Original paper

Isomaltodextrin Prevents DSS-induced Colitis by Strengthening Tight Junctions in Mice

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Tight junction (TJ) integrity is one of the key factors governing intestinal health. Isomaltodextrin (IMD), a highly branched α-glucan, has been characterized as a new type of soluble dietary fiber. Here, we evaluated the efficacy of IMD to improve the barrier function of TJs. Transepithelial electrical resistance (TER) and Lucifer Yellow (LY) leakage were measured after treating Caco-2 cells in vitro with IMD. A mouse model of dextran sulfate sodium (DSS)-induced colitis was used for the in vivo studies. IMD treatment in vitro maintained TER at higher levels and reduced LY leakage significantly. IMD preloading of mice reduced the signs of DSS-induced colitis compared with the Water group. Immunohistochemical and western blotting analyses of the colon in the IMD group revealed enhanced claudin-3 and ZO-1 expression, indicating the role of IMD-induced TJ strengthening in the alleviation of colitis. The ingestion of IMD has beneficial effects on colon health by improving TJ integrity.

Keywords: tight junction, isomaltodextrin (IMD), dextran sulfate sodium (DSS)-induced colitis, Caco-2

Introduction

The gastrointestinal epithelium acts as a boundary between the body and the external environment. It is known that tight junctions (TJs) within this selective membrane barrier play an important role in limiting the permeation of luminal noxious molecules, such as pathogens, toxins, and antigens. Disruption of the intestinal TJ barrier impacts the mucosal immune system and induces inflammation, thereby contributing to the development of intestinal and systemic diseases (Suzuki, 2013).

On the other hand, morbidity associated with inflammatory bowel disease (IBD) has been increasing recently in Japan (Kanai et al., 2014). A lack of dietary fiber is suggested to be one of the reasons, in addition to the increased consumption of Western foods containing animal fat. It has been reported that patients with IBD, including ulcerative colitis and Crohn’s disease, exhibit decreased expression of TJs in the large bowel (Das et al., 2012; Zeissig et al., 2013). For example, lowered protein levels of ZO-1 and claudin-3 have been noted, leading to an increase in intestinal epithelium permeability. Meanwhile, because the gastrointestinal epithelium is continuously exposed to food components in the lumen, TJ regulation may be modified by these components (Azuma et al., 2013). Therefore, in the present study, we investigated the effects of isomaltodextrin (IMD) as one such component on TJs.

Abbreviations: Tight junction (TJ); tight junction proteins (TJPs); isomaltodextrin (IMD); transepithelial electrical resistance (TER); Lucifer Yellow (LY); dextran sulfate sodium (DSS); inflammatory bowel disease (IBD); disease activity index (DAI); nondigestible saccharides (NDSs); difructose anhydride (DFA); fructo oligosaccharide (FOS); degree of polymerization (DP)

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IMD is a highly branched α-glucan with a molecular weight (MW) of 5000, which is produced from starch via the α-glucosyltransferase and α-amylase activities conferred by *Paenibacillus alginolyticus*. IMD is composed solely of glucose, including α-1,6-, α-1,4-, and α-1,3-glycosidic linkages (Tsusaki et al., 2009). IMD contains more than 80% dietary fiber and is water-soluble, thus it functions as a soluble dietary fiber. Nishimura et al. reported that the cecal contents of rats fed a diet containing 3.3 – 16.7% IMD exhibited a significant increase in bifidobacteria and a significant decrease in the Firmicutes/Bacteroidetes (F/B) ratio compared with the untreated group, indicating that IMD reached the large bowel and had a prebiotic effect (Nishimura et al., 2016). Moreover, 6 h after oral administration of IMD, we found that 14% of IMD remained in the cecum (unpublished data).

Generally, it has been reported that indigestible carbohydrates, including oligosaccharides (Suzuki et al., 2004) and dietary fiber (Mineo et al., 2001), open TJs and increase permeability. Therefore, our knowledge would be advanced if we can demonstrate the ameliorative effects of IMD on inflammatory colitis via the maintenance of TJ integrity to better regulate permeability.

In this study, our main objectives were to first examine the direct effect of IMD on TJs in Caco-2 cells and next assess whether IMD preloading results in the inhibition of DSS-induced colitis. The goal of this research was to determine if IMD, a new dietary fiber, has potential as a prophylactic material for colitis.

**Material and Methods**

**Strengthening effect of IMD on tight junctions in vitro** The cellular permeability across Caco-2 monolayers was estimated by measuring transepithelial electrical resistance (TER) and Lucifer Yellow (LY) transport in a transwell bicameral cell culture system. Epithelial monolayers of Caco-2 cells were prepared by seeding Caco-2 cells in a 24-well plate, at a concentration of 1×10^5 cells per well insert cup, and culturing in Dulbecco's Modified Eagle’s Medium containing 10% fetal calf serum for 14 d (medium exchange intervals, ranging from 1-2 d). The basolateral and apical chambers of the cells were bathed in 1.0 and 0.5 mL of prewarmed fresh medium at 37°C, respectively. After a 30-min stabilization period, the transport experiments were initiated by adding 1 – 4% of IMD to the apical chamber. An electric resistance system (Millicell-ERS; Millipore Corporation, Bedford, MA) was used to measure the TER values and LY (100 μmol/L addition) transport was assessed as the movement into the basolateral chamber during the 6 h incubation period. The LY concentration in the basolateral solution was measured fluorometrically at 428 nm for excitation and 536 nm for emission. IMD (Hayashibara Co., Ltd., Okayama, Japan) was used for the in vitro and in vivo experiments. The values of TER and LY transport are expressed as percentages of the control.

Caco-2 cells grown on the membrane filters, treated with or without 4% IMD during 6 h, were fixed and permeabilized in 1% paraformaldehyde and 0.1% Triton-X-100 in PBS. After washing with PBS thrice, the cell membranes were incubated in PBS containing 1% BSA for 30 min. The samples were rinsed with PBS and incubated with anti-claudin-3 antibody [1:50, ab15102; Abcam, Cambridge, UK] or anti-ZO-1 antibody [1:50, LS-B2129; LifeSpan BioScience, Inc., WA, USA] at 4°C overnight. The samples were rinsed with PBS and incubated with the secondary antibody (1:800, Alexa Fluor® 488; Thermo Fisher Scientific Inc., MA, USA) at room temperature for 2 h. After rinsing with PBS, the samples were mounted with glycerin and imaged using fluorescence microscopy (BX53; Olympus, Tokyo, Japan).

**Effects of IMD on DSS-induced colitis in mice** The R&D Center of the Hayashibara Company approved the protocol for the animal experiments in this study. The experiments were conducted in accordance with the guidelines for the Regulations of Animal Experimentation, and the use of experimental animals was approved by the Animal Care and Use Committee. Twenty-nine 9-wk-old female BALB/c mice were purchased from Charles River Laboratories (Atsugi, Japan) and housed under standard conditions in an animal facility (4 mice/cage; relative temperature: 23°C ± 1°C; relative humidity: 40 – 70%; 12 h light/12 h dark light cycle: lights on 07.00 – 19.00 h). After 1 wk of acclimation, the mice were divided into 3 groups (Water/DSS group: n=12; IMD/DSS group: n=11; Normal group: n=6) matched for body weight and fed the AIN-93G diet (Reeves et al., 1993) ad libitum at the same time; water or a 2% IMD solution was available ad libitum for another 2 wk. After 2 wk, the Water/DSS group received a 2.5% DSS solution (mean MW range 36,000 – 50,000) (MP Biomedicals, CA) and the IMD/DSS group received a mixture of 2.5% DSS and 2% IMD. The control group was fed the AIN-93G diet and water until the experiment ended (Figure 1). After determining the disease activity index (DAI) scores for signs of disease, body weight, and the amounts of food and water consumed daily, the mice were euthanized and samples were collected following the administration of DSS for 6 d. Under pentobarbital anesthesia (Somnopentyl; Kyoritsu Seiyaku, Tokyo, Japan) at the time of euthanization, whole blood samples were extracted from the caudal vena cava and the colon was removed from nonfasted mice. After measuring the length of the colon and rapidly cleaning the lumen with PBS, the lower 7 – 8 mm of the distal colon was immediately frozen using dry-ice ethanol for western blot analysis. The remaining distal, middle, and proximal colon were fixed using a neutral buffered formalin solution.

**Signs of colitis** We determined the levels of DSS-induced colitis using the DAI score slightly as we only determined gross bleeding without using a hemocult fecal occult kit. The scoring system is shown in Table 1. Briefly, the scoring system included the examination of stool
consistency, rectal bleeding, and weight loss.

Colon histopathology score  Formalin-fixed colons were washed with water, dehydrated using alcohol, cleared, and embedded in paraffin in the usual manner. Subsequently, 4-μm thin sections were stained with hematoxylin and eosin (H&E). Pathology scores for DSS-induced colitis were determined in accordance with the procedures outlined by Fitzpatrick (Fitzpatrick et al., 2006). Assessments made in this study were performed after modifying the evaluation criteria from the extent of inflammation to erosion (Table 2).

Immunostaining for tight junctions of the colon  The paraffin-embedded colons were immunostained using the claudin-3 or ZO-1 antibody mentioned above in the “strengthening effect of IMD on TJs in vitro” paragraph. After deparaffinizing the tissue samples, they were rinsed with Tris-Buffered Saline with Tween (TBST), as an antigen activation treatment, and incubated with the Histofine Antigen Retrieval Solution (pH 9 buffer; Nichirei Biosciences Inc., Tokyo, Japan) for claudin-3 antibody staining, and with a citric acid buffer (pH 6.0) for ZO-1 antibody staining, then autoclaved at 120°C for 5 min. The samples were subsequently rinsed with TBST. To remove endogenous peroxidases, the tissue samples were incubated with 3% H₂O₂ methanol for 10 min, rinsed with TBST and incubated with blocking solution (Block Ace) for 30 min. Then, the samples were incubated with the primary antibody (anti-claudin-3 antibody [1:50] or rabbit anti-ZO-1 polyclonal antibody [1:50]) at 4°C overnight. The tissue samples were rinsed with TBST and incubated with the secondary antibody (Envision Rabbit [1:2,DAKO Envision+® system Labelled Polymer-HRP Anti-Rabbit;Agilent Technologies, CA, USA]) at room temperature for 1 h. After rinsing with TBST, the samples with the claudin-3 antibody staining were visualized using True Blue (Histomark® TrueBlueTM Peroxidase System #54-78-00, KPL; Kirkegaard & Perry Laboratories, Inc., MD, USA) and the nuclei were stained with Contrast RED Solution (#71-00-05, KPL, MD, USA). The samples labeled with the ZO-1 antibody were visualized using a horseradish peroxidase (HRP) substrate (HistoMark® BLACK peroxidase System #54-75-00, KPL, MD, USA) and the nuclei were stained with a Contrast GREEN Solution (#71-00-11, KPL, MD, USA).

The immunostaining was evaluated in a blinded manner. Pathology experts observed the colon samples using an optical microscope, and scored the expression intensities using a range from 0 to 3+.

Western blotting for the distal colon  The lumens of the excised mouse colons were cleaned with PBS and an approximately 30-mg tissue sample was cut, weighed, and rapidly frozen in dry ice-ethanol. Subsequently, lysis buffer (Azuma et al., 2013) was added to the frozen tissues (1 mL/30 mg tissue), which were minced with scissors, disrupted using a Polytron-type homogenizer, heated at 95°C for 5 min, and centrifuged at 13,000 rpm and 4°C for 10 min. Finally, the supernatant was collected and the protein concentration was measured using the bicinchoninic acid assay (BCA) method (Pierce Biotechnology, Inc., IL, USA). The tissue extracts were combined with 0.5 times the amount of Laemmli

Table 1. Severity scale used to determine the disease activity index

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss</th>
<th>Stool consistency</th>
<th>Gross bleeding</th>
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<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>Normal</td>
<td>Negative</td>
</tr>
<tr>
<td>1</td>
<td>1-5%</td>
<td>Loose stool</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>5-10%</td>
<td>Loose stool</td>
<td>Slightly bloody</td>
</tr>
<tr>
<td>3</td>
<td>10-15%</td>
<td>Diarrhea</td>
<td>Slightly bloody</td>
</tr>
<tr>
<td>4</td>
<td>&gt;15%</td>
<td>Diarrhea</td>
<td>Bleeding from anus</td>
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Murthy SN.
sample buffer (3x concentration) and heated at 100°C for 5 min. Proteins (23 µg) were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After incubating with rabbit anti-ZO-1 antibody (1:4000, 61-7300; Thermo Fisher Scientific, MA, USA) or rabbit anti-claudin-3 antibody (1:10,000, 34-1700; Thermo Fisher Scientific, MA, USA) at 4°C overnight, the PVDF membrane was washed and the secondary antibody was incubated with HRP-conjugated goat anti-mouse IgG (1:10,000 or 1:40,000, P0448; Agilent Technologies, CA, USA). ß-actin was used as the protein-loading control. After incubating with the mouse anti-actin antibody (1:10,000,000, clone C4, MAB1501; Merck Millipore, MA, USA) at 4°C overnight, the PVDF membrane was washed and the secondary antibody was incubated with HRP-conjugated goat anti-mouse IgG (1:20,000, P0447; Agilent Technologies, CA, USA). Protein bands were detected using the enhanced chemiluminescence (ECL) method (RPN2232; GE Healthcare, Little Chalfont, UK). Detected bands were scored for concentration using Image J software and protein density was determined using the reference level of ß-actin as 100%.

Statistical analysis In vitro TER and LY transport was assessed using Dunnett’s test to compare mean values of each group to the control group (JMP 9.0; SAS, Cary, NC). The Wilcoxon test (JMP 9.0; SAS) was used to analyze the data for DAI, pathology score and TJ scores. The amount of water and food consumed, body weight, colon length, and protein expression detected by western blotting were assessed using the F test between the Water/DSS and IMD/DSS groups. The Student’s t test was used if homoscedasticity was observed. In order to confirm the establishment of the experimental system, the colon length and protein expression detected by western blotting were first assessed using a t-test between the Water/DSS and Normal groups, and then re-assessed between the Water/DSS and IMD/DSS groups using a t-test.

Results

**Strengthened tight junctions associated with IMD in vitro** We examined the direct effect of IMD on TJPs to assess the barrier function of Caco-2 cells. At first, we observed no viscosity effect of IMD at a high concentration (4%) on TER levels in the cell-free system (data not shown). When Caco-2 cells were treated with IMD, TER increased significantly in a dose-dependent manner, showing 103% (2% IMD group) and 107% (4% IMD group) compared with non-treated control cells (Figure 2A). Next, we used LY as a probe for the paracellular permeability assay. Consistent with the above-mentioned results of TER, the LY flux was significantly reduced 6 h after adding 2% (19% reduction) or 4% IMD (22% reduction) (Figure 2-B). We next examined the effect of IMD treatment on the protein expression of TJPs, such as claudin-3 and ZO-1, using immunostaining. Immuno-fluorescence observation demonstrated that claudin-3 and ZO-1 proteins were both detected at the boundary of each cell both in the control and IMD-treated cultures; however, the expression intensity was slightly (claudin-3) or remarkably (ZO-1) enhanced after 4% IMD treatment (Figure 2C-F). Together, IMD treatment of Caco-2 cells prevented LY paracellular leakage by enhancing TJPs expression.

<table>
<thead>
<tr>
<th>Table 2. Modified version of histological damage scoring system based on Fitzpatrick's system.</th>
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<td>Score</td>
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<td>-------</td>
</tr>
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<td>a</td>
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<td>d</td>
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Histologic score = (a×d) + (b×d') + (c×d'')

Fitzpatrick LR.
Fig. 2. IMD strengthened TJ in Caco-2 cells.
A. IMD significantly increased TER compared with untreated control at 6 h incubation period. Values are expressed as means ± SD, n=6 per group. Significant differences versus control were **p < 0.01.
B. The cell monolayers were incubated without or with 1%, 2%, and 4% IMD. Lucifer yellow (LY) transport was assessed as the movement into basolateral chamber during 6 h period. LY permeability was significantly lowered by 2% and 4% IMD. Values are expressed as means ± SD, n=6 per group. Significant differences versus control were *p < 0.05 and, **p < 0.01.
C. Untreated Caco-2 cells were immunostained by claudin-3 antibody. Bar=20 μm.
D. 4% IMD-treated Caco-2 cells were slightly increased claudin-3 protein expression compared with untreated. Bar=20 μm.
E. Untreated Caco-2 cells were immunostained by ZO-1 antibody. Bar=20 μm.
F. 4% IMD-treated Caco-2 cells were strongly increased ZO-1 protein expression compared with untreated. Bar=20 μm.
and strengthening TJ integrity.

**Effects of IMD on DSS-induced colitis in mice**

**DAI score, colon length** There were no differences between the IMD/DSS and Water/DSS groups in terms of body weight and the amounts of food and water consumed during the experiments (data not shown). From 4-6 d after DSS intake, the IMD/DSS group showed significantly lower DAI and diarrhea scores, which is an evaluation method described by Murthy et al. (Figure 3 A-B). In the less severe diarrhea model, there was no difference between these two groups in terms of bloody stools and body weight scores (data not shown). While colon length in the Water/DSS group was significantly shorter compared to the Normal group, the IMD/DSS group exhibited a significantly longer colon length than the Water/DSS group, demonstrating the ameliorating effect of IMD on colon length shortening (Figure 4).

**Pathology score** A total pathology score for the colon (based on the sum of tissue scores for the proximal, middle, and distal regions) in the IMD/DSS group was significantly lower than for the Water/DSS group (Figure 5 A). Although the tissue scores for the colonic sites, including the proximal and middle colon, were lower than for the Water/DSS group, the pathology score for the distal colon in the IMD/DSS group was significantly decreased (Figure 5 B). Histopathologically, IMD significantly suppressed the scores of crypt damage and inflammation compared with Water/DSS (Figure 5 C). Although a comparison of the H&E stained tissues showed marked inflammatory cell infiltration between epithelial cells as well as crypt disappearance in the area from the lamina propria to the epithelial layer in the Water/DSS group (Figure 6 A), the IMD/DSS group showed only slight inflammatory cell infiltration or normal colon structure, as depicted in Figure 6 B-C.

**Expression analysis of TJPs by immunostaining** The TJ structure plays an important role in the physiological function of the intestinal epithelium by regulating the permeability between epithelial cells (Lopetuso et al., 2015). Although the Water/DSS group showed markedly lower colonic expression of claudin-3 in the mucosal epithelium, from the luminal epithelial to the crypt, the IMD/DSS group revealed nearly equal or stronger expression in comparison with the Normal group (Figure 7). Furthermore, ZO-1 expression in the Water/DSS group was barely detected in the luminal epithelium, although it was comparably high in both the IMD/DSS and Normal groups (Figure 8).

To evaluate the expression results in a more quantitative manner, the scored expression intensities at the colonic sites were examined. The expression score for claudin-3 in the IMD/DSS group was significantly higher in the middle and distal colon (Table 3). The expression of ZO-1 in the IMD/DSS group was significantly greater in the middle colon and slightly higher (but not significantly) in the distal colon (Table 4).

**Western blot analysis of the distal colon** To confirm the
IMD Strengthens Tight Junctions

Fig. 5. Colon histopathology scores.
(A) Total histologic scores that are the sum of the proximal, middle, and distal colon scores.
(B) Histologic scores for each specific area of the colon.
Histologic scores were graded from 0 to 3 or 4 using Fitzpatrick’s formula and were based on the extent of inflammation, erosion, crypt damage, and the percentage occupied by lesions.
(C) Histologic scores for erosion, crypt damage, and inflammation.
IMD significantly suppressed crypt damage and inflammation score compared with Water/DSS.
Values represent the means ± SEM in the Water/DSS group (n=12), IMD/DSS group (n=11) and the Normal group (n=6). In the Normal group, total histologic scores, area specific scores and erosion scores, crypt damage scores and inflammation scores were 0.
Between Water/DSS versus IMD/DSS groups were **p < 0.01 (A and C) and *p < 0.05 (B and C).
immunostaining results, we performed a western blot study. The results of the western blot for the distal colon showed significantly higher protein expression, including ZO-1 and claudin-3, in the IMD/DSS group compared with the Water/DSS group (Figure 9 A-C).

**Discussion**

In this study, we first demonstrated, using in vitro culture of Caco-2 cells, that IMD treatment potentiated TJ integrity in a dose-dependent manner, which was verified by higher levels of TER, reduced permeability of LY, and enhanced expression of TJP. Disruption of the intestinal barrier has been identified as one of the most critical events for developing IBD. It is known that excessive interactions between the host and bacteria are induced in the initial stage of IBD (Tlaskaloá-Hogenová et al., 2011) and inadequate functioning of the intestinal barrier generally causes inflammation (Suzuki, 2013). Therefore, we reasoned that the in vitro effects of IMD on TJ integrity might also be valid under in vivo conditions. As a next step, we examined the in vivo effects of IMD using a mouse model of DSS-induced colitis.

Since a severe DSS-induced colitis model was not suitable for examination of TJP expression, due to disintegration of the intestinal epithelium, we employed a less severe diarrhea model by reducing the amount of DSS to 2.5%. We found that IMD treatment protected against the decrease in TJP expression caused by DSS intake and also ameliorated clinical and histopathologic signs related to colitis. To rule out the possibility that IMD absorbed DSS in a mixture form and then reduced DSS’s effect, we examined the possible molecular association between IMD and DSS using an HPLC equipped gel filtration column. Because the peak pattern of the DSS molecule showed no difference even in the presence of IMD, we concluded that the observed effects of IMD are not due to the absorbed effects of DSS by IMD (data not shown).

Each group of mice consumed similar amounts of water during the experiment, and it was assumed that there was no difference in the intake of DSS among all of the treated mice (data not shown). Furthermore, IMD intake resulted in mitigation of shortened colon
length, which is considered to be an indicator of the severity of
DSS-induced inflammation and dysfunction (Melgar et al., 2005).
Immunohistological examination of claudin-3 and ZO-1 proteins
revealed that their expression in the mucosal epithelium was
maintained from the lamina propria to the crypt in the IMD/DSS
group. On the other hand, in the Water/DSS group, a marked
decrease in expression was observed due to the DSS treatment.
Western blotting experiments confirmed the higher protein levels
of claudin-3 and ZO-1 in the IMD/DSS group than in the Water/
DSS group, confirming the immunostaining results. Together, these
results support our hypothesis that IMD could strengthen TJ
integrity and reduce DSS-induced colitis.

It was reported that a decrease in ZO-1 expression to 1/3 of the
normal expression in mice was detected 3 d after the start of DSS
intake, which is earlier than 7 d, or the time when the DSS-induced
tissue scores were significantly increased (Politz et al., 2007). In
other words, following DSS intake, the reduction of TJs precedes
the increase in permeability, penetration of external substances,
and intestinal epithelial inflammation. Thus, the change in TJs
appears to be a key mechanism by which DSS induces the
development of colitis.

Recently, a number of studies reported that enhanced
expression of TJs inhibited DSS-induced colitis. It was reported
that oral administration of Lactobacillus casei in DSS-induced
colitis mice prevented the loss of ZO-1 expression and reduced
intestinal hyperpermeability (Zakostelska et al., 2011). As a result,
treatment improved intestinal barrier function, protected against the
invasion of intestinal bacteria, and suppressed the excessive
interaction from the immune systems.

It was demonstrated that germ-free (GF) mice, compared with
specific pathogen-free (SPF) mice, exhibited a lower expression of
TJs in the colonic epithelium (claudin-7, occludin, and TJP-1) and
displayed nearly no intestinal indole. When colitis was induced by
DSS in GF mice with a lower expression of TJs, all SPF mice
survived on day 7 after initiating DSS administration while most
GF mice died. Feeding a capsule containing indole to GF mice for
2 wk resulted in significantly increased colonic expression of
occludin and claudin-7 and decreased DSS-induced mortality from

Fig. 7. Immunostaining of claudin-3 expression in the distal colon.
Claudin-3 was strongly stained from the surface epithelium to the crypts in the IMD/DSS group, but faintly stained in the
90% to 15% in GF mice, indicating that TJ preservation reduces DSS-induced colitis (Shimada et al., 2013).

Furthermore, it was described that DSS-induced colitis significantly inhibited the decreased expression of occludin and claudin-3 in the proximal and distal colon. However, DSS-induced diarrhea was significantly suppressed when TJs were preserved via the oral administration of 50 mg/kg somatostatin for 3 d after 8 d of DSS administration (Li et al., 2014).

**Table 3. Immunohistochemical detection of claudin-3 expression in the colon of DSS-colitis mice**

<table>
<thead>
<tr>
<th>group</th>
<th>Proximal</th>
<th>Middle</th>
<th>Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>IMD</td>
<td>Water</td>
</tr>
<tr>
<td>Expression</td>
<td>2.0</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>score</td>
<td>2.0</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
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<td>1.0</td>
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| Mean ± SEM | 1.7 ± 0.3 | 2.0 ± 0.4 | 1.3 ± 0.2 | **2.3 ± 0.2** | 1.7 ± 0.3 | **2.7 ± 0.2** |

Bold data is significantly different versus the Water/DSS group in the same region. *p* < 0.05.

*In each group, 6 mouse colons were randomly immunostained and the intensity of immunostaining in dark brown was scored as follows: 0, -; 0.5, ±; 1, +; 1.5, +++; 2.0, ++; 2.5, ++++; 3, +++.

**Fig. 8.** Immunostaining of ZO-1 expression in the distal colon. The strong expression of ZO-1 in the surface epithelium was detected in the IMD/DSS group, but not in the Water/DSS group. ▶: stained positive. Bar=100 μm.
It was reported that naringenin, a citrus polyphenol, reduced the loss of TJ barrier function and inflammation in the intestine. Although DSS administration impaired colonic TJ barrier function, increased colonic permeability, and increased the lipopolysaccharide (LPS)-binding proteins in blood plasma, naringenin suppressed these signs by protecting TJ barrier function, inhibiting colon length shortening as well as the expression of cytokines, such as IL-6 and IL-17A. As a result, the DSS-induced colitis was mitigated (Azuma et al., 2013).

These studies all supported the notion that substances which strengthen TJs, including probiotics, indoles, somatostatins, and polyphenols, have the ability to suppress the signs of DSS-induced colitis, inhibit colon length shortening, and suppress inflammation, and are in good agreement with our observations described in this paper.

In an in vitro study, Suzuki et al. reported that the addition of 100 mM/L of nondigestible saccharides (NDSs), including difructose anhydride (DFA) III, DFA IV, fructo oligosaccharide (FOS), and raffinose to Caco-2 cells and incubation at 37°C for 3 h significantly and dose-dependently reduced the TER of Caco-2 cells, increased LY permeability, and increased calcium absorption (Suzuki et al., 2004). In addition, Mineo et al. reported that polydextrose at concentrations ranging from 0.1 to 100 mM/L increased calcium absorption from the small bowel and colonic mucosa in rats, as assessed via the Ussing chamber technique (Mineo et al., 2001). In short, these NDSs increased transportation between cells rather than opening TJs. However, IMD differed from these NDSs, showing increased TJPs and decreased permeability in Caco-2 cells.

One of the mechanisms underlying the effect of IMD on DSS-induced colitis is its direct effect on TJ integrity of epithelial cells as demonstrated in Caco-2 models. In this case, IMD could strengthen TJ integrity via certain cell signals that up-regulate the expression of TJ-related molecules. We also speculate that TJ integrity might be strengthened through the modulation of TLR-4 signaling by IMD, since Dheer et al. reported an association between increased epithelial TLR-4 signaling and epithelial barrier impairment (Dheer et al., 2016).

Based on structural similarity to IMD, we next compared the effects of isomaltooligosaccharides on DSS-induced colitis with that of IMD. It was reported that the development of DSS-induced colitis was delayed in rats fed short-sized linear isomaltooligosaccharides (S-IMO, average degree of polymerization (DP)=3.3). Dietary S-IMO increased the concentration of n-butyric acid in the cecal contents and the level of glucagon-like peptide-2 in the colonic mucosa. Therefore, the mechanisms of S-IMO underlying the delayed development of DSS-induced colitis may involve fermentation in the large intestine (Iwaya et al., 2012).

It was reported that IMD (average DP=30) was fermented in the rat large intestine and also significantly increased cecal n-butyric acid concentrations (Nishimura et al., 2016). These results suggested that the effect of IMD on DSS-induced colitis is mediated not only by strengthening TJ integrity, but also by the fermentation, as in the case of S-IMO. These two mechanisms, the direct effect on TJ integrity and indirect effects of changing microbiota composition, could explain the anti-inflammatory effects on DSS-induced colitis. However, the definite mechanisms are unknown and require further research.

In conclusion, IMD directly strengthened TJ integrity and reduced permeability in Caco-2 cells. Furthermore, preloading mice with IMD strengthened the colonic TJs and reduced the signs of DSS-induced colitis. This could assist in better regulating colonic permeability so that bacteria and external antigen exposure is kept to a minimum and excessive inflammatory immunoreactions are suppressed. It is anticipated that further study of these effects may eventually show that the consumption of IMD in the daily diet of humans could help maintain TJs in the large intestine.
Fig. 9. Western blotting analysis of the tight junction proteins.

(A) Both claudin-3 and ZO-1 proteins extracted from the colonic tissue were detected by Western blotting using specific antibodies. Anti-ß-actin antibody was used as a loading control.

Intensities of both claudin-3 and ZO-1 of the Water/DSS group were remarkably decreased, but those of the IMD/DSS group were maintained at the same levels as those of the Normal group.

(B) Quantitative analysis of claudin-3 protein expression. Intensity of the claudin-3 expression was measured with a densitometer normalized by the intensity of the ß-actin bands. Values represent the means ± SEM in the Water/DSS group (n=10), IMD/DSS group (n=11) and the Normal group (n=6).

Whereas claudin-3 expression of the Water/DSS group decreased to some extent (p=0.10) compared with the Normal group, the IMD/DSS group maintained its expression at the same level as the Normal group.

(C) Quantitative analysis of ZO-1 protein expression.

The intensity of ZO-1 expression was measured as (B).

Values represent the means ± SEM in the Water/DSS group (n=10), IMD/DSS group (n=11) and the Normal group (n=6).

Whereas ZO-1 expression of the Water/DSS group were decreased significantly compared with the Normal group, the IMD/DSS group maintained its expression at the same levels as the Normal group.
IMD Strengthens Tight Junctions

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**References**


