Effects of Dietary Plant-Origin Glucosylceramide on Bowel Inflammation in DSS-Treated Mice

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Abstract: The effects of dietary plant-origin glucosylceramide (GlcCer) on symptoms similar to those of inflammatory bowel disease were investigated in dextran sulfate sodium salt (DSS)-treated mice. Dietary GlcCer suppressed decreases in body weight due to DSS administration. To determine its effects on the colon, we examined its surface under a microscope following toluidine blue staining. Dietary GlcCer decreased DSS-induced chorionic crypt injury and elevated myeloperoxidase levels. Moreover, dietary GlcCer significantly suppressed the production of cytokines by the intestinal mucosa. These results provide evidence for the suppression of DSS-induced inflammation by dietary GlcCer.

Key words: glucosylceramide, IBD, plant lipid, sphingolipid, cytokine

1 INTRODUCTION

Sphingolipids are composed of a sphingoid base backbone, amide-linked fatty acid (normal and 2-hydroxy fatty acids), and a polar head group, such as a phosphocholine or a hexose. Sphingolipids are frequently found in eukaryotes, with the mammalian sphingolipid trans-4-sphinganine (sphingosine) being the most prevalent. Others such as sphinganine (dihydrosphingosine) and 4-hydroxysphinganine (phytosphingosine) occur frequently in small amounts¹. Plant and fungal sphingolipids show diverse sphingoid base structures such as trans-8-sphinganine; cis-8-sphinganine; trans-4,trans-8-sphingadienine; trans-4,cis-8-sphingadienine; 4-hydroxy-trans-8-sphinganine; 4-hydroxy-trans-8-sphinganine; and 9-methyl-trans-4, trans-8-sphingadienine²–⁴. Sphingolipids and their metabolites function as intracellular mediators of cell differentiation, apoptosis, prevention of melanin formation, etc.⁵–⁸. Orally administered mammalian-origin sphingolipids undergo hydrolysis via the action of intestinal enzymes for subsequent uptake by mucosal cells. The intake of sphingomyelin and glycosphingolipid from bovine milk and plants exerts anti-cancer effects in vivo; this finding has prompted considerable research on dietary sphingolipids as functional lipids⁹–¹¹.

Sphingolipids used as ingredients in foods and cosmetics were isolated from bovine brain prior to the outbreak of bovine spongiform encephalopathy (BSE)¹²; however, the safety of their application for such purposes is yet to be confirmed.

Inflammatory bowel disease (IBD) is a term used for a group of disorders that cause inflammation of the intestine¹³. This inflammation is long lasting and usually recurs. The main symptoms of this disease include abdominal cramps and pain, diarrhea, weight loss, and intestinal bleeding. Two common types of IBD are Crohn’s disease, which usually causes ulcers (open sores) along the length of the small and large intestines, and ulcerative colitis, which usually causes ulcers in the lower part of the large intestine. In animal models of IBD, inflammation often starts in the rectum. Zugmond et al. reported the effects of intraperitoneal administration of β-glucosylceramide (GlcCer) in a trinitrobenzenesulfonic acid (TNBS)-induced colitis model¹⁴. However, the effects of dietary plant-origin GlcCer are still unclear.

In our previous study, we investigated the effects of dietary GlcCer on aberrant crypt foci formation in the large intestines of mice treated with 1,2-dimethylhydrazine (DMH)¹⁵. In this study, dietary GlcCer suppressed damage...
of the large intestines by DMH treatment. We investigated the effects of dietary GlcCer on bowel inflammation in dextran sulfate sodium salt (DSS)-treated mice, an important animal model of IBD.

2 EXPERIMENTAL PROCEDURES

2.1 Preparation of GlcCer
A commercial GlcCer-rich preparation (Nippon Flour Mills, Atsugi, Japan) was used as a source of maize-origin GlcCer. For isolation of GlcCer, the preparation was dissolved in chloroform, applied to a silica gel column, and purified by preparative thin layer chromatography (TLC), as described previously. The purity of GlcCer (>80%) was determined by HPLC, as described previously.

2.2 Animals and diets
Female BALB/c mice aged 4 weeks were obtained from CLEA Japan Inc. (Tokyo, Japan) and housed in isolator cages under a 20/12 h light/dark cycle. After test diet acclimation for 10 days, each mouse was kept in an aluminum cage, and water was supplied ad libitum; each mouse was administered 2% DSS in drinking water. In the feeding experiment, DSS intake was adjusted using drinking water. Experimental diets for mice were based on AIN-76 and supplemented with GlcCer (1 g/kg diet, 0.1%) (6). Glicosyl ceramide was dissolved in ethanol and added to corn oil. Ethanol was removed by rotary evaporator. This combined oil was used as an experimental diet oil. Mice were randomly divided into three groups of 10 mice each: Blank (−DSS, AIN-76), Control (+DSS, AIN-76), and 0.1% GlcCer (+DSS, AIN-76+0.1%GlcCer). After 5 or 15 days of feeding, mice were anesthetized, and their plasma and colon were collected. Mice fed for 5 days were used for determining cytokine levels. All protocols involving animals were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the Obihiro University Guidelines.

2.3 Histological analysis
The large intestine was excised from the mice under ether anesthesia, and the portion from the cecum to the vent was removed and rinsed with cold saline. This was followed by overnight cell fixation with 4% paraformaldehyde in phosphate-buffered saline. Subsequently, the specimens were embedded in paraffin wax, and transverse sections were cut and stained with toluidine blue.

2.4 Western blotting analysis
Myeloperoxidase levels were determined by western blotting analysis, as reported previously. Tissues were lysed with sodium dodecylsulfate (SDS) buffer and separated on 12.5% SDS-polyacrylamide gels under reducing conditions. Proteins on the gels were transferred onto nitrocellulose membranes (ATTO, Tokyo, Japan) and immunoblotted with the primary antibody (Sigma, St. Louis, MO) and a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences Corp., Piscataway, NJ). Bands were visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ), recorded on X-ray film, and identified based on comparison with commercially available protein standards (ATTO).

2.5 Quantitative analysis of cytokine levels in the mucosa of the large intestine
We used a cytokine array to determine the effects of inflammation-related colonic cytokine production by dietary sphingolipids in DSS-treated mice. This system can simultaneously detect 40 cytokines. Briefly, the mucosa of the large intestine was removed with a glass slide, homogenized with 1% Triton X-100 in phosphate buffered saline (pH 7.4), and centrifuged at 10,000 × g for 1 min. The supernatant was applied to a Cytokine Array kit (R&D Systems, Inc., Minneapolis, MN). The detectable cytokines were denoted as follows: B lymphocyte chemoattractant (BLC); chemokine (C-C motif) ligand 1 (CCL1); complement component 5a (C5/C5a); eotaxin; granulocyte macrophage colony-stimulating factor (GM-CSF); interferon-γ (IF-γ); interferon-γ-induced protein 10 (IP-10); interleukin-1α (IL-1α); interleukin-1β (IL-1β); interleukin-2 (IL-2); interleukin-3 (IL-3); interleukin-4 (IL-4); interleukin-5 (IL-5); interleukin-6 (IL-6); interleukin-7 (IL-7); interleukin-10 (IL-10); interleukin-12 (IL-12); interleukin-13 (IL-13); interleukin-16 (IL-16); interleukin-17 (IL-17); interleukin-23 (IL-23); interleukin-27 (IL-27); metalloproteinase inhibitor 1 (TIMP-1); monocyte chemoattractant protein 2 (MCP-2); macrophage inflammatory protein-1β (MIP-1β); macrophage inflammatory protein-1α (MIP-1α); monokine induced by gamma interferon (MIG); monocyte chemotactic protein 5 (MCP-5); monocyte-specific cytokine MCP-1 (CCL2); macrophage colony-stimulating factor (M-CSF); neutrophil-activating protein 3 (NSP3); interferon-inducible T cell alpha chemoattractant (I-TAC); regulated on activation, normal T cell expressed and secreted (RANTES); soluble intercellular cell adhesion molecule-1 (sICAM-1); stromal cell-derived factor 1 (SDF-1); thymus and activation regulated chemokine (TARC); triggering receptor expressed on myeloid cells 1 (TREM-1); and tumor necrosis factor-α (TNF-α).

2.6 Statistical analysis
The results were analyzed by ANOVA and Scheffe’s test. In all analyses, p < 0.05 was considered statistically significant.
**3 RESULTS and DISCUSSION**

Figure 1 shows the weight gain of mice during the experimental period (days). Before DSS administration, the body weight in the Control group increased daily, and dietary GlcCer had no effect on body weight (no significant difference from body weight on day 0). In contrast, after supplementation with DSS in drinking water, body weight decreased by over 10% until the feeding period compared to Blank group. Conversely, dietary GlcCer (0.1%/diet, dose the week before DSS administration) suppressed decreases in body weight in the early phase. To observe the status of the colon, we examined its colon under a microscope with toluidine blue staining (Fig. 2). The Blank group showed no unusual findings in crypts. Typical histological changes associated with acute DSS-colitis are mucin depletion and the disappearance of epithelial cells. In this study, similar phenomena were observed in the Control group (Fig. 2). Dietary GlcCer decreased injury to the chorionic crypts associated with DSS administration. Moreover, the levels of myeloperoxidase (MPO) induced by neutrophils were quantified by western blotting analysis. MPO levels were induced by DSS (approximately 2-fold higher as compared with the Blank group), while dietary GlcCer significantly suppressed MPO levels (Fig. 3). These results provided evidence for the suppression of DSS-induced inflammation by dietary GlcCer. A cytokine array was used to determine the effects of inflammation-related cytokine production by dietary sphingolipids in DSS-treated mice in the early stage of inflammation (5 days after DSS treatment). This system can simultaneously detect 40 cytokines. DSS administration induced cytokine production compared to the Blank group (Fig. 4).

The densities of positive and negative control spots in this kit were almost equal, indicating that this method is suitable for analysis of cytokine content. Inflammatory cytokine levels did not increase markedly with DSS administration, while the levels of IL-1α and IL-16 were increased. The levels of anti-inflammatory cytokines were unchanged. On the other hand, chemokines (MIG, IP-10)
and adhesion molecules (C5/C5α, sICAM-1) were induced by DSS administration. The levels of other inflammatory proteins did not show much increase. Only TIMP-1, a tissue inhibitor of metalloproteinases, showed increased levels. Dietary GlcCer recovered these cytokines to levels similar to those in the Blank group. Detailed analysis indicated that DSS administration induced inflammatory cytokines but not anti-inflammatory cytokines. Moreover, dietary sphingolipids suppressed the induction of inflammatory cytokines and chemokines induced by DSS administration.

Our findings suggest that colon inflammation was suppressed by dietary GlcCer in DSS-treated mice. DSS, a mucopolysaccharide, induced damage of colon epithelial cells after oral administration. Damage to the barrier system of the mucosa allowed DSS to reach the lower layer of mucosa, where it was phagocytosed by antigen-presenting cells. Activated antigen-presenting cells in turn induced the activation of T cells, resulting in a phenomenon similar to IBD. Dietary GlcCer suppressed the reduction of body weight, as observed in the early phase of DSS administration. In this study, the added amount of GlcCer was 0.1% (1 g/1 kg experiment diet). This amount was essentially the same as that used in an earlier study. Yunoki et al. reported an analysis of sphingolipid contents in sample Japanese meals. They reported that the total amount of sphingolipids in typical high- and low-calorie meal samples for healthy Japanese human subjects was 50–200 mg/day. The dose of GlcCer in the current study was about 5-10 fold higher than the level of intake/day found for healthy Japanese human subjects. Thus, the administration level of GlcCer in this study may represent a reasonable level for functional food materials. The relationship between dietary sphingolipid and colitis has not been elucidated. Some reports have previously indicated the induction of colitis by dietary sphingomyelin, while some other reports indicated suppression of colitis by dietary sphingomyelin. In the present study, dietary plant (maize)-origin GlcCer was shown to suppress colitis. Previous studies were performed using sphingolipids of mammalian origin (galactosylceramide, GlcCer, and sphingomyelin). Several questions regarding digestion and metabolic destiny remain unanswered. However, dietary sphingolipid would be...
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...indicated that dietary sphingolipid also suppressed cytokine formation. It was noteworthy that most chemokines were regulated to levels equivalent to those in the Blank group. These cytokines induce chemotaxis of neutrophils. These results suggest that dietary sphingolipid suppressed the accumulation of neutrophils.

In general, the interaction between sphingolipid metabolites and inflammation occurs as follows. Sphingolipid bases hydrolyzed from complex sphingolipid or de novo synthesis are converted to sphingosine-1-phosphate, which then accelerate inflammatory reaction via sphingosine-1-phosphate receptors in particular, consists of mainly phosphate receptors. The present findings revealed the anti-inflammatory effects of dietary plant-origin GlcCer in the large intestine, suggesting possible anti-IBD activity.

Recently, the interaction between sphingolipids, especially sphingosine phosphorylation, and colitis has attracted the attention of several research groups. Maines et al. reported that an inhibitor of sphingosine kinase suppressed inflammation of the large intestine. maize-origin GlcCer, in particular, consists of mainly cis-8 unsaturated type sphingoid base (trans-4,cis-8-sphingadenine). Generally, cis-type double bonds change physical properties compared to trans-type double bonds. Thus, plant sphingoid bases, especially cis-8-type, may show different regulation of sphingosine-related enzymes. Future studies should focus on analyzing the substrate specificity of sphingosine kinase. The present findings revealed the anti-inflammatory effects of dietary plant-origin GlcCer in the large intestine, suggesting possible anti-IBD activity.

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