Extraction of Lipophilic Fraction from Polished Rice Improves Its Ameliorative Effect on Intestinal Impairment

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Abstract: Glucosylceramide (GlcCer), a major sphingolipid in plants and fungi, is known to have food functions such as preventing intestinal impairment and enhancing the moisture content of skin. However, there is little information about functions of GlcCer in food sources as most of the studies on GlcCer functions are done using purified GlcCer. This study was performed to investigate the effects of GlcCer contained in food on intestinal impairment; polished rice flour (RF) and this ethanol extract (RE) were used as sources of GlcCer, and these were evaluated by studying the formation of aberrant crypt foci (ACF) in 1,2-dimethylhydrazine (DMH)-treated mice, which is a model of colon cancer. Mice were fed with either a control diet, a RF diet where RF replaces cornstarch (150 g/kg), or a plus RE diet (0.5 g/kg; RE was extracted from the same amount of RF present in the RF diet). The amount of GlcCer was similar in both the RF and RE diets (3.0 and 2.7 mg/kg, respectively). DMH treatment induced the formation of ACF and the production of inflammation-related cytokines. Both dietary RF and RE suppressed ACF formation and RE, in particular, showed a significant suppressive effect. Dietary RE inhibited the production of almost all of the inflammation-related cytokines studied, while RF suppressed only a few of these cytokines. The present study suggests that the lipophilic fraction including GlcCer, present in polished rice has protective effects against intestinal impairment, but it requires extraction since digestion alone is not enough to elicit its complete protective action.

Key words: polished rice, extract, glucosylceramide, colon cancer, colon inflammation

1 Introduction

Recently, the incidence rate of intestinal impairments, such as colon cancer and inflammatory bowel disease (IBD), has increased in East Asian countries, including Japan, while that in the Western countries still remains high. It is difficult to recover completely from IBD and these patients have an increased risk of developing colon cancer. In our previous research, we had suggested that the induction of inflammation-related cytokines has a connection with the formation of aberrant crypt foci (ACF), which are precursors of colon cancer. Epidemiological studies indicate that colon cancer is strongly associated with dietary intake and these inflammation-related diseases can be prevented through diet and supplements.

Glucosylceramide (GlcCer) is a sphingolipid found on the eukaryotic cell membrane, majorly in plants and fungi. The GlcCer molecule possess a sphingoid base with an amide-linked fatty acid (i.e., a ceramide) and a glucose. Plant GlcCer administration has been reported to prevent atopic dermatitis and improve skin-barrier function. Our previous studies showed that dietary GlcCer from plant sources alleviates colon inflammation in the IBD mice model treated with dextran sulfate sodium (DSS) and suppresses colon ACF formation and inflammation-related cytokine production.

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production induced by 1,2-dimethylhydrazine (DMH)\textsuperscript{4}. \textit{In vitro} experiments indicate the possibility that GlcCer protects the colon surface from the harmful effects of various drugs\textsuperscript{9}. In addition, sphingoid bases of plant origin have been shown to have an apoptosis-inducing effect on colon cancer cells \textit{in vitro}\textsuperscript{10}.

According to previous studies, we ingest an average of 26–77 mg/day of GlcCer from our diet\textsuperscript{11,12}. On the contrary, some studies report that an intake of 0.6–1.8 mg/day of GlcCer from supplements, such as extracts from rice or konjac, is enough to improve human skin health\textsuperscript{13,14}. There has been a lot of discussion about why supplementation with a much lesser amount of GlcCer than that obtained from diet can show this beneficial effect. Plant cell membrane contents such as glycolipids, including GlcCer, are difficult to extract due to the thick cell wall and therefore, animals may not be able to utilize these while digesting food of plant origin.

The purpose of this study was to evaluate our hypothesis about bioavailability of GlcCer from plant-based food sources. We investigated whether dietary polished rice and its ethanol extract, which contains GlcCer, alleviates intestinal disease, and whether these effects depend on the existence form of GlcCer. Mice treated with DMH were used as animal models of ACF, which are precursors of colon cancer, and the effects of dietary polished rice and its ethanol extract were evaluated by studying colon ACF formation and cytokine levels. In addition, we analyzed and discussed the relationships between GlcCer and other lipophilic functional components.

### 2 Experimental Procedures

#### 2.1 Preparation of rice flour (RF) and rice ethanol extract (RE)

We purchased polished rice \textit{(Oryza sativa cv. Koshikihari, polishing rate 90\%)} from a local market in Hokkaido, Japan. The rice was cooked using a rice cooker, freeze-dried, and crushed into rice flour (RF). The ethanol containing water was selected as the extract solution which can be applied as a food source because the cell membranes have been destroyed. RF was stirred in 80\% ethanol at 20°C for 1 h. After filtration, the residue was treated in the same way by using 90\% ethanol and 80\% ethanol successively. The amount of diluted ethanol used each time was three times the amount of RF. The filtrates were combined and dried using a rotary evaporator. The product was used as the rice ethanol extract (RE) and stored at −30°C until further use.

#### 2.2 GlcCer analysis in RF and RE

The levels of GlcCer in RF and RE added to experimental diets were analyzed (Table 1). We used the Folch procedure to extract total lipids from RF and RE. The total lipids were saponified with 0.4 M KOH in methanol at 38°C for 2 h to obtain the sphingolipid fraction. The amount of GlcCer was determined using thin-layer-chromatography (TLC) and high-performance-liquid-chromatography with evaporative light scattering detector (HPLC-ELSD)\textsuperscript{17}. The standards used for GlcCer and sterlyglycoside (SG) were prepared from maize by applying the extract onto a silica gel column and preparative TLC as described previously\textsuperscript{8}.

#### 2.3 Analysis of other lipophilic components in RF and RE

We quantified the levels of the other lipophilic components in RF and RE (Table 1). Ferulic acid (FA) levels were analyzed by modifying the previous method\textsuperscript{10}. The detection was done by HPLC with fluorescence detector (FLD; excitation 390 nm, emission 450 nm). Total FA including the esterified and the free forms were obtained by alkali saponification, while free FA was obtained by ethanol extraction. Tocotrienol (T3) composition was measured by alkaline hydrolysis and HPLC-FLD (excitation 294 nm, emission 326 nm)\textsuperscript{10}. Total fatty acid composition was determined by gas chromatography after direct methylation\textsuperscript{17}.

#### 2.4 Animals and their diet

Male BALB/c mice were obtained from Japan SLC, Inc. (Shizuoka, Japan) at 4 weeks of age, and housed in isolator cages at 22°C under a 12 h light/dark cycle. The mice were randomly divided into four groups (n = 7–8 in each group). The details of the diets of the various groups were as follows: blank group: control diet with intraperitoneal (i.p.) vehicle; control group: control diet with i.p. DMH; RF group: diet mixed RF instead of cornstarch with i.p. DMH; and RE group: plus RE diet with i.p. DMH. In addition, each of these groups was subdivided into two subgroups (n = 3–4) to determine either the ACF formation or cytokine levels in the colon. After acclimation to the experimental diet for 2 weeks, each mouse was administered i.p. 15 mg/kg body weight of DMH-HCl (Tokyo Chemical Industry Co., Ltd., Japan) once a week for 7 weeks\textsuperscript{18}. The control diet

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Contents of lipophilic substances in RF and RE.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RF 100 g</td>
</tr>
<tr>
<td>GlcCer, μmol</td>
<td>2.65</td>
</tr>
<tr>
<td>FA, μmol</td>
<td>46.7(24.3)\textsuperscript{b}</td>
</tr>
<tr>
<td>T3, μmol</td>
<td>1.72</td>
</tr>
<tr>
<td>Total fatty acid, μmol</td>
<td>1472</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>27.9</td>
</tr>
</tbody>
</table>

GlcCer: glucoclyceramide, FA: ferulic acid, T3: tocotrienol
\textsuperscript{a} 360 mg of RE was prepared from 100 g of RF.
\textsuperscript{b} Figures in parentheses show the contents of free FA.
was based on AIN-76, which does not contain sphingolipids. The RF group was given RF (150 g/kg) instead of cornstarch, and the RE group diet was supplemented with RE (540 mg extracted from 150 g of RF, i.e., 0.05%). All protocols involving animals were approved by the Animal Care and Use Committee and conducted in accordance with the Obihiro University Guidelines.

2.5 ACF identification

ACF from the large intestinal crypts were identified and quantified as previously described. The large intestine was excised from the mice under pentobarbitone anesthesia, and a portion of the intestine from the cecum to the vent was separated to the cold saline. This was followed by cell fixation overnight in PBS containing 4% paraformaldehyde and stained with 0.3% methylene blue solution in saline for 30 min at room temperature. ACF were counted throughout the large intestine under a microscope. ACF and AC in colon were divided by AC degree and counted; AC1, AC2, and AC3 indicate ACF formed by 1 crypt, 2 crypts, and 3 or more crypts, respectively.

2.6 Cytokine array assay

Cytokine secretion was examined using a Cytokine Array Kit (Mouse Cytokine Array Panel A; R&D Systems, Minneapolis, MN). Briefly, the colon mucosa was scraped, homogenized in PBS containing proteinase inhibitors (Protease inhibitor cocktail set III, Fujifilm Wako Pure Chemical Corp., Japan), and Triton X-100 was added. According to the manual, after freezing and thawing, the cytokines in the sample were bound to each antibody on the membrane, and the intensities were detected by chemiluminescence. The analyzed cytokines were as follows: B lymphocyte chemoattractant (BLC); chemokine (C-C motif) ligand 1 (CCL1); CCL3, CCL4, CCL5, CCL6, CCL7, CCL10, CCL11, CCL12, CCL13, CCL14, CCL16, CCL19, CXCL12, IL-1alpha, IL-1beta, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-16, IL-17, IL-23, and IL-27; and macrophage colony-stimulating factor (M-CSF), interferon-gamma (IFN-gamma); interferon-gamma-induced protein 10 (IP-10); interleukin-1beta (IL-1beta); IL-18; IL-27; matrix metalloproteinase inhibitor 1 (TIMP-1); monocoyte chemoattractant protein 2 (MCP-2); macrophage inflammatory protein-1 alpha (MIP-1alpha); monocyte chemoattractant protein-1 (MCP-1); monocyte-specific cytokine MCP-1 (JE); macrophage colony-stimulating factor (M-CSF) complex; neutrophil-activating protein 3 (KCN); 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-alpha (TNF-alpha).

2.7 Statistical analysis

The results in Tables 1 and 2, and Fig. 3 are expressed as mean ± standard deviation. The data in Tables 1 and 2 were analyzed by using variance analysis and Scheffe’s test. Further, the data in Fig. 3 was evaluated by Student’s t-test. In all analyses, differences were considered statistically significant when p<0.01 or p<0.05.

3 Results

3.1 The composition of the lipophilic fraction in RF and RE

Table 1 shows amounts of lipophilic components in RF and RE that were added to experimental diets. It is known that FA and T3 have anti-inflammatory and anticarcinogenic effects, and a low n-6/n-3 ratio in diets suppresses inflammation.

We obtained 360 mg of RE from 100 g of RF. Figure S1 shows RF and RE contents measured by TLC, after saponification. The amount of RE was almost identical to RF. HPLC-ELSD analysis revealed that the efficiency of GlcCer extraction by ethanol was 90% and the RF diet and plus RE diet contained 3.0 and 2.7 mg/kg of GlcCer, respectively. The extraction efficiencies of total FA including the esterified form, T3, and total fatty acids were 63, 9, and 40%, respectively, while that of free FA was 100%. Gamma-T3 was the prominent T3 species in RF (67%) and RE (90%). The predominant fatty acids in RF and RE were 18:2n-6, 18:1n-9, and 16:0.

Table 2  Body, liver and spleen weights, and colon length of DMH-treated mice.

<table>
<thead>
<tr>
<th></th>
<th>Final body weight (g)</th>
<th>Liver weight (g)</th>
<th>Spleen weight (g)</th>
<th>Colon length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>28.1 ± 3.0</td>
<td>1.06 ± 0.03</td>
<td>0.10 ± 0.01</td>
<td>10.5 ± 1.2</td>
</tr>
<tr>
<td>Control</td>
<td>25.1 ± 0.8</td>
<td>1.04 ± 0.06</td>
<td>0.11 ± 0.01</td>
<td>10.4 ± 0.9</td>
</tr>
<tr>
<td>RF</td>
<td>25.3 ± 2.3</td>
<td>0.92 ± 0.06**</td>
<td>0.11 ± 0.01</td>
<td>10.7 ± 1.0</td>
</tr>
<tr>
<td>RE</td>
<td>25.6 ± 1.3</td>
<td>0.97 ± 0.06</td>
<td>0.12 ± 0.01</td>
<td>10.4 ± 0.8</td>
</tr>
</tbody>
</table>

Mean ± standard deviation, n = 7–8.

** p<0.01 vs. Control group.
Table 3  Effects of dietary RF and RE on ACF formation and growth of AC in DMH-treated mice.

<table>
<thead>
<tr>
<th></th>
<th>AC1</th>
<th>AC2</th>
<th>AC3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.1 ± 0.1**</td>
<td>0.0 ± 0.1*</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.2**</td>
</tr>
<tr>
<td>Control</td>
<td>21.4 ± 8.3</td>
<td>9.3 ± 5.6</td>
<td>2.9 ± 2.7</td>
<td>34.0 ± 16.3</td>
</tr>
<tr>
<td>RF</td>
<td>12.2 ± 2.4</td>
<td>6.1 ± 1.3</td>
<td>1.2 ± 0.5</td>
<td>19.5 ± 4.0</td>
</tr>
<tr>
<td>RE</td>
<td>4.7 ± 2.6*</td>
<td>3.2 ± 1.9</td>
<td>1.0 ± 1.3</td>
<td>8.9 ± 5.5*</td>
</tr>
</tbody>
</table>

(Number of crypts/cm² colon)

Mean ± standard deviation, n = 3–4.
ACF and AC in colon were divided by AC degree and counted; AC1, AC2, and AC3 indicate ACF formed by 1, 2, and 3 or more crypts, respectively.
** p<0.01 *p<0.05 vs. Control group.

3.2 Body, liver, and spleen weights, and colon length of the mice
No differences in the final body, liver, and spleen weights, and colon length were noted between control and blank groups (Table 2). After 9 weeks of following either RF or plus RE diets with DMH treatment, there were no significant differences between control and other groups, except for the liver weight between RF and control groups.

3.3 Effects of dietary RF and RE on the incidence of DMH-induced ACF in mouse colon
Table 3 shows the degree and number of ACF in each experimental group. ACF and AC in colon were divided by the AC degree and counted; AC1, AC2, and AC3 indicate ACF formed by 1, 2, and 3 or more crypts, respectively (Fig. S2). The observed numbers of AC1, AC2, and total AC in the control group were significantly higher than those in the blank group (Fig. 1). In contrast, the number of AC1 in the RF group tended to decrease when compared...
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Effects of dietary RF and RE on the induction of inflammation-related cytokines in the colon of DMH-treated mice

We have shown the tendency of inflammation-related cytokine production in colon mucosa by DMH treatment and diets (Fig. 2). The cytokine array was performed using a pool of 2 mice, was repeated twice, and the average was indicated. Cytokines with over 1.4-fold change were identified as differentially expressed cytokines.

In the control group, treatment with DMH induced cytokine production when compared to the blank group with over 1.4-fold increase in production of IFN-γ, IL-13, IP-10, I-TAC, KC, JE, MIG, MIP-1α, MIP-2, M-CSF, and TIMP-1. On the other hand, over 1.4-fold decrease, IL-23 and IL-27 was also seen. Hence, we conclude that the levels of inflammation-related cytokines except anti-inflammatory cytokines increased with DMH administration. Notably, the levels of IP-10, I-TAC, and MIP-1α as chemokines were increased considerably. On the contrary, dietary RE suppressed the levels of most inflammation-related cytokines induced by DMH administration such as TNF-α, IFN-γ, IL-7, IL-13, IL-16, I-309, IP-10, I-TAC, KC, JE, MIG, MIP-1α, IL-4, M-CSF, TIMP-1, and TREM-1, by almost over 1.4 fold. On the other hand, dietary RF suppressed the levels of only those cytokines that were strongly induced by DMH; I-TAC and TIMP-1 by over 1.4-fold decrease while increasing the levels of IL-1β, IL-2, IL-27, and GM-CSF by over 1.4 fold.

Further, to explain the differences between cytokine production by dietary RF and RE, we compared the RF and RE groups in further detail (Fig. 3). The RE group showed significantly lower values for 17 cytokines including TNF-α out of the 33 cytokines measured. The levels of IL-13 and GM-CSF in RE group tended to be lower (**P < 0.01 and 0.05 vs. RF group. Columns and error bars indicate mean and standard deviation, respectively. n = 4.

4 Discussion

Dietary GlcCer is known to have several food functions, e.g., prevention of intestinal impairment, improving skin moisture, and relieving atopic dermatitis. However, there is little information about the functions of GlcCer from food sources. In this study, we investigated whether GlcCer in polished rice and the GlcCer extracted from its products would affect ACF formation and the levels of inflammation-related cytokines in the colons of DMH-treated mice. In these experiments, dietary RE, which is the lipophilic fraction extracted from polished rice, suppressed ACF formation induced by DMH treatment, while dietary RF, derived from crushed polished rice, showed less suppressive effect than RE (Table 3). In addition, dietary RE suppressed most of the inflammation-related cytokines induced by DMH, while dietary RF suppressed only a few cytokines (Fig. 2). In our previous study performed on DMH-treated mice, we have shown that dietary GlcCer suppressed ACF formation and cytokine levels. The patterns of cytokine suppression by RE in this study are very similar to those by GlcCer. Therefore, we suggested that suppression of ACF formation by RE might be related to the GlcCer content in RE. Although RF contained higher levels of GlcCer, FA, and T3 than RE, RF showed less suppressive effect on ACF formation and inflammation-related cytokine production (Tables 1 and 3, and Fig. 3).
known that GlcCer is a component of rice cell membrane, and because of the presence within the cell wall, it is not able to display its complete activity in the colon during digestion.

The GlcCer level in RE was close to that in RF, while the levels of FA and T3 in RE, especially T3, were much lower than those in RF (Table 1). FA exists in ester-forms mainly along with arabinoxylans in the cell walls. T3 is localized on rice bran and it mainly exists in the free form, while T3 in polished rice is at a much lower level than rice bran and it is mainly in the ester-form. Glycosides from the FA ester-form can be easily hydrolyzed, therefore, RF and RE had free FA released during the rice cooking process. On the other hand, the T3 ester-form needs saponification with high concentration of a strong base to release free T3 and T3 is non-polar compound. Therefore, RE contained a small amount of T3.

In this study, although the GlcCer content in plus RE diet was much less compared to the purified GlcCer content in the diet used in the previous study (1 g/kg diet), dietary RE showed almost the same anti-ACF and anti-inflammatory effects. RE also contains small amounts of FA and T3, which are known to have anti-cancer and anti-inflammatory properties (Table 1). It was recently reported that T3 controls the synthesis and metabolism of intrinsic sphingolipids, including GlcCer, in the colon and pancreatic cancer cells in vitro. Additionally, when both T3 and free FA are added to the colon and pancreatic cancer cells in vitro, intracellular T3 concentrations are higher than when T3 is added alone. This increases the apoptotic effects on cancer cells. Therefore, extrinsic GlcCer may act together along with the other components contained in RE to show additional and/or synergistic anti-cancer effects on colon. Further studies done with an in vitro model of the intestines are required to determine the molecular mechanisms underlying these effects.

In terms of suppression of inflammation by the lipophilic fraction, there have been many discussions about the n-6/n-3 ratio of the fatty acid composition in diets. The predominant fatty acid in both RF and RE is 18:2n-6 elevating the n-6/n-3 ratio in them. The lipid amount is much less than in corn oil used in diets (corn oil: 50 g/kg diet, n-6/n-3 ratio = 69). Hence, it is hard to determine if the fatty acid composition of RE influences the observed anti-ACF and anti-inflammatory effects.

Dietary RF was able to suppress only a few cytokines at a level comparable with RE (Fig. 2). The anti-ACF mechanisms of RF may differ from those of RE because of the difference in the suppression pattern of cytokines such as TIMP-1 and IL-27. In addition, only the RF group showed a significantly lower liver weight than the control group (Table 2). It has been reported that dietary resistant starch and alkali-extracted protein from rice ameliorate colon inflammation and improve lipid metabolism in the liver. Polished rice products, without their lipophilic fraction, might also have beneficial effects on DMH-treated mice. Thus, a processing method is required that enhances the properties of all the constituents of the lipophilic and hydrophilic fractions of rice.

Our in vitro and in vivo studies indicate that GlcCer purified from food sources protects the colon epithelium from a variety of stressors (DSS, DMH, LPS, and TNF-α). Dietary RE suppressed the initial stages of inflammatory response (Table 2), and our results support this view. Further, daily GlcCer intake in RE group was 0.4 mg per kg body weight, and this value is converted to 1.9 mg/60 kg for human using a formula based on the body surface area. Similar dosage of GlcCer was seen to be effective in improving the moisture content of human skin. Previous studies done on improving atopic dermatitis and skin moisture suggest that GlcCer intake activates enteric canal immunity and ceramide metabolism in the skin, rather than the direct reutilization of dietary sphingolipids. Thus, we conclude that the maintenance of intestinal homeostasis by dietary GlcCer might be indirectly related to these mechanisms.

In conclusion, the lipophilic fraction of polished rice containing GlcCer exhibits in vivo suppressive effects on ACF formation in DMH-treated mice. However, it is not usually bioavailable through digestion, and needs to be extracted beforehand. There is a possibility that the beneficial effect of the lipophilic fraction is not only due to the presence of one compound but due to the additional and/or synergistic actions by different components.

Supporting Information
This material is available free of charge via the Internet at http://dx.doi.org/jos.68.10.5650/jos.ess.19013

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