ORIGINAL

Protective effects of glycoglycerolipids extracted from spinach on 5-fluorouracil induced intestinal mucosal injury

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Abstract : Glycoglycerolipids are mostly found in plants, however the beneficial effects of the glycoglycerolipids on mammalian body have not been understood. In this study, we investigated the effects of glycolipid extracts from spinach, which highly contained glycoglycerolipids, on mucosal injury induced by 5-fluorouracil (5-FU) in rats. Preadministration of glycolipid extracts from spinach (20 mg/kg body weight) prevented villous atrophy, misaligned crypts, and increased inflammatory cytokines in rat jejunum treated with 5-FU (300 mg/kg body weight) compared with the extracts from soybean. The glycolipid extracts from spinach highly contained monogalactosyl-diacylglycerol (MGDG) and diglactosyl-diacylglycerol (DGDG). In Caco-2 cells, MGDG and DGDG inhibited the production of reactive oxygen species induced by phorbol ester. We concluded that glycolipid extracts from spinach has anti-oxidative and anti-inflammatory effects, and the extract may be useful for prevention of drug-induced mucosal injury and other inflammatory diseases. J. Med. Invest. 57 : 314-320, August, 2010

Keywords **:** *glycoglycerolipid, 5-Fluorouracil, mucositis, oxidative stress*

INTRODUCTION

Glycolipids are divided in two classes, one is glycosphingolipids, and the other is glycoglycerolipids. The former is a constituent of plasma membrane of mammalian cells (1), the latter is mostly found in plastid thylakoid membrane of higher plants (2, 3). Sphingolipids play variety roles in mammalian cells. For instance, sphingolipids are major molecules consist of membrane microdomains such as caveolae and lipid rafts (4). Their metabolites such as sphingosine, sphingosine 1-phosphate, and ceramides are utilized as signal transduction molecules or biofactors (5). On the other hand, only a little is known about the function of glycoglycerolipids in mammals (6-8).

Some plants and seaweeds are good source of glycolipids (3). We have examined physiological effect of glycolipids from spinach because it is easy to prepare glycolipids from spinach at low cost and efficiently (9). Glycolipids extracted from spinach mainly include mono-galactosyl-diacylglycerol (MGDG), digalactosyl-diacyl glycerol (DGDG) and sulfoquinovosyl-diacylglycerol (SQDG) [9]. In previous studies, it is reported that these glycoglycerolipids can inhibit cell proliferation by decreasing DNA polymerase activity and have anti-tumor effect (10). On the other hand, glycolipids from bacteria also have cell protective effect (11, 12). In this study, we found that plant glycoglycerolipids have preventive effect on gastrointestinal mucosal injury induced by anti-tumor drug in rat.

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MATERIALS & METHODS

Preparation of glycolipid extracts from spinach and soy beans.

Glycolipid extracts from spinach (SPN) were prepared according to established procedures as we reported previously (9). The extracts contained over 80% of glycoglycerolipids and the composition of three major glycolipids, mono-galactosyl-diacylglycerol (MGDG), di-galactosyl-diacylglycerol(DGDG) and sulfoquinovosyl-diacylglycerol(SQDG), were approximately 5 : 4 : 1(data not shown). For an experimental control, the extracts from soybeans (SOY) were prepared by the same method as the case of spinach. The extracts from soybean contained over 70% of phospholipids (most of these was phosphatidylcholine), and the rest was almost glycolipids (MGDG, DGDG and steryl glycosides).

Preparation of rat model for 5-FU-induced intestinal mucosal injury

7-week-old male Sprague-Dawley rats received AIN93G diet and water *ad libitum* during the experiments. Rats were randomly divided into four groups (control group (n=4), 5-FU group (n=8), 5-FU+SPN group (n=8), and 5-FU+SOY group (n=8). In the groups administrated with 5-FU, rats were orally administrated 5-FU (300 mg/kg, Sigma) on the day 5 as previously described (13). For the group administrated glycolipid extracts, rats were daily given glycolipid extracts from spinach (SPN) or soybean (SOY) at 20 mg/kgBW together with AIN93G diet from the beginning to the end of the experimental period. This study was approved by the University of Tokushima Animal Use Committee, and the rats were maintained according to guidelines of the University of Tokushima for care of laboratory animals.

Histochemical analysis

Rats were sacrificed under deep anesthesia and bleed the whole blood from the postcaval vein. The small intestine was excised and measured the unstretched length. Jejunal segment was rinsed in PBS (pH 7.4), incised longitudinally, Swiss-rolled and fixed in 4% paraformaldehyde for histochemical analysis (10 cm long). Segments of jejunum were placed in formalin for 24 h, dehydrated from 70% to 100% Ethanol gradually, penetrated xylene and embedded in paraffin wax. Tissue sections were cut at 4 μm and stained with haematoxylin and eosin.

Measurement of alkaline phosphatase (ALP) activity

Jejunal mucosal homogenate was appropriately diluted with PBS and was subjected to measurement of ALP activity. ALP activity was measured by using Lab assay™ ALP (WAKO, Osaka Japan) according to manufacture's protocol.

Real time Reverse Transcription-Polymerase Chain Reaction (Real-Time RT-PCR) analysis

Total RNA was isolated using Trizol (Invitrogen life technologies) from jejunal mucosal homogenate. Complementary DNA synthesis was performed by using 1st strand cDNA synthesis kit (Invitrogen life technologies) from 1 μg of the extracted total RNA. Then, we performed real-time RT-PCR with the specific primers and SYBR™ green dye using a LightCycler real-time PCR system (Roche Diagnostics, Tokyo, Japan). The forward (fwd) and reverse (rev) primer sequences are as follows : IL-1α (forward, 5'-TTTGTGAGTGCTCAGGGAGA-3' ; reverse, 5'-GAAAGCTGCGGATGTGAAGT-3'), TNF-α (forward, 5'-ATGGATCTCAAAGACAACCA-3' ; reverse, 5'-TAGAGCCACCAATCCACACA-3'), β-actin (forward, 5'-TGACAGGATGCAGAAGGAGA-3' ; reverse, 5'-TAGAGCCACCAATCCACACA-3'). The reaction mixture containing reverse-transcribed cDNAs was preheated for 10 min at 95°C to activate Taq polymerase. A 50-cyclic three-step PCR, consisting of a 10 s (denaturation) step at 95°C, a 15 s (annealing) step at 60°C and a 15 s (extension) step at 72°C, was performed. Throughout the real-time PCR analysis, product identities were confirmed by melting curve analysis and PCR amplification products were visualized on 2% agarose-ethidium bromide gels to ensure the proper amplification products as single bands. The quantification of each gene was relative to a standard curve generated from a serially diluted sample. The ratios of the amounts of target mRNA to the amount of the internal standard (β-actin) mRNA was shown as an arbitrary unit. We confirmed that the immobilization itself did not change the amounts of β-actin transcripts identified by real-time RT-PCR.

Cell Culture and NBT assay

MGDG and DGDG were dissolved in DMSO (10 mg/ml). 0.2% Nitroblue tetrazolium chloride (NBT) was prepared in Hank's buffered salt solution (HBSS) containing 1% of bovine serum albumin (BSA) by adding NBT powder (Invitrogen). Human colon carcinoma (Caco-2) cells were grown in

DMEM with 4,500 mg/L of glucose medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin, and 100 mg/ml of streptomycin, and were routinely passaged before the cells reached confluence. Caco-2 were pre-incubated with DMEM containing 1% of BSA with glycolipids overnight at 37°C, and incubated with 100 nM PMA, NBT solution and glycolipids for 2 hours. After incubation, cells were washed with warmed HBSS, then once with methanol, and air-dried. The NBT deposited inside the cells were then dissolved, by adding 30 mL of 2 M KOH to solubilize cell membranes and then by adding 35 mL of DMSO to dissolve blue formazan with gentle shaking for 10 min at room temperature. Absorbance at 620 nm was measured by a conventional micro-plate reader.

Statistical analysis

The data are expressed as the mean ± SEM. Statistical analysis was determined by analysis of variance (ANOVA). When the *F*-ratios were statistically significant ($P < 0.05$), mean values were compared by Fisher's PLSD test at a 5% significance level.

RESULTS

Effects of glycolipid extracts from spinach and soybean on changes of food intake, body weight and symptom of digestive tract in 5-FU treated rats

To investigate the effect of glycolipid extracts from spinach or soybean on the intestinal mucosal injury, we utilized the 5-FU induced mucosal injury model as previously reported (13). As same as previous report, administration of 300 mg/kg 5-FU to rats extremely decreased in food intake. The decreased food intake was not recovered in all groups until the end of experiment (Table 1). However, there is no significant difference in food intake among 5-FU-treated groups. Following diminished appetite, normal increases in body weight in 5-FU-treated rats were inhibited (Table 1). Thus, administration of glycolipid extracts was not able to ameliorate the diminished appetite and weight gain. On the other hand, the extracts from spinach and soybean ameliorated diarrhea (Table 1).

Effects of glycolipid extracts from spinach or soybean on morphological changes in 5-FU treated rats

As shown in Table 1, 5-FU treatment significantly reduced small intestinal length ($p < 0.01$, control group vs. the other groups). However, administration of glycolipid extracts did not improved (5-FU group vs. 5-FU+SPN or 5-FU+SOY group). Therefore, we examined the effect of the extracts on 5-FU-induced villous atrophy by histochemical analysis. As shown in Fig. 1, 5-FU treatment was markedly involved in villous atrophy (representative images were shown as control (Fig. 1A) and 5-FU (Fig. 1B)). Administration of glycolipid extracts from spinach ameliorated the villous atrophy and misaligned crypts in jejunum caused by 5-FU treatment

Table 1 Food intake change, body weight change, small intestinal length and frequency of diarrhea after oral administration of 5-fluorouracil(300 mg/kg) or saline in rats treated with 20 mg/kg/day glycolipid extracts from spinach (SPN), soybean (SOY) or vihicle for 7 days.

	treatment group			
	control	5-FU	5-FU+SPN	5-FU+SOY
food intake change (g)				
day1	20.2± 0.7	19.3± 0.8	19.8± 0.7	18.8± 0.9
day2	21.7± 0.8	20.6± 0.6	20.4± 0.6	18.2± 0.7
day3	21.8± 0.7	20.7± 0.6	20.9± 0.6	19.5± 0.7
day4	21.1± 1.1	19.7± 0.7	20.4± 0.7	18.8± 0.6
day5	20.7± 0.3	3.2± 0.7*	5.1± 0.9*	4.2± 0.7*
day6	21.2± 0.7	4.5± 2.18*	7.5± 1.64*	3.8± 2.0*
day7	19.3± 1.4	6.8± 1.5*	8.8± 1.5*	5.3± 2.2*
body weight change (g)				
day1	260.0± 3.9	250.0± 5.1	254.3± 4.4	252.1± 4.0
day5	284.7± 4.4	274.3± 4.6	282.0± 6.0	274.9± 4.9
day8	303.6± 5.1*	232.1± 7.3	244.8± 9.1	226.0± 7.1
small intestinal length (cm)	126.3± 1.3	105.4± 3.0*	108.9± 1.7*	107.0± 2.4*
frequency of diarrhea (%)	0	75	0	25

Values are means± S.E (control group ; n=4,other groups ; n=8) *$p < 0.01$ vs. control group.

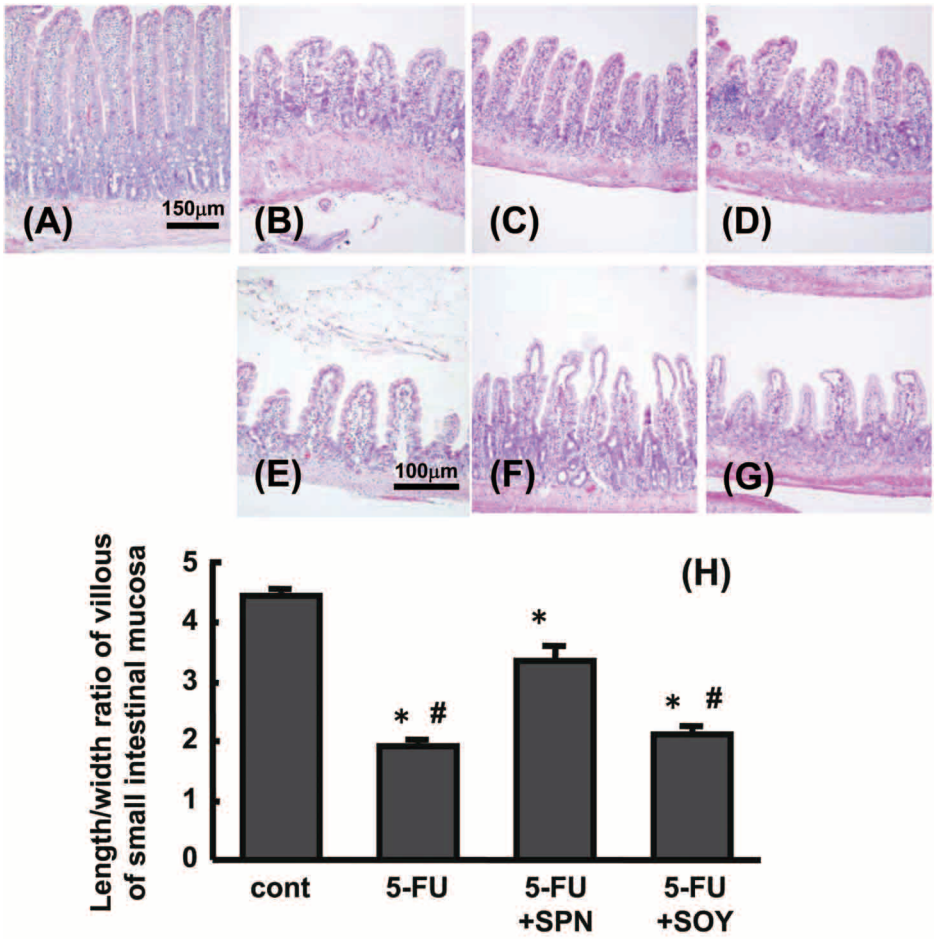

Fig. 1 Effects of glycolipid extracts from spinach or soybean on intestinal morphology in the 5-FU-induced mucositis rats. Rats were fed diet contained 20 mg/kg/day of glycolipid extracts from spinach (SPN) or soybean (SOY) for 7 days. On day 5, the rats were orally administrated 300 mg/kg of 5-FU (5-FU). Small intestine were subjected to HE staining (A-G) and estimated length/width ratio of villous (H). A : control, B and E : 5-FU, C and F : 5-FU+SPN, D and G : 5-FU+SOY. Values are mean± SEM. *$p < 0.01$ vs. cont, #$p < 0.01$ vs. 5-FU+SPN

(Fig. 1C), but the effect of the extracts from soybean was not observed (Fig. 1D). For quantitative assessment, we estimated the length/width ratio of villous in jejunum. As shown in Fig. 1E, 5-FU treatment significantly decreased the length/width ratio, however administration of glycolipid extracts from spinach significantly prevented the decrease in the ratio. On the other hand, the preventive effect of the extracts from soybean was not observed.

Effect of glycolipid extracts from spinach on the decreased alkaline phosphatase activity by 5-FU treatment

From the above studies, glycolipid extracts from spinach can prevent small intestinal functions from 5-FU induced mucosal injury. Next, we investigated the effect of glycolipid extracts from spinach on the alkaline phosphatase which is a representative digestive enzyme localized on the mucosal surface. As shown in Fig. 2, 5-FU significantly decreased ALP

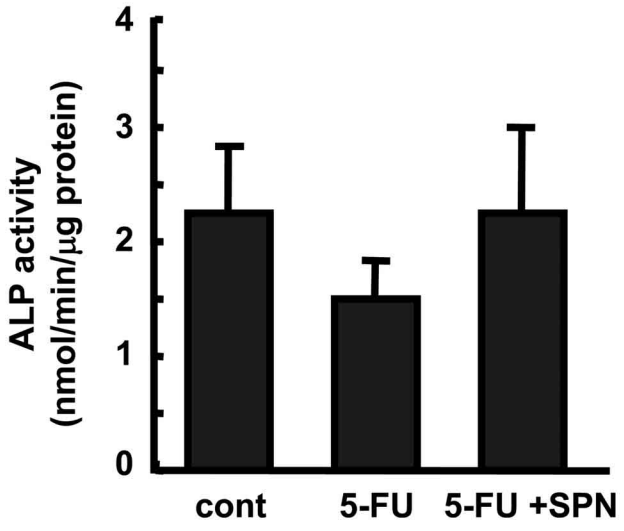

Fig. 2 Effects glycolipid extracts from spinach (SPN) on intestinal alkaline phosphatase activity in the 5-FU-induced mucositis rats. Rats were treated as same as figure 1. Small intestinal alkaline phosphatase (ALP) activity was estimated. Values are mean± SEM.

activity, however, administration of glycolipid extracts from spinach prevented 5-FU mediated reduction of ALP activity.

Inhibitory effects of glycolipid extracts from spinach on the 5-FU mediated induction of inflammatory cytokine mRNA expression

While 5-FU causes villous atrophy, inflammatory cytokines such as IL-1α and TNF-α have been induced. Thus, we examined whether the glycolipid extracts can inhibit the 5-FU induced gene expression of IL-1α and TNF-α in the jejunal mucosa by quantitative real-time RT-PCR. Compared with control group, 5-FU treatment significantly increased both IL-1α and TNF-α mRNA expressions (Fig. 3). On the other hand, administration of glycolipid extracts from spinach significantly inhibited the induction of IL-1α mRNA, and tend to decrease the TNF-α mRNA expression despite statistical insignificance.

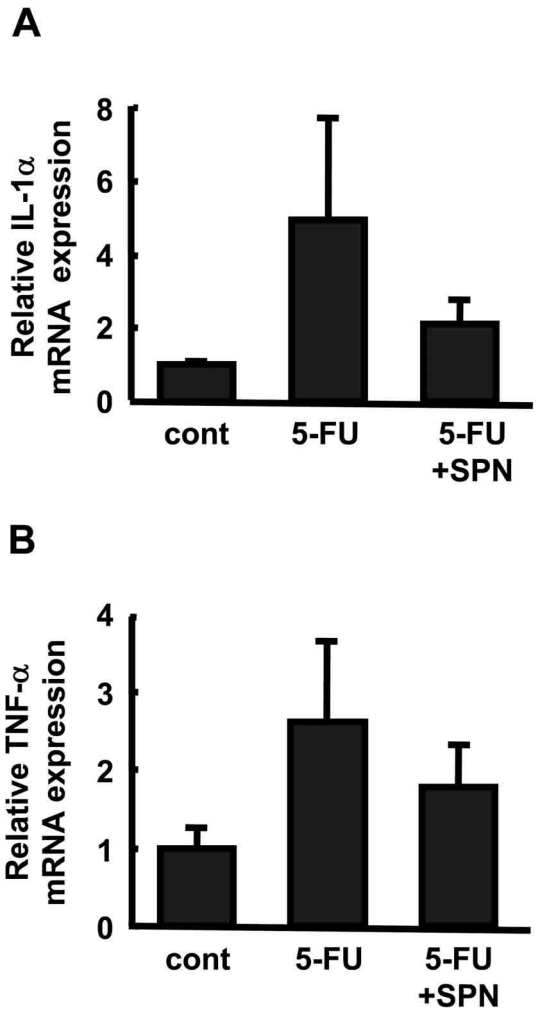

Fig. 3 Effects glycolipid extracts from spinach on IL-1α and TNF-α mRNA expressions in the 5-FU-induced mucositis rats. A : IL-1α, B : TNF-α. The amount of each target mRNA was normalized to the amount of the β-actin as internal standard mRNA, and then were calculated relative amount to control amount as 1.

Effect of glycoglycerolipids on the production of reactive oxygen species (ROS) in Caco-2 cells

Previous study demonstrated that oxidative stress would be a principal mediator of mucosal injury induced by 5-FU (14). Therefore, we investigated whether MGDG and DGDG, which are major components of glycolipid extracts from spinach, have anti-oxidant activity by NBT assay with Caco-2 cells. As shown in Fig. 4, PMA, a phorbol ester that can induce ROS production, increased ROS production in Caco-2 cells. Both MGDG and DGDG significantly inhibited the PMA-induced ROS production. The inhibitory effect of DGDG was much potent than that of MGDG. DMSO was used as solvent for PMA, but DMSO had no effect on ROS production at used concentrations.

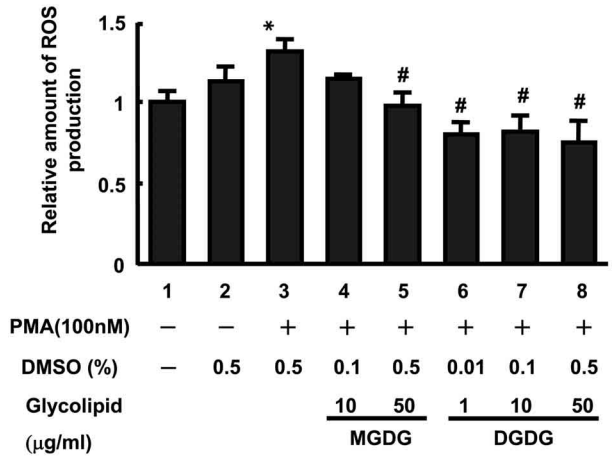

Fig. 4 Effects of MGDG and DGDG on PMA-induced reactive oxygen species (ROS) production in Caco-2 Cells. NBT assay was performed as described in Materials and Methods. Values are mean± SEM. *$p < 0.05$ vs lane 1, #$p < 0.05$ vs lane 3.

DISCUSSION

Glycolipid extracts from spinach ameliorated rat intestinal mucosal injury induced by 5-FU, but glycolipid from soybean had only small effect. Glycolipid extracts from spinach consist of the greater part of glycolipids and nearly 20% of phospholipids, whereas glycolipid extracts from soybean consist of over 70% of phospholipids. Therefore, the difference between spinach and soybean extracts in the ameliorative effect on the intestinal mucosal injury may be partly due to the composition of glycolipid

extracts.

5-FU has been widely used for treatment of advanced colorectal cancer. 5-FU inhibits thymidylate synthase and both RNA and DNA synthesis, and consequently induces marked apoptosis (15). In addition, several anticancer agents including 5-FU have shown to induce ROS generation in normal tissue as well as cancer cells (16-18). 5-FU induces mitochondrial ROS production in the p53-dependent pathway that induces mitochondrial ferredoxin reductase expression (14). Induction of ROS production is a major cause of intestinal mucosal injury. As shown in this study, MGDG and DGDG have anti-oxidative effect. Matsufuji et al. also reported that MGDG from *Bacillus subtilus* (M874B) is a radical scavenger and has a cell protective effect (11). On the other hand, there is no report that soybean phospholipids (mainly phosphatidyl choline) that are major components of glycolipid extracts from soybean can have radical scavenger activity or anti-oxidative activity according to our knowledge.

In addition, 5-FU can change the brush border membrane fluidity of the intestinal epithelial cells (19), and this is considered as a part of the mechanism of intestinal mucosal injury by 5-FU. Glycoglycerolipids may affect the membrane fluidity. Slomiany et al. reported that GM_1 ganglioside increased mucus gel viscosity and hydrophobicity, and protect from ethanol-induced gastric mucosal injury by the enhancement of physicochemical characteristics of mucus layer (20). MGDG and DGDG have sugar group and hydrophobic lipids as same as gangliosides. These characteristics of glycoglycerolipids may also contribute to ameliorate the mucosal injury by 5-FU. Further investigation is required to clarify the mechanism in detail.

In conclusion, glycolipid extracts from spinach have protective effects against mucosal injury by 5-FU. MGDG and DGDG are primary components of the extracts, and have anti-oxidative and anti-inflammatory effects. Glycoglycerolipids enriched extracts from vegetables may be useful not only for the prevention of drug-induced mucositis, but also for the amelioration of chronic inflammatory diseases such as inflammatory bowel disease.

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