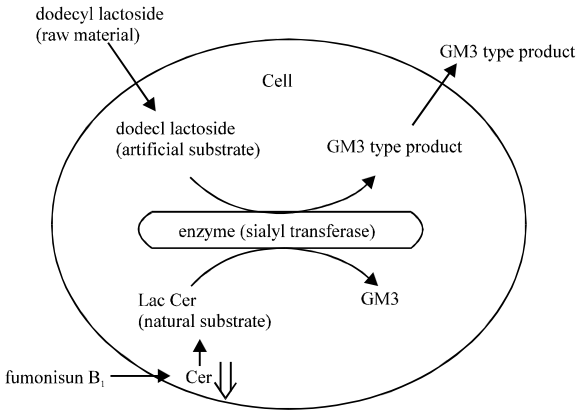

Fig. 3: Inhibition of ceramide synthesis by fumonisin B1 controled the competitive reaction with sialyl transferase and enhanced the productivity from exogenous artificial substrate

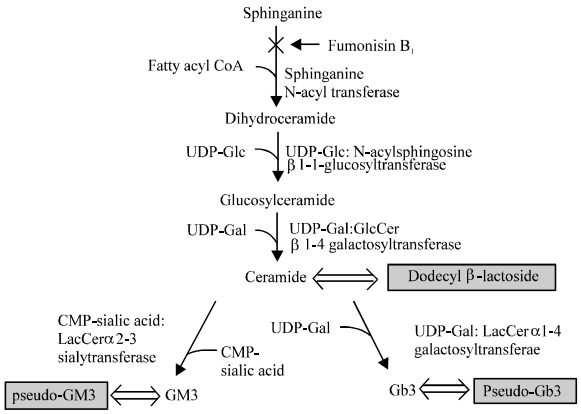

Fig. 4: Pathway of glycolipids and biosynthetic conversion of *n*-dodecyl β-lactoside to pseudo-glycolipids by the cells. Inhibition of ceramide synthesis by incubation with fumonisin B1 promotes pseudo-glycolipids production in the cells

(Fig. 3). If LacCer is abundant in cells, the glycosylation of oligosaccharide primer may be interrupted due to the competition of *n*-dodecyl β-lactoside with LacCer. It was reported that fumonisin B_1 inhibited *de novo* sphingolipid biosynthesis by neuronal cells[23]. On the other hand, glycosylation of saccharide primer was promoted by fumonisin B_1. The mouse melanoma B16 cells, which synthesize abundant endogenous glycolipids, were induced to produce pseudo-glycolipids from saccharide primer by ceramide synthesis inhibition rather than the African green-monkey kidney (Vero) cells, which prepare a small amount of endogenous glycolipids. The oligosaccharide primer was utilized as a favorable substrate for glycosylation, which was catalyzed by the cellular enzyme, i.e. glycosyltransferase, at low concentration of ceramide, which is the natural substrate for glycolipids synthesis (Fig. 4). Generally, the amount of oligosaccharide products obtained by the saccharide primer method is limited because the concentration of the saccharide primers in the culture medium is low due to its toxicity and the efficiency of glycosylation is relatively low.

In general condition of B16 cell culture, a certain amount of GM3 expressed and the activity of the GM3 synthase (sialyl transferase) is controlled. On the other hand, the presence of synthetic substrate (dodecyl lactoside) caused competitive inhibition of the glycosylation of endogenous lactosyl ceramide. Most of the sialylated synthetic substrate was released to the culture medium, while the endogenous GM3 mostly remained in the cell. Consequently, it was considered that GM3 synthase was activated by the addition of synthetic substrate in order to hold the certain amount of GM3. Moreover, the inhibition of ceramide synthesis by fumonisin B_1 caused the significant decrease in amount of endogenous GM3 and it might result in the considerable activation of GM3 synthase. Therefore, in the presence of fumonisin B_1, the synthetic substrate was glycosylated by the considerably activated enzyme in the cell and the sialylated product was efficiently prepared.

In Vero cell culture, two kinds of glycosyl transferases predominantly act on lactosyl ceramide. Those are sialyl transferase and galactosyl transferase. Similarly to B16 cell, in the case of Vero cell also we may expect both enzymes to be considerably activated by the addition of dodecyl lactoside and fumonisin B_1. Both activated sialyl transferase and galactosyl transferase scramble for dodecyl lactoside which is the common substrate. With increasing the amount of fumonisin B_1, the amount of galactosylated products (Gb3-type and Gb4-type) more increased than that of sialylated product (GM3-type) (Fig. 2c), indicating that the galactosyl transferase can preferentially act on the dodecyl lactoside compared with sialyl transferase in Vero cell.

As described above, the amount of oligosaccharide products obtained by the saccharide primer method could be increased by shingolipid synthesis inhibition. This strategy is expected to augment oligosaccharides production using saccharide primers.

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