

Polar Lipid Fraction from Golden Oyster Mushrooms (*Pleurotus citrinopileatus*) Suppresses Colon Injuries from Inflammatory Stresses *in vivo* and *in vitro*

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Abstract: The rising incidence of inflammatory bowel disease (IBD) in East Asian countries has necessitated the implementation of preventive methods in the form of dietary supplementation and changes in dietary habits. We have previously reported that dietary golden oyster mushroom (Pleurotus citrinopileatus) ethanol extract (GOMEE) suppresses intestinal inflammation in mouse models of IBD induced by dextran sulfate sodium salt (DSS). Here, we investigated the components of GOMEE that exert suppressive effects on colon inflammation in vivo and in vitro. The total lipid fraction was extracted from GOMEE, and the polar and neutral lipid fractions were subsequently separated via solvent fractionation. Mice were assigned to dietary groups-control, 1% total lipid, 1% polar lipid, or 1% neutral lipid diet-and fed the respective diets for one week; mice were administered 1.5% DSS in drinking water ad libitum for 20 days. Dietary supplementation with the total or polar lipid fraction alleviated DSS-induced chorionic crypt injury as determined by morphological observation, while dietary supplementation with the neutral lipid fraction did not produce such effects. In the in vitro study, using differentiated Caco-2 cells as the colon model, treatment with the total or polar lipid fraction suppressed cell decrease by lipopolysaccharide (LPS)-induced apoptosis whereas treatment with the neutral lipid fraction did not. Moreover, accumulation of glucosylceramide (GlcCer), a fungal sphingolipid, was observed in the intestinal cells after treatment with polar lipid fraction. These results suggest that the active components of GOMEE that suppress colon inflammation are polar lipids, especially GlcCer. The structure of mushroom GlcCer differs from that of the plant counterpart and is therefore expected to exert different food functions.

Key words: apoptosis, colitis, inflammation, mushroom, polar lipid, sphingolipid

1 Introduction

The incidence of intestinal impairments, such as colon cancer and inflammatory bowel disease (IBD), is increasing in East Asian countries, while in the Western countries it remains high^{1, 2)}. Complete recovery from IBD is difficult, and patients with this disease are at an increased risk of developing colon cancer³⁾. We have previously suggested that the induction of inflammation-related cytokines has a connection with the formation of aberrant crypt foci (ACF), which are precursors of colon cancer⁴⁾. Epidemiological studies indicate that colon cancer development is strongly associated with diet and thus, diseases related to inflammation in the colon can be prevented through dietary supplementation and changes in dietary habits⁵⁾.

Pleurotus citrinopileatus, commonly known as the golden oyster mushrooms has recently become a popular delicacy in East Asia due to its flavor and health benefits. This mushroom and its extract have been shown to exert various biological effects⁶⁻¹⁰. We have previously reported that dietary golden oyster mushroom ethanol extract (GOMEE) suppresses intestinal injury in a mouse model of IBD and that it is rich in sphingolipids, including glucosylceramide (GlcCer)¹¹.

GlcCer is found on the eukaryotic cell membrane, mostly in plants and fungi¹²⁾. The GlcCer molecule possesses a sphingoid base with an amide-linked fatty acid (i.e., a ceramide) and glucose. Dietary GlcCer from plant sources is reported to alleviate colon inflammation in the IBD mouse

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Accepted March 29, 2020 (received for review February 20, 2020) Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs models¹³⁾ and suppress colon ACF formation and inflammation-related cytokine production in the mouse models of colon cancer⁴⁾. *In vitro* experiments indicate that GlcCer protects the colon surface from the damage caused by various drugs¹⁴⁾.

The sphingolipid composition of mushrooms has been reported to differ from those of plants and mammals¹⁵⁾. The major sphingoid base in GlcCer is a 9-methyl-*trans*-4,*trans*-8-sphingadienine (9-Me d18:2^{*i*t,*8t*}), which is found only in fungi such as mushrooms. Moreover, the main sterol in mushrooms is ergosterol, which is not found in plants or mammals¹⁶⁾.

In this study, we investigated the components in GOMEE that exert suppressive effects on colon inflammation. The total lipid, polar lipid, and neutral lipid fractions were prepared from GOMEE. These lipid fractions were supplemented in the diets of mice with colitis induced by dextran sulfate sodium salt (DSS) (mode of colitis) and used to treat intestinal cells stimulated with lipopolysaccharide (LPS) (model of intestinal tract inflammation).

2 Experimental Procedures

2.1 Preparation of lipid fraction from GOMEE

GOMEE was purchased from Three-B Corporation (Sapporo, Japan). The total lipid fraction was extracted from GOMEE using the Folch method and separated to the polar lipid and the neutral lipid fractions by solvent fractionation with cold acetone (Fig. 1A)¹⁷⁾.

2.2 Animals and their diet

Male BALB/c mice were obtained from Japan SLC, Inc.

(Shizuoka, Japan) at 4-week old and housed in isolator cages at 22°C under a 12 h light/dark cycle. Mice were provided with tap water and CE-diets (CLEA Japan, Inc., Tokyo, Japan) ad libitum. After acclimation for one week, the mice were randomly divided into five groups(n=5). The blank and control groups were fed a control diet based on AIN-76, which does not contain sphingolipids¹³⁾. Groups of total lipid, polar lipid, or neutral lipid were each fed the AIN-76 diets containing 1% total lipid, 1% polar lipid, or neutral lipid. After following the experimental diet for one week, mice, excluding those in the blank group, were administered 1.5% DSS in drinking water. The mice were killed and dissected after 21 days. All protocols involving the animals were approved by the Animal Care and Use Committee and were conducted in accordance with the Obihiro University Guidelines (Permit Number, 29-196).

2.3 Histological analysis

The large intestines of the mice were excised under anesthesia using sodium pentobarbital (50 mg/kg BW, i.p.), and the portion from the cecum to the vent was removed and rinsed with cold saline. This was followed by an overnight cell fixation with 4% paraformaldehyde in phosphate-buffered saline. The specimens were subsequently embedded in paraffin wax, and transverse sections were cut and stained with hematoxylin and $eosin^{11}$.

2.4 Cell culture

Caco-2 cells, obtained from Riken Gene Bank (Tsukuba, Japan), were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, and 0.1 mM nonessential amino acids. For routine maintenance, Caco-2 cells on one dish were trypsinized and passaged to three new dishes, and



Fig. 1 Preparation (A) and profiles (B) of lipid fractions from golden oyster mushroom ethanol extract.
 1. Maize GlcCer fraction; 2. Total lipid fraction from GOMEE; 3. Hydrophilic fraction from GOMEE; 4. Polar lipid fraction; 5. Neutral lipid fraction. Mobile phase, Chloroform/Methanol (95/12, v/v); Detection, 50% H₂SO₄. TLC, thin-layer chromatography; GlcCer, glucosylceramides; SG, sterylglycosides; FFA, free fatty acids.

then maintained in culture to reach confluency. For spontaneous differentiation, the time at which the cells initially reached confluency, as determined by light microscopy, was designated as day 0, and the cells were transfected 21 days later.

2.5 Cell count

Caco-2 cells were seeded at a density of 2.0×10^5 cells/ mL in 24-well plates (Nunc, Rochester, NY) in 1 mL of culture medium and incubated for 21 days at 37°C and 5% CO₂. The medium was then replaced with the test medium containing 0.1% BSA instead of 10% FCS. LPS (50 µg/mL) as an inflammatory stimulator and lipid fractions were added for 48 h. After stimulation, cells were rinsed with PBS (Nissui, Tokyo, Japan), trypsinized, and counted using a counting chamber (improved Neubauer chamber, EM-Techcolor; Hirshmann, Eberstadt, Germany).

2.6 Apoptosis assay

Caco-2 cells were seeded at a density of 1.0×10^5 cells/ mL on Lab-TekTM Chamber Slides (Thermo Scientific, Waltham, MA) in 0.5 mL of culture medium, and subsequently treated as described above. Apoptotic cells were detected by characteristic fragmented nuclei with 4',6-diamidino-2-phenylindole (DAPI) staining, under a fluorescence microscope¹⁴.

2.7 Thin-layer chromatography (TLC) analysis

To determine the movement of exogenous lipid fractions, TLC analyses of cells, cell surface, and medium were conducted¹⁴⁾. Briefly, after 48-h cell incubation with 50 μ M of each lipid fraction in medium or medium only, the total

lipids in cells, on the cell surface, and in the medium were extracted. The alkaline-stable lipid fraction was prepared and subjected to TLC with 95:12 (v/v) chloroform/methanol as the solvent.

2.8 Statistical analysis

Differences among all data groups were assessed by using one-way ANOVA with Fisher's least significant difference test; P values of <0.05 were considered to be statistically significant. All data were analyzed using BellCurve for Excel(Social Survey Research Information Co., Ltd., Tokyo, Japan) and expressed as means \pm standard error of the mean(SEM).

3 Results

3.1 Profiles of lipid fractions from GOMEE

The total lipid fraction was obtained at 75 wt% of GOMEE and was divided to 25 wt% polar lipid fraction and 49 wt% neutral lipid fraction of GOMEE (33 wt% and 65 wt% of the total lipid fraction, respectively). Lipid composition of each GOMEE fraction was determined by TLC (Fig. 1B). The major components of the polar lipid fraction were GlcCer and sterylglycoside (SG) derivatives, while those of the neutral lipid fraction were free and acyl sterols, free fatty acids (FFA), ceramides, and SG derivatives.

3.2 Effect of dietary lipid fractions from GOMEE on weight gain and other factors by DSS

Figure 2A shows the weight gain of mice during the ex-



Fig. 2 Effects of dietary lipid fractions on weight gain (A) and large intestinal villi (B) in DSS-treated mice. After feeding experimental diets for one week, mice were administered 1.5% DSS in drinking water for 20 days and dissected on day 21. The colons were stained with hematoxylin and eosin. 1. Blank (non-treated with DSS); 2. Control; 3. Total lipid group; 4. Polar lipid group; 5. Neutral lipid group. Mean \pm SEM (n = 5). ^{a,b}Values with different superscript letters in the same column differ significantly (p < 0.05). Body weight of each mouse at 0 day starting to drink DSS = 1.0. B. × 10 objective magnification. Scale bar = 100 µm. DSS, dextran sulfate sodium salt.

perimental period. Before DSS administration, body weight of animals in all groups increased daily. Although the addition of 1.5% DSS to the drinking water did not affect body weight for 12 days of treatment, body weights of the control group tended to decrease in thereafter. The body weights of mice from the groups administered the dietary lipid fractions of GOMEE were not significantly different from those of animals in the control group; however, animals in the dietary neutral lipid fraction-supplemented group showed significantly decreased body weight compared with that of blank animals.

In general, acceleration of colon inflammation induced colon constriction and swelling in the spleen. DSS administration significantly reduced the length of the large intestine and increased the spleen weight, and dietary lipid fractions of GOMEE did not significantly suppress these factors by DSS (data not shown). Because the dietary neutral lipid fraction reduced the body weight of DSS-treated mice at an early stage, the DSS administration period in this study was shorter than the 26-day period in our previous study on GOMEE¹¹ and there was no significant difference in the colon length or the spleen weight

among groups treated with DSS.

3.3 Effects of dietary lipid fractions of GOMEE on colon villus damage by DSS

To observe the status of the colon, we examined specimens under a microscope following hematoxylin and eosin staining (Fig. 2B). The blank group showed normal crypts in the mucosa layer, whereas the control group showed mucin depletion and the loss of epithelial cells due to DSS administration. Supplementation with the dietary total or polar lipid fraction from GOMEE alleviated injury to the chorionic crypts associated with DSS administration, while supplementation with dietary neutral lipid fraction did not.

3.4 Effects of lipid fractions of GOMEE on LPS-induced cell number decrease and apoptosis of intestinal cells

Under the culture conditions used herein, Caco-2 cells were adherent. Therefore, to determine cell viability and inflammatory stress, the numbers of adherent cells were assessed. Addition of LPS as an inflammation inducer decreased the number of adherent cells, and treatment with the total or polar lipid fraction of GOMEE suppressed this



Fig. 3 Effects of lipid fractions from GOMEE on the viability(A) and apoptosis(B and C) of differentiated Caco-2 cells under LPS-induced inflammation.

After cultivation for 48 h in the presence of LPS and/or 50 μ M of each lipid fraction, the number of viable cells were counted (A), and apoptotic bodies (characteristic of apoptosis initiation) were observed following DAPI staining (B) and counted (C). 1. Blank (non-treated with LPS); 2. Control; 3. Total lipid group; 4. Polar lipid group; 5. Neutral lipid group. A & C. Mean ± SEM (n=4).^{a,b,c,d}Values with different superscript letters in the same coulmn differ significantly (p < 0.05). Blank = 1.0. B. × 20 objective magnification. Scale bar = 50 μ m. LPS, lipopolysaccharide; DAPI, 4',6-diamidino-2-phenylindole.

decrease (Fig. 3A). Treatment with the neutral lipid fraction did not significantly suppress the LPS-induced cell numbers decrease. Without LPS treatment, the cell numbers in the group treated with the neutral lipid fraction were also lower when compared to the blank group (data not shown).

Induction of the apoptotic pathway leads to the formation of apoptotic bodies characterized by cell fragmentation around the nucleus. Cells in the blank group had normal sized nucleus, whereas the control group, treated with LPS, had normal and small nuclei (Figs. 3B and C). The total or polar lipid fraction of GOMEE suppressed the formation of small nuclei, while the neutral lipid fraction did not. Without the presence of LPS, treatment with the neutral lipid fraction induced the formation of small nuclei and the numbers of apoptotic cells significantly increased about fourfold (data not shown).

3.5 Accumulation of GlcCer in differentiated Caco-2 cells incubated with lipid fractions determined by TLC

To confirm localization of lipid components in additional lipid fractions, Caco-2 cells were divided to the medium, surface, and cell parts at 48-h after the addition of each lipid fraction. In the medium and cell parts, mushroom GlcCer from GOMEE was detected after the addition of polar lipid fraction (Fig. 4). Mushroom GlcCer was also detected in the medium and cell parts after the addition of total lipid fractions, but it was not observed in all the parts after the addition of the neutral lipid fraction (data not shown).

4 Discussion

Dietary golden oyster mushrooms and its hydrophilic fraction are known to exert several food functions⁶⁻¹¹⁾. We have previously reported that dietary GOMEE, which is mushroom lipophilic fraction, alleviated intestinal injury in mice with DSS-induced colitis via suppression of intestinal inflammation. In this study, we investigated the components of GOMEE that exert the suppressive effects on colon injuries from inflammatory stresses *in vivo* and *in vitro*. The polar lipid fraction of GOMEE alleviated DSS-and LPS-induced colon injuries *in vivo* and *in vitro*, respectively. However, the neutral lipid fraction did not exert suppressive effects *in vivo* or *in vitro*, but instead affected the mice and the cells adversely (Figs. 2, 3, and not shown data).

According to TLC analysis, the major component of the polar lipid fraction was GlcCer, whereas those of the neutral lipid fraction were FFA and ceramides (Fig. 1B). GlcCer from plants has been reported to suppress intestinal inflammation *in vivo* and *in vitro*^{13, 14)}. In this study, the polar lipid fraction containing GlcCer also suppressed intestinal inflammation *in vivo* and *in vitro* (Figs. 2 and 3). However, the main sphingoid base $(9-\text{Me d}18:2^{4t,8t})$ of mushroom GlcCer is different from that of plants, and the specific mushroom GlcCer was reported to exert different effects from plant GlcCer on the fluidity of phospholipid liposomes¹⁸⁾. GlcCer from golden oyster mushrooms localized in the cell portion (Fig. 4), while wheat GlcCer, which has 8-cis-sphingenine (d18:1^{8c}) as the main sphingoid base, localized in the cell surface portion¹⁴⁾. Therefore, GlcCer from golden oyster mushrooms may have a food function different from that of plant GlcCer.



In contrast, it is well-known that high level of FFA is cy-

Fig. 4 Accumulation of sphingolipids in differentiated Caco-2 cells incubated with polar lipid fraction determined by TLC. Cells were cultured for 48 h in the presence of 50 μM polar lipid fraction, and then fractionated (3, 4, and 5). Mobile phase, Chloroform/Methanol (95/12, v/v); Detection, 50% H₂SO₄. 1. Wheat GlcCer standard; 2. Medium incubated with polar lipid fraction of GOMEE; 3. Medium incubated with Caco-2 cells and polar lipid fraction; 4. Surface of the cells incubated with polar lipid fraction; 5. Cells incubated with polar lipid fraction; 6. Medium incubated with cells; 7. Surface of cells; 8. Cells.

totoxic¹⁹⁾ and ceramide is an apoptotic signal in cells^{20, 21)}. In addition, sphingosine-1-phosphate(S1P), a ceramide metabolite, is thought to be a therapeutic target for colitis because the inhibition of sphingosine kinase 1, which generates S1P from ceramides, reduces immune responses in DSS-induced colitis^{22, 23)} and the deficiency of ceramide synthase 6 protects against the development of colitis induced by an adoptive transfer method²⁴⁾. However, when extrinsic ceramides are added to cells to induce apoptosis, short-chain ceramides (C2 to 8) are used because ceramides usually have a chain length of C18 to 26 and low cellpermeability²⁵⁾. Therefore, it is thought that ceramides in the neutral lipid fraction, made only marginal contributions to the progression of intestinal inflammation. However, it is reported that a small part of long-chain ceramides can enter the intestinal cells²⁶; in addition, intestines treated with DSS have increased nonspecific cell permeability. Moreover, a part of ceramides is digested to sphingoid bases by the digestive enzymes and enteric bacteria, which induce apoptosis in weak cells (e.g. cancer cells and damaged cells)²⁷⁾. Overall, we think that an overdose of FFA and ceramides in the neutral lipid fraction adversely affected the intestinal condition.

In conclusion, the total lipid or polar lipid fraction of GOMEE exerts anti-inflammatory effects *in vivo* and *in vitro*. These results suggest that the active contents in GOMEE in the suppression of colon inflammation are polar lipids, especially GlcCer. The structure of mushroom GlcCer is different from that of the plant counterpart and is therefore expected to exert different food functions.

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Conflict of Interest

The authors declare no conflict of interest.

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