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Oral glucosylceramide reduces 2,4-dinitrofluorobenzene induced inflammatory response in mice by reducing TNF-alpha levels and leukocyte infiltration

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**Running title**: Anti-inflammatory Property of Orally Administered Glucosylceramide

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Abstract

Sphingolipids are constituents of cellular membranes and play important roles as second messengers mediating cell functions. As significant components in foods, sphingolipids have been proven to be critical for human health. Moreover, diverse metabolic intermediates of sphingolipids are known to play key roles both in proinflammatory and in anti-inflammatory effects. However, the effect of dietary sphingolipids on inflammation is a complicated field that needs to be further assessed. Our study evaluated the effects of orally administered maize glucosylceramide (GluCer), one of the most conventional dietary sphingolipids, on inflammation using the 2, 4-dinitro-1-fluorobenzene (DNFB)-treated BALB/c murine model. Oral administration of GluCer inhibited ear swelling and leukocyte infiltration to the inflammatory site, suggesting that dietary GluCer has anti-inflammatory properties. ELISA analyses revealed that oral administration of GluCer for 6 days was not modified the Th1/Th2 balance, but significantly down-regulated the activation of TNF-α at the inflammatory site. Based on these results, the down-regulation of TNF-α by dietary GluCer may suppress vascular permeability and reduce the migration of inflammatory cells. Our findings increase understanding of the actions dietary sphingolipids on the balance of the immune response.
Keywords
Sphingolipids, Dietary supplements, glucosylceramide, Anti-inflammatory agents, DNFB, BALB/c mice, TNF-α, immune response

Abbreviations
DNFB 2, 4-dinitro-1-fluorobenzene
GluCer glucosylceramide
IFN-γ interferon-gamma
IgE immunoglobulin E
IL-1β interleukin-1beta
IL-4 interleukin-4
IL-6 interleukin-6
NF-κB receptor activator of nuclear factor-kappa B
Th1 T-helper 1
Th2 T-helper 2
TNF-α tumor necrosis factor-alpha
Introduction

Sphingolipids are commonly believed to protect the cell surface against harmful environmental factors by forming the mechanically stable and chemically resistant outer leaflet of the plasma membrane lipid bilayer [1-4]. Sphingolipids generate diverse metabolic intermediates, notably ceramide, sphingosine, sphingosine-1-phosphate and ceramide-1-phosphate, which serve as important mediators in the signaling cascades involved in apoptosis, proliferation, and stress responses [5-8]. Although we have already demonstrated that dietary sphingolipids are poorly absorbed by the intestine [9], sphingolipids that are significant components of foods have gained considerable attention for their potential and essential roles in human health [10-14]. It has been reported that dietary supplementation with sphingolipids has diverse physiological effects, such as lowering plasma lipids [15], improving skin barrier function [16], preventing melanin formation [17], contributing to central nervous system myelination [18] as well as protecting the colon against inflammation [19-25]. However, the functional, regulatory, and physiological significance of the immune regulating effects of dietary sphingolipids is an appreciably complicated field that is not well understood.

One hypothesis of immune regulation involves the balance between T-helper 1 (Th1) and T-helper 2 (Th2) cells, which direct different immune response pathways. Th1 cells drive the "cellular immunity" pathway to fight viruses and other intracellular pathogens, eliminate cancer cells, and stimulate delayed-type hypersensitivity skin reactions. Th2 cells are involved in "humoral immunity" and up-regulate antibody production to fight extracellular organisms. Either pathway can down-regulate the other. Disruption of the Th1/Th2 balance can cause immunological diseases [26, 27]. Via the actions of sphingolipid degrading enzymes, such as sphingomyelinase, glycolipidases and ceramidase, dietary sphingolipids are hydrolyzed to various kinds of metabolic intermediates which are critical for the activation and mediation of various types of immune cells. Metabolites of sphingolipids initiate and maintain diverse aspects of immune cell balance and functional responses by regulating cell migration and inflammatory pathways [8, 20, 28-31]. For instance, sphingolipid hydrolysis products regulate
cyclooxygenase-2, interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and nuclear factor kappa B (NF-κB) via the sphingosine kinase 1/ sphingosine-1-phosphate and ceramide kinase 1/ceramide-1-phosphate pathways, and thus cause the activation of mast cells, control thymocyte maturation and regulate the balance of lymphocyte subpopulations [32-37].

The goal of this study was to evaluate the effects of orally administered glucosylceramide (GluCer), one of the most important dietary sphingolipids, against DNFB-induced ear swelling in the BALB/c murine model, to provide further understanding of how dietary sphingolipids act on the balance between proinflammatory and anti-inflammatory responses.

Materials and Methods

Maize GluCer preparation

GluCer from maize was kindly donated by Nippon Flour Mills Co. Ltd. (Atsugi, Japan). The purity of this GluCer was 96%, which was determined by HPLC equipped with an evaporative light-scattering detector, as described previously [12].

Animals

Female BALB/c mice (6 weeks old, 15–20 g body weight) were purchased from Japan SLC Inc. (Shizuoka, Japan). Animals were group-housed at 6 mice per cage, and were bred at the Institute's animal facilities at 25 °C with a 12-hour light/dark cycle. Pure water and AIN-93G diet (Oriental Yeast Co., LTD., Tokyo, Japan) were available ad libitum. All experiments were performed according to the guidelines of Kyoto University for the use and care of laboratory animals.

Contact hypersensitivity induced by DNFB

After a 2-week acclimatization period, allergic contact dermatitis was induced by DNFB in BALB/c mice according to a previously published method with minor modifications [38]. Briefly, mice were sensitized on day 0 by application of 100 µl 0.5% DNFB in acetone-soybean oil (4:1, v/v) on their shaved dorsal skin. The mice were divided into control, low dose (5 mg)
and high dose (50 mg) groups (n=12 in each group). An identifying mark was made on the tail of each mouse.

The maize GluCer was suspended in 0.5% carboxymethyl cellulose (CMC) (Nacalai Tesque Co. Ltd., Kyoto, Japan) solution and was orally administered at 5 or 50 mg to each mouse daily for six days. One hour after the final treatment, mice were challenged with 20 µL 0.5% DNFB in acetone–soybean oil (4:1) on both ears. The thickness of the right ear of each mouse was measured with a Dial Thickness Gauge (Mitutoyo Co., Kanagawa, Japan) at 0, 6 and 24 hours after the DNFB challenge. Ear swelling was calculated as the difference in thickness before and after challenge [39].

Six hours (n=6) and 24 hours (n=6) after DNFB treatment, blood was collected and mice were sacrificed under anesthesia. The right ear and spleen of each mouse was immediately excised and frozen in liquid nitrogen, then stored at -80°C until use.

**Morphological analysis**

The left ear of each mouse was fixed in 10% neutral buffered formalin solution and was then processed routinely into paraffin wax. Formalin fixed paraffin sections were stained with hematoxylin and eosin (H&E) to observe morphological changes using a microscope (Keyence Co., Osaka, Japan).

**Measurement of cytokine production and serum immunoglobulin E (IgE)**

Amounts of IFN-γ, interleukin-4 (IL-4), TNF-α and interleukin-10 (IL-10) in homogenates of tissues were quantified using Murine IL-4 (Diaclone Research, Besancon, France), Murine IFN-γ (Diaclone Research), Mouse TNF-α (Pierce Biotechnology Inc., Rockford, IL, USA), and Murine IL-10 (Diaclone Research) ELISA kits, respectively, according to the manufacturer’s instructions. Levels of those cytokines in each supernatant were normalized to total protein content, which was determined using a DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Total serum IgE levels of DNFB-challenged mice were quantified using a Mouse IgE ELISA kit (Immunology Consultants Laboratory, Newberg, Oregon, USA) according to the
manufacturer’s instructions.

**Statistical analysis**

Data are reported as means ± SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Fisher's PLSD method to identify levels of significance between the groups.

**Results**

**GluCer suppresses DNFB-induced ear swelling of BALB/c mice**

After challenge with DNFB, typical allergic contact dermatitis was provoked in ears of BALB/c mice, which was characterized by an initial increase of ear thickness and visible congestion of blood vessels. Oral treatment with maize GluCer suppressed DNFB-induced inflammatory symptom (redness and thickness) of ears. As shown in Fig. 1, a significant depression of ear thickness was observed at 6 h in both low (5mg/day) and high dose (50mg/day) GluCer treated groups ($p<0.05$). At 24 h, the average value of ear thickness was also reduced by GluCer, but there were no statistical differences among the three groups, which may due to large individual differences. The reduction of DNFB-induced ear swelling implies dietary GluCer has anti-inflammatory property.

**GluCer inhibits inflammatory infiltrates in the ears of BALB/c mice**

Histological specimens of ears were prepared at 6 h and 24 h after topical application of DNFB in BALB/c mice. In the control group, typical allergic contact dermatitis with congested blood vessels and apparent edema could be observed by H&E staining. As shown in Fig. 2A and Fig. 2D, microvascular dilations and dense leukocytes infiltrating the connective tissue, which are characteristics of inflammatory reactions, were clearly observed in the control group. At higher magnification, various kinds of migrated inflammatory cells could also be observed, including fibrocytes, mononuclear cells, degranulated mast cells and other leukocytes. These
results confirm that DNFB induces severe inflammation in the ears of BALB/c mice and that a
variety of lymphocytes migrated out from blood vessels during this contact sensitivity procedure.
In both the low (5 mg/day) and the high (50 mg/day) dose GluCer-treated groups, microvascular
dilation and leukocytes in inflammatory infiltrates were inhibited at 6 h (Fig. 2 B&C) and 24 h
groups (Fig. 2 E&F). These results show that dietary GluCer inhibits microvascular dilation
and inflammatory infiltration of DNFB-induced BALB/c mice.

GluCer inhibits inflammation by reducing TNF-α production in the ear

To clarify the effect of GluCer on DNFB-induced inflammation, especially on Th1/Th2
balance, levels of IFN-γ as an indicator of Th1 cells and IL-4 as an indicator of Th2 cells were
measured by ELISA assay. IFN-γ and IL-4 levels of GluCer-treated group were not
significantly altered (Table 1). In other words, Th1/Th2 balance was not modified by oral
administration of GluCer for 6 days.

For further evaluating the effect of GluCer on DNFB-induced inflammation, IL-10 was
determined. In ear, IL-10 was significantly decreased at 6 h both low and high dose groups,
whereas this effect did not prolong to 24 h (Table 1). However, in spleen, IL-10 was increased
by GluCer treatment at 24 h, but not reached a statistical significance in 6 h because of the
relatively large individual differences (Table 1).

TNF-α as the most important proinflammatory cytokine and IgE as the most important
antibody in the serum were also measured. As shown in Fig. 3A, the TNF-α level in the ear
was significantly suppressed both in the low and high dose GluCer groups ($p<0.05$). Moreover,
TNF-α level was also significantly down-regulated at 24 h by the effect of high dose GluCer (Fig.
3B). In contrast, IgE in the serum was almost at the same level among the control, low and
high GluCer dose groups (Fig. 3 C&D). The anti-inflammatory effect of dietary GluCer on
contact dermatitis in DNFB-induced BALB/c mice is via regulation of the level of TNF-α
secreted by inflammatory cells in the ear.

Discussion
The findings presented here indicate that dietary plant GluCer, one of the most important and abundant sphingolipids in food [12], suppresses the DNFB-induced ear swelling of BALB/c mice, and inhibits the microvascular dilation and inflammatory infiltration response via down-regulating levels of TNF-α, but not modifying the balance of Th1/Th2. Meanwhile, over-expressed IL-10 in inflammatory ear skin was suppressed by dietary GluCer. This anti-inflammatory effect of dietary GluCer increases our understanding of biofunctional sphingolipids.

Dietary GluCer is known to be hydrolyzed to ceramide, sphingosine and free fatty acids in the intestinal lumen. In mucosal cells, exogenous free sphingosine and dihydrosphingosine are rapidly absorbed and metabolized to palmitic acid [40]. A smaller portion of the sphingoid bases is reincorporated into ceramide and more complex sphingolipids. Our recent findings revealed that dietary GluCer originating from higher plants can be hydrolyzed in the intestine and that the intact plant form of sphingoid bases is barely absorbed by the tissues [9,41]. Ono et al. reported that dietary maize and yeast GluCer did not alter the sphingoid base composition in the skin of NC mice [42]. We speculate that dietary maize GluCer accomplishes its anti-inflammatory effect, not only by producing bio-active metabolic intermediates through the sphingolipid metabolic pathways, but also via the activation of sphingolipid metabolic enzymes that affect endogenous sphingolipids at the inflammatory site, because diverse metabolic intermediates of sphingolipids, including ceramide, sphingosine, sphingosine-1-phosphate and ceramide-1-phosphate are well known important and highly bioactive endogenous regulators, which are involved in a complex metabolism network and play critical roles in inflammation [43-46].

In the case of our study, dietary maize GluCer accomplishes its inhibition of inflammation via the down-regulation of TNF-α level at the inflammatory site. TNF-α, produced by mononuclear phagocytes and other inflammatory cells (neutrophils, lymphocytes, natural killer cells and mast cells) or non-inflammatory cells (endothelial cells), is known to be one of the most important inflammatory mediators [47,48]. TNF-α facilitates the formation of adhesion
molecules, vascular permeability and migration of leukocytes to sites of inflammation by affecting endothelial cells [49-51]. Our results reveal that dietary maize GluCer down-regulates levels of TNF-α in inflammatory ears. This down-regulation of TNF-α may affect endothelial cells and inflammatory cells, and also can inhibit vascular permeability at the site of inflammation. As a result, leukocyte migration is reduced. In the inflammatory cells, DNFB activates NF-κB by depredating the inhibitor of NF-κB (IκB) [52]. In addition, it has been reported that sphingolipids down regulated TNF-α via inactivating of NF-κB in histamine-induced mouse skin tissues [53]. Thus, dietary GluCer seems to inhibit the DNFB activated NF-κB and down-modulate TNF-α expression in this study.

Moreover, IL-10 levels were increased in spleens but suppressed in ears by dietary GluCer. This well known anti-inflammatory cytokine, IL-10, has been reported over-expressed during the antigen-specific type of skin inflammation [54] and DNFB-challenged ear [55, 56]. IL-10 is released by CD4+ T helper 2 (Th2) cell clones and a variety of other cells, including keratinocytes, macrophages, B lymphocytes, and mast cells [56]. The suppressive effect of dietary GluCer on IL-10 expression in DNFB-challenged ears might be caused by the inhibition of leukocytes infiltrating to inflammatory site.

Ono et al. demonstrate that supplementation of 0.1% GluCer diet for 7-week prevented atopic dermatitis-like symptoms in a mouse model by regulating the Th1/Th2 balance [42]. However, IFN-γ, IL-4 and IgE levels, the markers of Th1/Th2 balance, were not notably affected by GluCer administration for 6 days in the present study. It appears that the period of treatment is important for immunological response of dietary GluCer.

Furthermore, allergic inflammatory skin disease is associated with a loss of ceramide in the extracellular lamellar membranes which causes an abnormal barrier function of the stratum corneum [57]. Application of ceramide on diseased skin could significantly reduce allergic inflammatory reactions by improving the severity score, stratum corneum cohesion and hydration [58-60]. Dietary GluCer might also improve the stratum corneum cohesion and hydration of ear skin to reduce the skin inflammation.
In summary, our data provide evidence that maize GluCer has anti-inflammatory effects on the DNFB-induced inflammation of BALB/c mice. We confirmed that dietary GluCer inhibits ear swelling and leukocyte infiltration. Furthermore, our results indicate that this effect is accomplished mainly by down-regulating TNF-α, but does not significantly affect Th1/Th2 balance or IgE levels in the serum. We hypothesize that dietary GluCer accomplishes its anti-inflammatory effect by down-regulating TNF-α to suppress vascular permeability. This reduces the migration of inflammatory cells, affects endogenous sphingolipids through metabolic pathways by activating sphingolipid-related enzymes, and moreover, by hydrolysis to ceramide to improve skin barrier function at the dermatitis site. Our findings increase the comprehensive understanding of the actions of dietary sphingolipids on the balance of immune responses.

Acknowledgment

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**Figure Legends**

Fig. 1 Effect of orally administered GluCer on DNFB-induced ear swelling in BALB/c mice.

The thickness of the left ear of each mouse was measured both before and after the DNFB challenge. Ear swelling values are presented as the difference in thickness at 6 h (A) and at 24 h (B): Ear swelling = ear thickness after challenge (6/24 h) – ear thickness before challenge (0 h).

Con, 6 days 0.5% CMC (vehicle) orally administered; Low, 6 days low dose (5 mg/day) maize GluCer orally administered; High, 6 days high dose (50 mg/day) maize GluCer orally administered. Values are means ± SD, n = 6. Values with different superscript letters are significantly different (p<0.05).

Fig. 2 Histopathological analysis of orally administered maize GluCer on DNFB-induced ear swelling in BALB/c mice.

Morphological changes in the left ear of 6 h and 24 h after DNFB-challenged BALB/c mice were observed. Ear sections from control mice (A), low dose (5 mg/day) maize GluCer administered mice (B) and high dose (50 mg/day) maize GluCer administered mice (C) were stained with hematoxylin and eosin (H&E). Microvacular (asterisk marks) and leukocyte (arrowheads) were pointed out in the histological sections. Sections are representatives of more than five observations.

Fig. 3 TNF-α levels in the ears and IgE levels in the serum of DNFB-challenged BALB/c mice.

TNF-α (A,B) levels in the right ear homogenates and IgE levels in the serum (C,D) of control, low dose (5 mg/day) and high dose (50 mg/day) maize GluCer administered mice were measured both 6 (A,C) and 24 h (B,D) after the DNFB challenge. These data represent the means ± SD for groups of six mice. Values with different letters differ significantly (p<0.05).
Fig. 1
Fig. 2
Fig. 3
Table 1. IFN-γ, IL-4 and IL-10 levels in ears and spleens of DNFB-challenged BALB/c mice.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Ear</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 h mg/day</td>
<td>24 h pg/mg protein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>482.4±106.3</td>
<td>85.9±16.7</td>
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<tr>
<td>5</td>
<td>362.0±89.4</td>
<td>89.1±18.3</td>
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<tr>
<td>50</td>
<td>373.2±107.1</td>
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</tr>
<tr>
<td>IL-4</td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>25.3±4.4</td>
<td>10.3±2.5</td>
</tr>
<tr>
<td>5</td>
<td>30.6±6.5</td>
<td>10.7±2.3</td>
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<tr>
<td>50</td>
<td>29.8±8.4</td>
<td>9.0±2.1</td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3137.7±881.3 a</td>
<td>698.7±212.5</td>
</tr>
<tr>
<td>5</td>
<td>1326.7±297.7 a,b</td>
<td>543.0±158.9</td>
</tr>
<tr>
<td>50</td>
<td>1578.5±352.3 b</td>
<td>618.0±264.9</td>
</tr>
</tbody>
</table>

These data represent the means ± SD for groups of six mice. Values with different superscript letters in the same series differ significantly (p<0.05).