Inhibitory effect of rice bran-derived crude glycosphingolipid on colon preneoplastic biomarker lesions induced by azoxymethane in male F344 rats

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Abstract. The aim of the present study was to examine whether crude glycosphingolipid (cGSL) has short-term chemopreventive effects on the preneoplastic biomarker lesions involved in carcinogen-induced rat colon carcinogenesis. We also examined whether cGSL affects cell proliferation and apoptosis in these lesions. The crude preparation was obtained by the simple ethanol extraction method. Five-week-old male F344 rats were divided into 6 groups. Rats in groups 1-4 were given subcutaneous injections of azoxymethane (AOM) (20 mg/kg body weight) once a week for 2 weeks. Starting 1 week before the first injection of AOM, the rats in groups 2, 3 and 4 were fed a diet containing 250, 1,000 and 3,000 ppm cGSL, respectively, for 5 weeks. The experiment was terminated 5 weeks after the start date, and the number of aberrant crypt foci (ACF) and mucin-depleted foci (MDF) was counted. Dietary cGSL significantly inhibited the induction of ACF (group 3, P<0.01; group 4, P<0.001) and MDF (groups 2 and 3, P<0.001; group 4, P<0.005) as compared to group 1 treated with AOM alone. In groups 3 and 4, proliferating cell nuclear antigen-positive indices of epithelial cells were significantly lower than in group 1 (group 3, P<0.05; group 4, P<0.005). Caspase-positive indices were significantly higher in groups 3 and 4 than in group 1 (group 3, P<0.01; group 4, P<0.001). These results suggest that dietary cGSL had a potent chemopreventive effect in the present short-term colon carcinogenesis bioassays, and that this effect may be associated with the inhibition of ACF and MDF and the induction of apoptosis.

Introduction

Sphingolipids are a group of structural and bioactive derivatives that have a long chain backbone called a sphingoid base (sphingosine). These compounds have been regarded as inert structural components of cell membranes. However, in recent years sphingolipids have emerged as an important group of signaling molecules involved in cellular events such as immunoresponse, differentiation, cell proliferation and apoptosis. It was demonstrated that ceramide causes growth inhibition and induces morphological differentiation in HL-60 human myelocytic leukemia and neuroblastoma cell lines (1,2). Obeid et al reported that tumor necrosis factor-α stimulates the production of ceramide and the induction of apoptosis by the hydrolysis of sphingomyelin (3). Other reports have indicated that the proapoptotic effect of ceramide is induced by the activation of Fas or radiation (4,5). As a result, sphingolipids are attracting attention as a promising target of cancer chemoprevention and therapy.

Aberrant crypt foci (ACF) were proposed as colon preneoplastic lesions by Bird (6,7) and are widely used as reliable biomarkers of colon carcinogenesis (8). Mucin-depleted foci (MDF) are lesions observed as mucin defects in rat colonic mucosa stained with high iron diamin-Alcian blue, and their histological characteristics demonstrate dysplastic features (9). Additionally, MDF are significantly correlated with the development of colon tumors in azoxymethane (AOM)- or 1,2-dimethylhydrazine (DMH)-treated rats (9-11). Furthermore, both ACF and MDF are valuable biomarkers for short-term colon carcinogenesis bioassays, and are useful for the examination of possible chemopreventive effects of a wide variety of candidate agents (12,13).

Complex sphingolipids such as glycosphingolipid and sphingomyelin display biological activities similar to those of ceramide. Recent studies have shown that dietary intake of synthesized or naturally-occurring sphingomyelin and glycosphingolipids prevents colon carcinogenesis in DMH-treated or multiple intestinal polyposis (Min) mice (14-18).

Sphingolipids can be found in milk, eggs and soybeans; foods that are commonly consumed in everyday life as a dietary supplement. Although sphingolipids exhibit a variety
of biological activities, the precise mechanisms by which the complex causes tumor suppression are not known.

In the present study, we examined whether crude glycosphingolipid (cGSL) obtained from rice bran actually inhibits the development of carcinogen-induced preneoplastic lesions such as ACF and MDF in the rat colon. Furthermore, to investigate its mechanisms of action on cell proliferation and apoptosis, immunohistochemical analysis of proliferating nuclear cell antigen (PCNA) and cleaved caspase-3 was performed.

Materials and methods

Animals, diets and chemicals. Four-week-old male F344 rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). All animals were housed in wire cages (3 rats/cage) with free access to drinking water and the control diet (CE-2, Clea Japan Inc., Tokyo, Japan) under controlled humidity (50±10%), lighting (12-h light/dark cycle) and temperature (23±2°C) conditions. Composition of the CE-2 diet was water (8.6%), protein (24.9%), fat (4.6%), fiber (3.7%), ash (6.7%) and nitrogen-free extract (51.4%). cGSL was provided by Oryza Oil & Chemical Co. Ltd. (Owari-Ichinomiya, Japan), and was extracted from rice bran by the ethanol extraction method. High performance liquid chromatography (HPLC) analysis revealed that the cGSL contained monoglucosylceramide (8.6%), sterol (5.0%), triglyceride and diglyceride (26.4%), calcium carbonate (53.0%) and starch (7.0%).

Experimental design. Animal experiments were approved by the Animal Welfare Committee of the University of the Ryukyus. After being quarantined for 1 week, 42 male F344 rats were divided into 6 groups. Starting at 5 weeks of age, the rats in groups 1 to 4 (n=9/group) received AOM (Nard Institute Ltd., Hyogo, Japan, 20 mg/kg body weight, s.c. injection) once a week for 2 weeks. Rats in groups 2, 3 and 4 were fed a diet containing 250, 1,000 and 3,000 ppm cGSL, respectively, throughout the experiment. The concentration of cGSL was determined according to our previous study (19). Rats in group 5 (n=3) were fed a diet containing 3,000 ppm cGSL to examine the adverse side effects of the complex. Rats in group 6 (n=3) were fed the control diet alone and served as negative controls. At 5 weeks from the start date, all animals were euthanized under CO₂ anaesthesia. Colon tissues were removed, washed with saline, opened longitudinally and fixed with 10% buffered formalin.

Detection of aberrant crypt and mucin-depleted foci. The staining procedure for ACF and MDF was performed as described in our previous study (11). The fixed colon tissues were stained in an Alcian blue solution (Sigma Chemical Co., St. Louis, MO) for 5 min and immediately washed with distilled water, then placed on a glass plate with the mucosal side up. Using a light microscope at a magnification of x40, ACF and MDF were counted. Briefly, ACF were identified according to the following criteria: larger than and elevated above the adjacent normal crypts, with thickened cell walls lining the crypt and increased pericryptal area (11). MDF were identified as focal lesions characterized by the absence or very small production of mucins (11).

Immunohistochemical analysis. After ACF and MDF counting, colon tissues were rolled up, embedded in a Swiss roll form and subjected to immunohistochemical staining of PCNA and cleaved caspase-3. Immunohistochemical staining of PCNA is widely applied to examine cell proliferation (20). Caspase-3 is a pivotal apoptotic protein. Its active form, cleaved caspase-3, is generated by the proteolysis of its zymogen and exists only in the activated apoptotic cascade. Therefore, the detection of cleaved caspase-3 is a sensitive indicator of apoptosis (21). Sections (4-μm) were deparaffinized and rehydrated, then incubated in 3% H₂O₂ for 20 min in order to block endogenous peroxidase activity. Sections were boiled in 1 mM EDTA (pH 8.0) for 10 min in a pressure cooker. Subsequently, the sections were incubated with primary antibodies of PCNA (1:100 dilution, Dako Co., Ltd., Kyoto, Japan) or cleaved caspase-3 (1:200 dilution, Cell Signaling Technology Inc., Beverly, MA) at room temperature for 60 min, then secondary antibody treatment was performed using Histofine Simple Stain (Nichirei, Tokyo, Japan). Visualization of immunoreactivity was performed with the Liquid DAB Substrate Chromogen System (Dako Co., Copenhagen, Denmark). To determine the PCNA-positive (PCNA PI) and caspase-3-positive indices (caspase-3 PI), 15 visible crypts per colon were chosen, and at least 1,500 epithelial cells were counted. Positive indices were calculated as the percentage of positive cells with respect to the total number of cells counted.

Statistical analysis. One-way ANOVA was performed to analyze the data, and statistical differences were determined by Dunnet’s test. All statements of significance are P<0.05.

Results

General observations. A total of 42 rats survived to the end of the experiment, and none developed colon tumors. To ascertain whether the dietary administration of cGSL caused any toxic adverse side effects on body weight gain, the rats were monitored on a weekly basis. AOM and/or cGSL were not observed to have any significant effects on body, liver or kidney weight (data not shown). Dietary cGSL at 250, 1,000 and 3,000 ppm caused no symptomatic side effects in any of the rats.

Inhibition by cGSL of the occurrence of aberrant crypt and mucin-depleted foci. All rats in groups 1–4 developed ACF and MDF in their colonic mucosa. No ACF or MDF were observed in any of the rats in groups 5 and 6. The total number of ACF was significantly lower in AOM-treated rats fed a 1,000 and 3,000 ppm cGSL diet than in the control rats (1,000 ppm, P<0.01; 3,000 ppm, P<0.05; Table I), and the total number of MDF was significantly lower in AOM-treated rats fed a cGSL diet than in the control rats (250 ppm, P<0.001; 1,000 ppm, P<0.001; 3,000 ppm, P<0.05; Table I). The number of ACF or MDF that contained >4 crypts was not modified by the treatments. These results indicate that dietary cGSL inhibits the occurrence of ACF and MDF induced by AOM.

Inhibition of proliferating cell nuclear antigen-positive index by cGSL on the colonic epithelial cells. To determine whether cGSL affects cell proliferation in carcinogen-treated colonic
mucosa, we measured the PCNA PI in epithelial cells by immunohistochemistry (Fig. 1). As shown in Table II, treatment of rats with 1,000 and 3,000 ppm cGSL caused a significant decrease in the PCNA PI (1,000 ppm, P<0.05; 3,000 ppm, P<0.005) as compared to the control group (group 1). This inhibition occurred in a dose-dependent manner. The PCNA PIs of groups 1 and 6 were 5.28 and 3.25, respectively. In groups 2, 3 and 4, the PCNA PI was 3.99, 3.92 and 3.47 in AOM-treated rats fed 250, 1,000 and 3,000 ppm cGSL, respectively. These findings suggest that dietary cGSL normalizes the increase in cell proliferation induced by AOM.

Effect of cGSL on apoptosis. Since cGSL is able to normalize increases in cell proliferation in carcinogen-treated colonic epithelial cells, we examined whether this inhibition by cGSL is due to the induction of apoptosis. After the injection of AOM, treatment of rats with 1,000 and 3,000 ppm cGSL

Table I. Inhibition of the occurrence of aberrant crypt and mucin-depleted foci by crude glycosphingolipid.

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>Total no. of ACF</th>
<th>No. of ACF containing &gt;4 ACs</th>
<th>Total no. of MDF</th>
<th>No. of MDF containing &gt;4 ACs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM</td>
<td>138.2±24.8a</td>
<td>13.8±4.9</td>
<td>13.9±4.1</td>
<td>0.6±0.7</td>
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<tr>
<td>2</td>
<td>AOM+250 ppm cGSL</td>
<td>116.1±16.8</td>
<td>13.4±4.3</td>
<td>8.2±1.9b</td>
<td>0.3±0.5</td>
</tr>
<tr>
<td>3</td>
<td>AOM+1,000 ppm cGSL</td>
<td>97.9±17.7c</td>
<td>10.7±4.3</td>
<td>7.8±2.5b</td>
<td>0.4±0.5</td>
</tr>
<tr>
<td>4</td>
<td>AOM+3,000 ppm cGSL</td>
<td>105.9±37.6d</td>
<td>10.8±5.0</td>
<td>9.8±2.4d</td>
<td>0.3±0.7</td>
</tr>
<tr>
<td>5</td>
<td>3,000 ppm cGSL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</table>

*aMean ± SD. Significantly different from group 1: bP<0.001, cP<0.01, dP<0.05; Dunnet's test. cGSL, crude glycosphingolipid; ACF, aberrant crypt foci; MDF, mucin-depleted foci; ACs, aberrant crypts; AOM, azoxymethane.

Table II. Inhibition of the proliferating nuclear cell antigen-positive index by crude glycosphingolipid in colonic epithelial cells.

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>PCNA PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM</td>
<td>5.28±1.18a</td>
</tr>
<tr>
<td>2</td>
<td>AOM+250 ppm cGSL</td>
<td>3.99±0.74</td>
</tr>
<tr>
<td>3</td>
<td>AOM+1,000 ppm cGSL</td>
<td>3.92±0.35b</td>
</tr>
<tr>
<td>4</td>
<td>AOM+3,000 ppm cGSL</td>
<td>3.47±0.42c</td>
</tr>
<tr>
<td>5</td>
<td>3,000 ppm cGSL</td>
<td>3.29±0.50</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>3.25±0.34</td>
</tr>
</tbody>
</table>

*aMean ± SD. Significantly different from group 1: bP<0.05, cP<0.005; Dunnet's test. PCNA PI, proliferating nuclear cell antigen-positive index; cGSL, crude glycosphingolipid; AOM, azoxymethane.

Figure 1. Representative results of immunohistochemical staining of PCNA (A) and caspase-3 (B and C) in the rat colonic epithelium. PCNA immunoreactivity is positive in the nucleus (arrow heads). Caspase-3-positive cells are mainly localized in the upper half of the crypt (arrows), and immunoreactivity shows the cytoplasmic pattern at high power magnification (C).
caused a significant increase in the caspase-3 PI as compared to the control group (group 1) (1,000 ppm, P<0.01; 3,000 ppm, P<0.001; Table III). This increase occurred in a dose-dependent manner. These results indicate that cGSL induces apoptosis in colonic epithelial cells treated with AOM.

Discussion

Complex sphingolipids display a wide spectrum of biological activities including cell proliferation, differentiation and apoptosis. However, the precise mechanisms by which this complex exerts a cancer preventive effect are unknown. This study provides the first detailed examination of the inhibitory effects of rice bran-derived cGSL, which was extracted with safe solvents only, on 2 different categories of carcinogen-induced rat colon preneoplastic lesions, ACF and MDF. In the present study, we found that dietary administration of cGSL significantly inhibited the occurrence of ACF and MDF induced by the carcinogen AOM. We also found that the PCNA PI in colonic epithelial cells was inhibited in a dose-dependent manner by the treatment of rats with cGSL. Furthermore, dietary cGSL increased the caspase-3 PI in AOM-treated colonic epithelial cells. These findings suggest that cGSL prevents colon preneoplastic lesions, and that this effect may be associated with the inhibition of cell proliferation and the induction of apoptosis. Similar results have been reported in previous studies, which demonstrated that dietary intake of synthesized ceramide and milk-derived sphingolipids inhibited colonic cell proliferation in DMH-treated or Min mice (18,22). As well, sphingosine, sphinganine and C2-ceramide induced apoptosis in HT29 and HCT116 human colon carcinoma cell lines (23). These findings, together with the results of the current study, suggest that cGSL has cancer preventive potential in a short-term colon carcinogenesis bioassay.

In the present study, the suppressive effect of cGSL on the occurrence of ACF and MDF was not statistically significant at a lower dose (250 ppm). At higher doses (1,000 and 3,000 ppm), the inhibitory effect of cGSL on the occurrence of ACF and MDF was not dose-dependent. It is possible that a dose level of 250 ppm cGSL was not sufficient to suppress the formation of these lesions, while at 1,000 ppm cGSL had already reached its maximum effect. It seems likely that had we used a larger number of animals, and had the experiment continued for a longer period, the number of ACF or MDF would have decreased further in the high-dose cGSL-treated groups.

Ceramide, sphingosine and sphinganine are considered to play a pivotal role in tumor suppression (24,25). Schmelz et al have suggested that complex sphingolipids in food are hydrolyzed to ceramide in the intestinal tract and taken up by colonic cells, thus performing its biological activity (16,17). Most sphingolipids are hydrophobic, and are thus insoluble in aqueous solution. There have been no studies investigating the colon cancer preventive effect of cGSL, and no epidemiological data are available regarding the relation between the daily consumption of sphingoid-rich food (e.g., rice bran, milk, eggs and soybeans) and colon cancer risk. Therefore, to clarify whether its effect is confined to colon cancer requires further investigation. The ceramide-mediated signaling pathways involved in tumor suppression are extensive (24,25). The induction of apoptosis and the inhibition of cell proliferation are considered to be key events in tumor suppression (23,26). Molecules such as RB, Bcl-2, p53, mitogen-activated protein kinases and protein kinase C are the downstream targets of ceramide (24,25). Ceramide activates Bcl-2, and this proapoptotic protein eventually activates caspase family molecules through the release of cytochrome c from mitochondria (27,28). Schmelz et al demonstrated that sphingolipids normalize the localization of the β-catenin protein in the colonic mucosa (29). Aberrant expression of the β-catenin protein has been demonstrated in carcinogen-induced MDF in rats (10,11). We found that the development of MDF was inhibited by cGSL treatment, suggesting that the alteration of the signaling pathway via β-catenin may play an important role in the modulation of carcinogen-induced colon carcinogenesis. However, this aspect is in need of further study.

In summary, we demonstrated the inhibitory effect of dietary cGSL on 2 different biomarker lesions, ACF and MDF, in azoxymethane-treated F344 rats. The conceivable mechanism of action of cGSL might be the inhibition of cell proliferation and the induction of apoptosis in the colonic mucosa. With these considerations, cGSL may have had a potent chemopreventive effect in our short-term colon carcinogenesis bioassay system. Further studies are in progress to determine whether dietary cGSL suppresses tumor formation in long-term experiments.

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References


