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Effects of dietary ethanol extract from fruiting bodies of golden oyster mushroom (*Pleurotus citrinopileatus*) on chronic colon inflammation in mice treated with dextran sulfate sodium salt

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Abstract

In recent years, the incidence of intestinal impairments, such as colon cancer and inflammatory bowel disease (IBD), has been increasing in East Asian countries. Healthy dietary habits, as well as taking dietary supplements, are seen as important to preventing these impairments. Mushrooms and the hydrophilic fraction are commonly recognized for multiple nutritious functions, while the function of the lipophilic fraction remains uncertain. In this study, we investigated the effects of the lipophilic fraction containing sphingolipids, extracted from *Pleurotus citrinopileatus* (golden oyster mushroom), on mice suffering from chronic colon inflammation by treatment of dextran sulfate sodium salt (DSS) as IBD models. In the experiment, mice were fed with (1) control AIN-76 standard diet or (2) AIN-76 diet supplemented with 1% ethanol extract from fruiting bodies of golden oyster mushroom (GOMEE) or (3) AIN-76 diet supplemented with 5% GOMEE. Subsequent to experimental diets for 10 days, mice were drinking water *ad libitum* supplemented with DSS. We found that in mice ingesting DSS for 26 days, dietary GOMEE suppressed the body weight reduction and the spleen weight increase by administration of DSS. Dietary GOMEE decreased DSS-induced chorionic crypt injury, and the ameliorative effect by 5% GOMEE diet was stronger when compared to the 1% GOMEE diet. Moreover, we researched the impact of GOMEE on the early/middle stage of inflammation in colon mucosa, by assessing levels of inflammation-related cytokines in mice ingesting DSS for 18 days. The levels of almost inflammatory cytokines and chemokines examined in the colon were significantly increased due to DSS ingestion. Dietary 5% GOMEE was correlated with a significant decrease in the levels of 5 inflammatory cytokines and 5 chemokines. These results suggested that dietary GOMEE contributes to suppression of colon inflammation and the effect is dose-dependent.

Key words: Colitis, Cytokine, Golden oyster mushroom, Inflammatory bowel disease, Sphingolipid

Introduction

In recent years, the incidence rate of intestinal impairments, such as colon cancer and inflammatory bowel disease (IBD), has increased in East Asian countries, including Japan, and remains alarmingly high in the Western countries[1, 2)]. IBD is a persistent disease difficult to treat and achieve full recovery. Moreover, IBD patients carry an increased risk of developing colon cancer[3)]. It is believed that colon cancer in IBD patients can be induced due to dedifferentiation-associated epigenetic regulations by chronic inflammation[4)]. Numerous epidemiological studies indicate that colon cancer is strongly associated with diet. Subsequently, it is suggested that these inflammation-related diseases can be prevented by following dietary habits and taking dietary supplements[5)].

In Japan in the 1960s, dietary mushrooms were introduced into the health food market, and there has been increasing research interest in the properties of *Agaricus blazei* Murill (Almond mushroom)[6)], *Hericium erinaceus* (Monkey head mushroom)[7)], *Grifola frondosa* (Maitake)[8, 9)], and *Pleurotus citrinopileatus* (Golden oyster mushroom)[10-15)]. Golden oyster mushroom recently became a popular delicacy in East Asia, including China, Japan, and Taiwan, due to its flavor and potential health benefits. Ingestion of whole fruiting bodies of golden oyster mushrooms, as well as extracts obtained from them, have been demonstrated to have a number of biological effects, including, antioxidative effects[10, 11)], cardiovascular disease prevention[11)], reduction of blood sugar levels[12)], and immune regulation[13, 14)]. In addition, numerous bioactive components have been isolated from the fresh fruiting bodies, e.g., novel lectin with potent antitumor, mitogenic, and HIV-1 reverse transcriptase inhibitory activities[15)].

Glucosylceramide (GlcCer) is a sphingolipid found on the eukaryotic cell membrane, mainly in plants and fungi. The GlcCer molecule consists of a sphingoid base with an amide-linked fatty acid (i.e., a ceramide) and one glucose. Administration of GlcCer derived from plants has been reported to prevent atopic dermatitis[16)] and

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improve skin-barrier function[17]. Our previous studies showed that dietary GlcCer from plant sources alleviates colon inflammation in the IBD mice model treated with dextran sulfate sodium (DSS)[18] and suppresses colon aberrant crypt foci formation and inflammation-related cytokine production induced by 1,2-dimethylhydrazine (DMH)[19]. *In vitro* experiments indicate the possibility that GlcCer protects the colon surface from the harmful effects of various drugs[20]. In addition, sphingoid bases induce apoptosis in colon cancer cells *in vitro*[21].

It has been shown that sphingolipids found in the lipophilic fractions extracted from mushrooms are different from those found in plants and mammals[22]. The main difference is 9-methyl-4,8-sphingadienine being the major sphingoid base in the structure of GlcCer found in mushrooms. It is well-unknown how the specific chemical structure of GlcCer is related to its function. Moreover, the main sterol found in the lipophilic fractions extracted from fruiting bodies of mushrooms is ergosterol, which is absent in plants or mammals[23]. This motivated our interest in the food functions of the lipophilic fraction extracted from fruiting bodies of golden oyster mushrooms.

In this study, we investigated the effects of ethanol extract from the fruiting bodies of golden oyster mushroom (GOMEE) - lipophilic fraction - on colon inflammation in DSS-treated mice, which served as a model of colitis. Prior to this experiment, we had determined the optimal DSS concentration for inducing chronic colon inflammation in mice. To research the role of dietary GOMEE in initiation of inflammation in the colon, we measured the expression of inflammation-related cytokines in colon mucosa at the early/middle stage of inflammation, when blood is not present in feces.

Materials and Methods

Materials

GOMEE was purchased from Three-bee Corporation (Hokkaido, Japan). Briefly, golden oyster mushroom (culture strain Elm Mush 291) was cultured in a 850 mL-size polypropylene bottle with 450 g of medium (containing 65% of water, and dried wheat bran and dried sawdust from *Larix kaempferi* (Japanese larch) mixed at 32 : 31 ratio). One culture bottle yielded 100 g of fruiting bodies. As shown in Fig. 1, after removing the hydrophilic fraction from 30 kg of fruiting bodies of golden oyster mushrooms, 210 g of GOMEE was obtained from the residue by 3 series of extractions with 150 L of ethanol. Lipid composition in GOMEE was analyzed on the silica gel thin-layer chromatography (TLC)[24]. We used the Folch procedure to extract total lipids from GOMEE. The total lipids were saponified with 0.4 M KOH in methanol at 38℃ for 2 h to obtain the alkali-resistant fraction. GlcCer, sterols, and ceramides were determined by the position and the sterol-specific dye. GlcCer standard was prepared from maize by applying to a silica gel column and preparative-TLC, as described previously[18]. All reagents used in the following experiments were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan) unless stated otherwise.

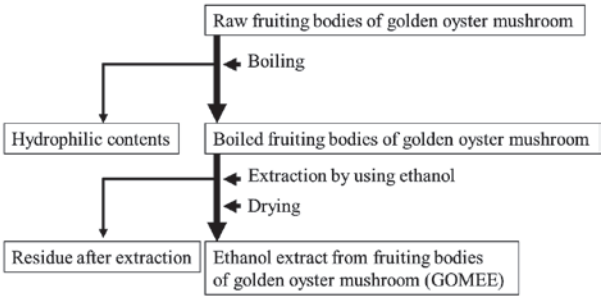

Fig. 1. Preparation of ethanol extract from fruiting body of golden oyster mushroom (GOMEE).

Animals and their diet

Male BALB/c mice were obtained from Japan SLC, Inc. (Shizuoka, Japan) at 4 weeks old and housed in isolator cages at 22℃ under a 12-hour light/dark cycle. Mice were provided tap water and CE-diets (CLEA Japan, Inc., Tokyo, Japan) *ad libitum*. After one-week acclimatization, mice were randomly divided into 4 groups (blank, control, and two test groups), with 10 mice in each group. The blank and control groups were fed control AIN-76 diet, which does not contain sphingolipids[24]. "1% GOMEE" test group was fed with AIN-76 diet supplemented with 1% GOMEE and "5% GOMEE" test group was fed with the AIN-76 diet supplemented with 5% GOMEE. After feeding the experimental diet for 10 days, all groups, except for the blank one, were administrated DSS using drinking water as a vehicle. Samples for the cytokine array assay were collected post mortem after 18 days of ingesting DSS, with 5 mice in each group. Samples for all other tests were collected after 26 days of ingesting DSS, with 5 mice in each group. All protocols involving work on animals were approved by the Animal Care and Use Committee and were conducted in accordance with the Obihiro University Guidelines (Permit Number, 28-164).

Sample preparation for histological analysis

The large intestine was excised under anesthesia, and a portion of the large intestine from the cecum to the vent was isolated and rinsed with cold saline. Next, the histological samples were fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS). Subsequently, the specimens were embedded in a paraffin wax, and transverse sections were cut and stained with hematoxylin and eosin[18].

Cytokine array assay

Cytokine secretion was examined using a commercial cytokine array in accordance with the manufacturer's protocol (Mouse Cytokine Array Panel A; R&D Systems, Minneapolis, MN). Briefly, the mucosa of the large intestine was collected using a glass slide. Next, it was homogenized with 1% Triton X-100 in PBS (pH

7.4), centrifuged at 10,000 × *g* for 1 minute, and the supernatant was collected and examined using the cytokine array assay. Detected cytokines were denoted as follows: B lymphocyte chemoattractant (BLC); chemokine (C-C motif) ligand 1 (I-309); complement component 5a (C5/C5a); eotaxin; granulocyte macrophage colony-stimulating factor (GM-CSF); interferon-γ (IFN-γ); interferon-γ-induced protein 10 (IP-10); interleukin (IL)-1ra; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; IL-10; IL-12; IL-13; IL-16; IL-17; IL-23; IL-27; metallopeptidase 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 (JE); macrophage colony-stimulating factor (M-CSF); neutrophil-activating protein 3 (KC); 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).

Statistical analysis

The significance of data collected from all four groups was assessed using one-way ANOVA with Tukey's post hoc test. For the results to be considered significant, p values were set to be < 0.05. Further data analysis was performed using BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan) and all of the results are reported as means ± standard error of the mean (SEM).

Results

Lipid profiles of GOMEE

Lipid composition of GOMEE was obtained by TLC analysis for specimens obtained before and after alkali-treatment. As presented in **Fig. 2**, the lipid profiles of GOMEE from both conditions look alike, with sphingolipids (i.e., ceramides and GlcCer), sterylglycoside (SG) derivatives, and free and acyl sterols (alkali-resistant lipids) being the most abundant.

Effects of dietary GOMEE on weight gain associated with DSS ingestion

Figure 3 shows a progression of weight gain in mice over the course of the experiment. In the initial phase, before DSS was administered, body weight increased daily, and that was true for all groups. Within the initial 14 days of ingesting 1.5% DSS, the previously observed pattern of body weight gain remained unaffected. In the later phase of the experiment, weight loss was recorded within the control group and the body weight of the control group indicated significantly lower level when compared to those of the blank group. On the other hand, dietary 1% or 5% GOMEE suppressed this reduction in body weight when compared to the control group.

Effects of dietary GOMEE on colon constriction and spleen swelling caused by DSS ingestion

It is widely agreed that acceleration of colon inflammation induces other symptoms, such as colon constriction and spleen swelling[25]. As shown in **Fig. 4**, after treatment with DSS, the length of the large intestine was reduced, and the weight of the spleen was significantly higher. Respectively, ingesting dietary GOMEE

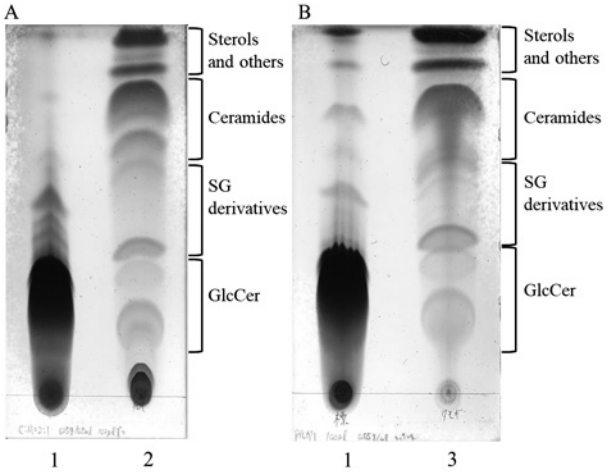

Fig. 2. Lipid profiles of GOMEE on silica thin-layer chromatography.
A: Lipid composition of GOMEE; B: Composition of alkali-resistant lipids in GOMEE.
To remove lipid components except for alkali-resistant lipids (sphingolipids and sterols), GOMEE was treated with alkali.
1, Maize GlcCer fraction; 2, GOMEE; 3, GOMEE treated by alkaline.
Mobile phase, Chloroform:Methanol (95:12, v/v); Detection, 50% H_2SO_4.
GlcCer, glucosylceramides; SG, sterylglycosides.

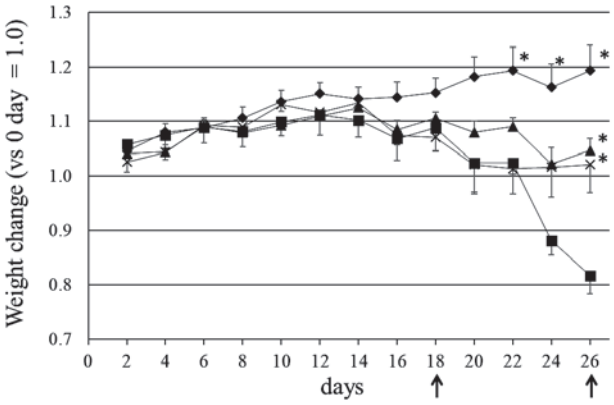

Fig. 3. Effects of dietary GOMEE on weight gain in DSS-treated mice.
After feeding experimental diets for 10 days, mice were given 1.5% DSS in drinking water *ad libitum*. Numbers on the horizontal axis indicate day when DSS treatment was terminated.
◆, Blank; ■, Control treated by DSS; ▲, 1% GOMEE treated by DSS; ×, 5% GOMEE treated by DSS.
↑: Day of dissection; first for cytokine array; second for other analyses.
*: Significant difference ($p<0.05$) vs Control.

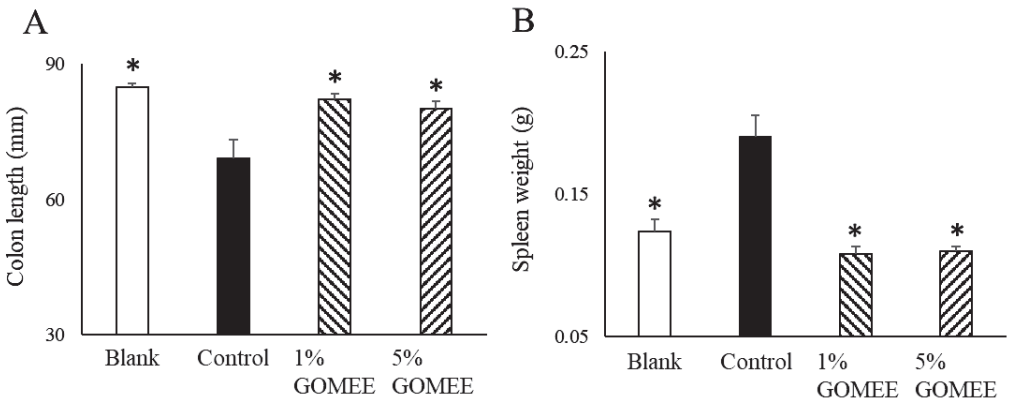

Fig. 4. Effects of dietary GOMEE on colon length (A) and spleen weight (B) in DSS-treated mice.
*: Significant difference ($p<0.05$) vs Control.

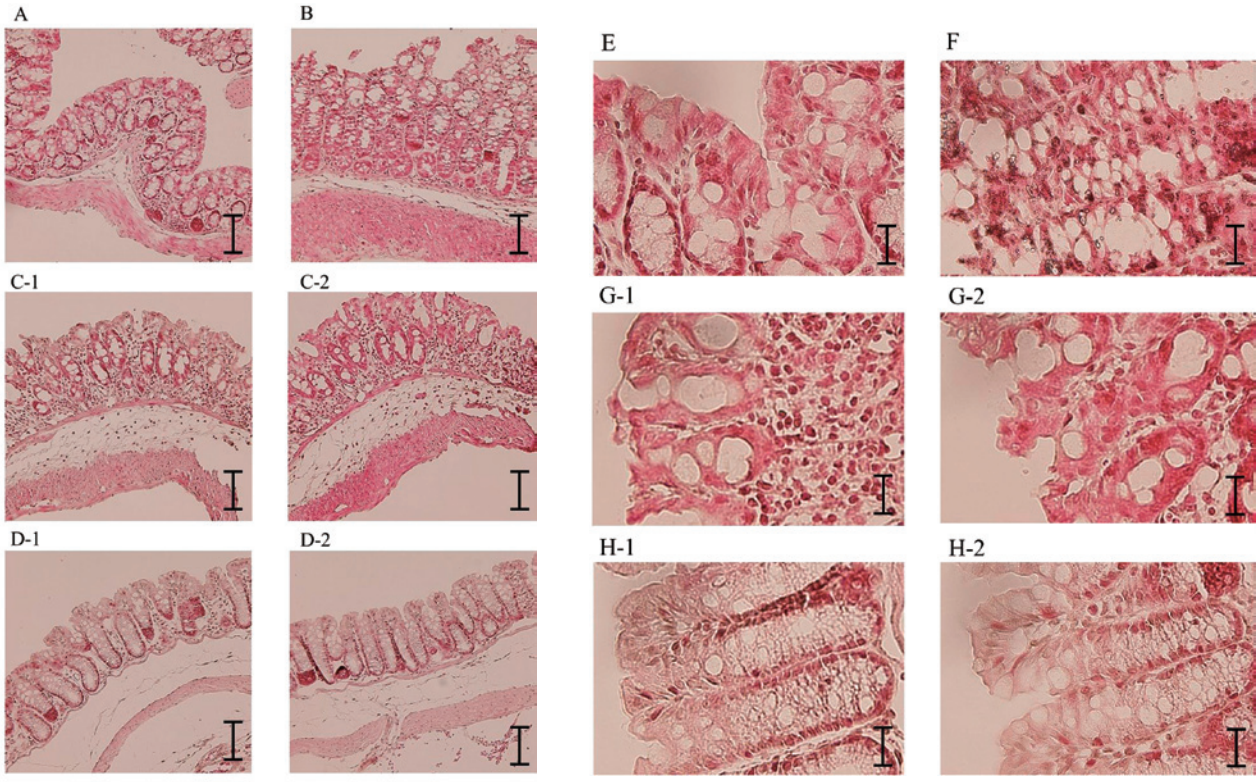

Fig. 5. Effects of dietary GOMEE on large intestinal villi in DSS-treated mice.
A & E, Blank; B & F, Control treated by DSS; C & G, 1% GOMEE treated by DSS; D & H, 5% GOMEE treated by DSS.
A-D, ×10 objective magnification. Scale bar = 100 μm.
E-H, ×40 objective magnification. Scale bar = 25 μm.

significantly suppressed the length reduction in the large intestine and the increase in the spleen weight.

Effects of dietary GOMEE on colon villi damage caused by DSS ingestion

We assessed the status of the colon by microscopic examination of the histology samples stained with hematoxylin and eosin (Fig. 5). Colon samples from the blank group had normal morphology of crypts. Typical histological changes associated with acute DSS-colitis are mucin depletion and the disappearance of epithelial cells. Similar morphological changes were observed in the histological samples collected from the control group. Ingestion of dietary GOMEE limited damage of the chorionic crypts associated with DSS treatment and the effect was more visible in samples collected from the group fed with diet supplemented with 5% GOMEE in comparison to the "1% GOMEE" group.

Effects of dietary GOMEE on colon cytokine induction caused by DSS ingestion

Cytokine array assay was performed to determine

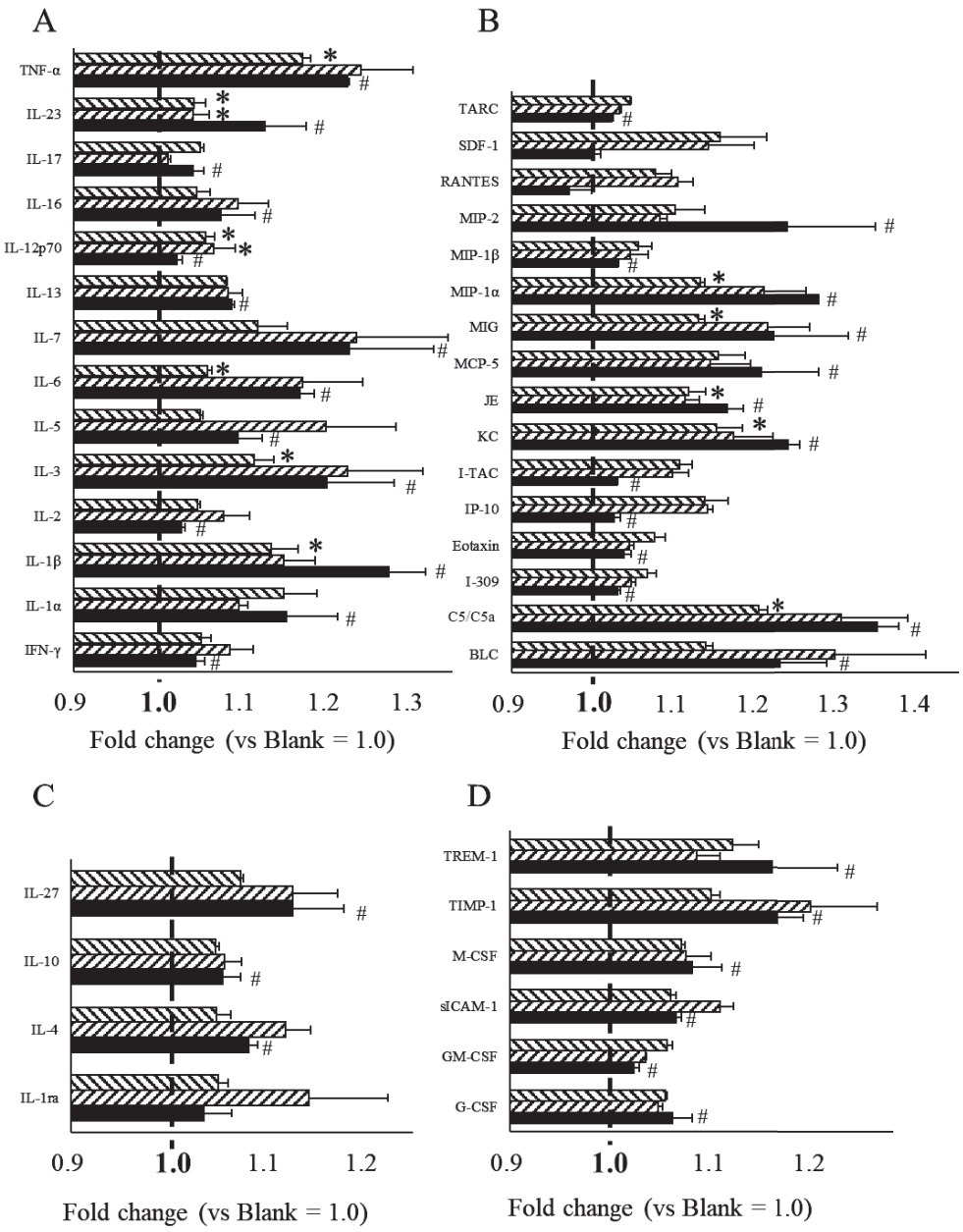

Fig. 6. Effects of dietary GOMEE on cytokine levels in the colonic mucosa of DSS-treated mice.
(A) Inflammatory cytokines. (B) Chemokines. (C) Antiinflammatory cytokines. (D) Others.
Control treated with DSS, ■; 1% GOMEE treated with DSS, ▨; 5% GOMEE treated with DSS, ▨.
Each cytokine expression was adjusted the values to the blank group = 1.0.
: Significant difference ($p<0.05$) between blank and control.
＊: Significant difference ($p<0.05$) vs control.

the influences of dietary GOMEE on production of inflammation-related cytokine in DSS-treated mice in the early/middle stage of inflammation (18th day of DSS treatment). The cytokine array simultaneously detects 40 different cytokines. The suitability of this assay was validated by comparing the densities of positive and negative control spots in this array.

Figure 6 shows cytokine production levels in the colon in the group treated with DSS in comparison to the blank group. Levels of most inflammation-related cytokines examined were significantly increased by DSS ingestion. In comparison to the control group, ingestion of dietary 5% GOMEE significantly decreased levels of 5 inflammatory cytokines and 5 chemokines and significantly increased levels of IL-12p70. The levels of anti-inflammatory cytokines were not altered by ingesting dietary GOMEE. Other cytokines, i.e., adhesion molecules, were stimulated by DSS intake and remained unaffected by GOMEE intake.

Discussion

Dietary mushrooms and the hydrophilic fraction extracted from dietary mushrooms are known to have numerous food functions, e.g., antioxidative effects, cardiovascular disease prevention, immune regulation, and reduction of blood sugar level[6-15]. However, there is still little known about the functions of the lipophilic fraction extracted from dietary mushrooms. In this study, we investigated the impact of GOMEE (lipophilic fraction extracted from fruiting bodies of mushrooms) ingestion on chronic colon inflammation and the colon inflammation-related cytokines production in DSS-treated mice. In this work, we demonstrated that in DSS-treated mice, dietary GOMEE ingestion suppresses body weight loss, colon constriction, and spleen swelling (**Fig. 3** and **4**), as well as preventing colon villi damage and reducing production of inflammation-related cytokines (**Fig. 5** and **6**). These findings are in line with our previous study on DSS-treated mice, where we demonstrated that dietary plant GlcCer ingestion limited colon villi damage and decreased the levels of inflammatory cytokines and chemokines[18]. Because mushroom GlcCer, which possess a different sphingoid base than plant GlcCer, is a major type of lipid found in the GOMEE (**Fig. 2**), we hypothesize that suppression of colon inflammation by GOMEE is related to the presence of mushroom GlcCer.

In our previous study, we examined the anti-inflammatory effects of dietary plant GlcCer in mice treated with 2% DSS[18]. This method has the benefit of yielding results within a short time, but it is not suitable for long-term estimation because of the heavy symptoms of colon inflammation. To examine the long-term effects of dietary functional food products, the dose-dependency of the effect of DSS was examined. DSS was investigated in mice at concentrations ranging from 0% to 1.5%. The effects of DSS were only observed at the highest 1.5% dose, where body weight gain was stopped, and blood in feces was observed, but there were no heavy symptoms of inflammation such as bleeding from the vent (data not shown).

In this study, we reported that an intake of 5% GOMEE suppresses the production of inflammation-related cytokines (**Fig. 6**) and limits the colon villi damage (**Fig. 5**) when ingestion of 1% dietary GOMEE is not sufficient to trigger these effects. In contrast, there were no significant differences in body weight, colon length, and spleen weight in samples collected from groups fed diets supplemented with both 5% and 1% dietary GOMEE (**Fig. 3** and **4**). The mechanism of DSS action remains unclear. Probably, DSS has a direct toxic impact on epithelial cells of the basal crypts, causing the surfactant effect, induction of colon inflammation, decreasing intestinal barrier activity, and worsening of colon injury and inflammation. This result in changes of body parameters (e.g., body weight loss, colon constriction, and spleen swelling)[25]. Therefore, it is believed that ingesting 5% GOMEE can have a strong protective effect in the early stages of damage caused by DSS treatment. The next logical step would be to study the dose-dependent effects of GOMME on suppression of body weight gain. However, because DSS is a potent inducer of colon inflammation, continuous GOMEE intake, even small doses, is expected to decrease the risk of colon inflammation in our life.

The lipophilic fraction of GOMEE contains considerable amounts of mushroom GlcCer, as well as several lipids such as ergosterol and SG. Moreover, in the context of inflammation suppression by the lipophilic fraction, the n-6/n-3 ratio in the fatty acid composition in the diet has been widely discussed[26]. The most common fatty acid in fruiting bodies of golden oyster mushrooms is linoleic acid (C18:2n-6)[27], which has the potential to stimulate inflammation[26]. In addition, the fatty acid content in GOMEE is lower than that in corn oil used in experimental diets (corn oil: 50 g/kg diet, n-6/n-3 ratio = 69)[24]. Therefore, it is unlikely that the observed anti-inflammatory effects of GOMEE are related to fatty acid composition.

The anti-cancer and anti-inflammatory effects of dietary mushrooms and the hydrophilic fractions extracted from the dietary mushroom have been reported previously[28, 29]. It has been demonstrated that dietary fruiting bodies of mushrooms can prevent and/or treat DSS-induced ulcerative colitis by modulation of immune function, e.g., downregulation of cytokines such as TNF-α, IL-10, and IFN-γ[29]. Dietary GOMEE suppresses the increase in chemokine production in inflammatory tissues. In our previous study, we demonstrated that dietary GlcCer extracted from plants suppresses the production of inflammatory cytokines and chemokines in DSS-treated mice[18]. Therefore, we speculated that mushroom GlcCer having a specific sphingoid base present in GOMEE also suppresses inflammation by regulation of inflammatory cytokine and chemokine levels. However, there are slight differences between GOMEE and plant GlcCer; e.g., dietary GOMEE increased levels of IL-12p70 while dietary plant GlcCer had no effect on IL-12p70 levels. Differences between structures of plant and mushroom GlcCer might influence the modulation of cytokines. In addition, candidates for anti-inflammatory components in GOMEE also included other lipophilic components. This is supported by the fact that fungal sterols such as ergosterol and their glycosides have several unique functions in comparison to sterols found in plants and mammalians[30-32]. Further studies are required to separate the lipid classes and this could be achieved by using solvent distribution methods to identify the active compounds.

It has been reported that in humans, GlcCer (which is a component of the cell membrane) as well as other nutrients in plants and mushrooms are not able to display complete activity in the colon during digestion,

because of the lack of cellulase which digests the cell wall composed of cellulose[23, 33]. Mushroom GlcCer and other lipophilic compounds in GOMEE have already been released from mushroom fruit bodies; therefore, GOMEE is thought to have higher bioavailability in comparison to lipophilic compounds existing in the cell when consuming the whole fruiting bodies.

In conclusion, dietary GOMEE is rich in sphingolipids, and it exerts anti-inflammatory effects in DSS-treated mice. The present results suggest that the dietary lipophilic fraction extracted from fruiting bodies of golden oyster mushrooms displays the potential to regulate colon homeostasis and that the benefits of ingesting GOMEE are dose-dependent. Continuous dietary GOMEE intake, even small doses, is expected to alleviate colon injuries caused by common weak stressors.

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和 文 摘 要

DSS 誘導潰瘍性大腸炎モデルマウスにおけるタモギタケ(*Pleurotus citrinopileatus*) 子実体エタノール抽出物摂取の効果

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近年，日本を含む東アジアにおいて過敏性腸炎や潰瘍性大腸炎に代表される炎症性腸疾患の増加は深刻になりつつある．これら慢性的な腸管炎症の継続は，下痢等による生活の質の低下だけでなく，大腸ガン発症リスクを上昇させる可能性が報告されている．タモギタケを含むきのこ類，またその水溶性画分において，抗腫瘍活性などの機能性が報告されおり，その摂取による腸疾患予防が期待されている．本研究では，きのこ子実体の脂溶性画分の腸疾患に対する機能性を明らかにするために，タモギタケ脂溶性画分としてタモギタケ子実体エタノール抽出物（GOMEE）を用い，食餌性 GOMEE が慢性大腸炎モデルマウスに及ぼす影響を調査した．1.5% デキストラン硫酸ナトリウム（DSS）飲水により誘導された慢性大腸炎に対し，食餌性 GOMEE は，飼育期間後期の体重減少を抑制するとともに，DSS 飲水 26 日目では脾臓重量の増加並びに大腸の収縮を有意に抑制した．また，GOMEE 摂取は DSS により誘導される大腸絨毛の損傷を摂取量依存的に抑制することが観察された．DSS 飲水 18 日目の炎症初期・中期においては，GOMEE 摂取は大腸粘膜の炎症関連サイトカインレベルの上昇を摂取量依存的に抑制した．以上の結果は，タモギタケの脂溶性成分には，抗炎症活性を有する腸管保護成分が存在する可能性を示唆する．

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