An Herbal Medicine Orengedokuto Prevents Indomethacin-Induced Enteropathy

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Prostaglandin E2 (PGE2) is a key regulator of gastrointestinal, immunological, and mucosal homeostasis. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the prostaglandin-producing enzyme cyclooxygenases (COXs), and can induce serious complications, such as gastrointestinal damage, with long-term treatment. Orengedokuto (OGT), a Japanese traditional herbal medicine (Kampo medicine), is effective in various animal models of enteropathy. In the present study we examined whether OGT prevents enteropathy induced by NSAIDs in mice. Ulceration in the small intestine was induced with 2 subcutaneous injections of indomethacin (20 mg/kg body weight). Orally administered OGT prevented or reduced lethality, intestinal lesions, bleeding, increased serum nitrate/nitrite levels, and reduction of mucosal PGE2 induced by indomethacin. These beneficial effects of OGT were accompanied by increased production of PGE2 and interleukin 10 by isolated lamina propria mononuclear cells; COX-2 in these cells may be a major source of PGE2 in normal intestines. These findings suggest that OGT could be an effective therapeutic agent for the treatment of inflammatory bowel disease and adverse reactions to NSAIDs.

Key words nonsteroidal anti-inflammatory drug; interleukin 10; herbal medicine; inflammatory bowel disease; prostaglandin E2

Prostaglandin E2 (PGE2) is not only a key mediator of inflammation but also a regulator of gastrointestinal mucosal homeostasis through its influence on various functions and mediators.[1—4] PGE2 enhances interleukin 10 (IL-10) synthesis and modulates the intestinal immune response to dietary antigen.[5,6] The breakdown of systemic tolerance to luminal antigen is reportedly involved in the development of indomethacin-induced enteropathy.[7] Indomethacin is one of the non-steroidal anti-inflammatory drugs (NSAIDs) that reduce PGE2 production *via* inhibition of the cyclooxygenases (COXs)[2], COX-1 and COX-2. Although NSAIDs are useful for their analgesic and anti-inflammatory properties, gastrointestinal damage is major limitation of their use. For example, long-term NSAIDs treatment causes small intestinal inflammation similar to Crohn's disease in 70% of patients receiving these drugs.[8—10] Furthermore administration of NSAIDs may cause immediate relapse of quiescent intestinal bowel disease (IBD) and other diseases accompanying mucosal injuries.[11—14] Similar observations have been made in animal models.[15,16] Recently, although selective COX-2 inhibitors have been developed that were expected to produce less gastric injury, they retain the ability to cause bowel damage.[8] These reports indicate that the methods for reducing the enteropathy are needed and important in NSAIDs therapy.

A Japanese traditional herbal medicine (Kampo medicine) Orengedokuto (OGT, Huang-Lian-Jie-Du-Tang in Chinese) has been ethically used for the therapy of various diseases including gastric ulcers, gastritis, and melena. OGT decreases intestinal injury in dextran sulfate sodium (DSS)- and trinitrobenzene sulphonic acid (TNBS)-induced animal models of enteropathy.[17—19] In the present study, we describe the effect of OGT on lethality, intestinal injury, and PGE2 depletion induced by subcutaneous indomethacin injection, and we discuss OGT's possible therapeutic value for the treatment of IBD and adverse reactions to NSAIDs.

MATERIALS AND METHODS

Drugs, Antibody, and Chemicals The Kampo medicine (Japanese traditional herbal medicine), Tsumura OGT extract powder ("TJ-15"), is manufactured with a standardized quality and quantity of ingredients and is approved as an ethical drug by the Ministry of Health, Labour and Welfare of Japan. OGT consists of crude ingredients extracted with boiling water from the following four medicinal herbs in the ratio given in parentheses: Ogon (Scutellariae radix; 3.0), Oren (Coptidis rhizoma; 2.0), Sanshishi (Gardeniae fructus; 2.0), Obaku (Phellodendri cortex; 1.5). Spray-dried extract powders of the Kampo medicine, OGT, was prepared by Tsumura & Co. (Tokyo, Japan).

PGE2 enzyme immunoassay (EIA) kit was purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Anti-COX-2 polyclonal antibody was obtained from Alexis Biochemicals (San Diego, CA, U.S.A.). Indomethacin and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise specified.

Treatment of Mice Female BALB/c mice (7-weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were housed in an air-conditioned room with a 12-h light–dark cycle under specific pathogen-free conditions. All mice were given AIN-93M powder diet (CLEA Japan, Inc., Tokyo, Japan) and water *ad libitum*. Before starting all experiments, mice were fasted for 24 h and then re-fed the normal diet or diets containing OGT at concentrations of 0.5%, 1%, or 2% which correspond to the effective doses established in our previous study.[20] These diets were administered throughout the experimental period.

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Enteropathy was induced as described in a previous study.[21] In brief, 1 h after re-feeding, mice were subcutaneously injected with freshly prepared indomethacin (20 mg/kg) once a day for 2 d. For preparation of tissue samples, mice were sacrificed by severing their carotid artery under diethylether anesthesia at arbitrary time points after the first injection of indomethacin. All animal experiments were conducted in accordance with the institutional guideline for the care and use of laboratory animals for research, which conform to the guideline of Science Council of Japan.

Morphological Studies The whole small intestine was rinsed with saline, opened longitudinally, spread flat on filter paper, and fixed in 15% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Using a stereoscopic microscope, we counted the number of ulcers and quantified the sum of ulceration areas by tracing the outline of the areas using Image Processor for Analytical Pathology software (Sumika Technoservice Co., Hyogo, Japan).

Formalin-fixed intestine samples were stained with hematoxylin & eosin (H&E) and the intestinal damage was scored as follows: 1) disruption of the epithelial cells lining the villous tip; 2) disruption of the lamina propria; 3) depletion of lamina proprial cells and denudation of muscularis mucosae; 4) damage localized to the submucosa; 5) damage localized to the muscularis mucosae and thinning or perforation of the intestinal wall.

For immunohistology, the sections were stained with diaminobenzidine (Nichirei Co., Tokyo, Japan) by a streptavidin–biotin complex method using a Histomouse-Plus kit (Zymed Laboratories, Inc., South San Francisco, CA, U.S.A.) or a Histofine Mouse Stain kit (Nichirei Co.) and then counterstained with hematoxylin according to the manufacturer's protocol.

Quantitation of Fecal Hemoglobin and Serum Nitrate/Nitrite The hemoglobin concentration of stool in the cecum was quantified by measuring oxihemoglobin using a Biochemical Analyzer TBA-40FR (Toshiba Lab Medical Co., Ltd., Tokyo, Japan). Serum nitrate and nitrite (NOx) concentration was measured using a colorimetric assay kit (Cayman Chemical Company) according to the manufacturer's protocol.

Quantitation of Intestinal PGE2 Levels and Effect of COX inhibition *ex Vivo* Intestinal PGE2 levels were determined using a modification of a previously described protocol.[22,23] Briefly, the whole intestine was washed *via* the lumen with ice cold saline and carefully stripped of surrounding fat. The mucosa was gently scraped off the muscularis propria and mixed with a spatula. The muscularis propria was minced well with scissors. The mucosal scraping and the minced muscularis propria were incubated 5 min in Tyrode's solution (Sigma Chemical Co.) at 37 °C to convert arachidonic acid to PGE2 and then homogenized for 30 s. The tissue homogenate was then centrifuged at 10000 rpm for 15 min and the amount of PGE2 in the supernatant was immunoassayed using an EIA kit (Cayman Chemical Company).

To determine which COX isozyme contributed to PGE2 production, we performed COX inhibition assays *ex vivo*. The mucosal scrapings were incubated for 5 min in Tyrode's solution containing of 1×10^{-6} M of COX inhibitor, indomethacin, or NS-398, at 37 °C. The supernatant was collected and the amount of PGE2 was measured as described above.

Cell Preparation and Culture Intestinal tissue samples were taken from mice that fasted for 24 h and then re-fed the OGT-containing or normal diet for 48 h with or without indomethacin injection. Lamina propria mononuclear cells (LPMC) were isolated from small intestine according to a modified method described in previous studies.[5,24,25] In brief, the small intestine was cut into short segments and incubated in RPMI1640 medium for 30 min at 37 °C. LPMC were isolated from the remaining fragments that were incubated in digestion medium (RPMI1640 containing 10% fetal bovine serum; FBS; ICN Biomedicals Inc., Aurora, OH, U.S.A.) and 120 units/ml collagenase S-1 (Nitta Gelatin Inc., Osaka, Japan) for 75 min at 37 °C. Single cell suspensions were passed through a glass wool column and then refined using a 75%/40% Percoll (GE Healthcare, Chicago, IL, U.S.A.) gradient. When 2 doses of indomethacin were injected, it was not possible to obtain a sufficient amount of Percoll-fractionated cells for analysis, so semi-purified cells collected after passage through a glass wool column were used. Cells were counted for viability by trypan blue (Life Technologies Inc., Rockville, MD, U.S.A.) exclusion and were greater than 70% viable.

Cells were cultured without OGT or any chemicals at a density of 2.5×10^{6} cells/ml in RPMI1640 containing 2 mM Glutamax I (L-alanyl-L-glutamine; Life Technologies Inc.), 10 mM HEPES, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 mg/ml gentamicin (Life Technologies Inc.), 50 μM β-mercaptoethanol, 10% FBS, and 0.5% normal mouse serum after the initial 10-h isolation procedure. Supernatants were removed and stored at −80 °C until analysis. PGE2 was measured using an EIA kit (Cayman Chemical Company). Cytokines were measured using a Bio-Plex Suspension Array System (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's protocol.

Statistical Analysis Values of $p<0.05$ were considered to be statistically significant in this study. Values in the text are expressed as mean±standard error (S.E.). For statistical analysis, the Kaplan–Meier method, log-rank test, two-way analysis of variance (ANOVA), Scheffe's *post hoc* analysis, and Mann–Whitney U test were performed using StatView Version 5 software (SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Protection from Indomethacin-Induced Death by OGT In mice treated with indomethacin the lethality rate was 80% on day 9 (Fig. 1). Seventeen of the 20 mice treated with 2% OGT (equivalent to a dosage of 2.8 g/kg body weight) survived the indomethacin-treatment and were still alive at the end of the experiment (1 month after the first injection of indomethacin) with no sign of disease. The effect of OGT was dose-dependent (Fig. 1). OGT concentrations of 0.5% and 1% were equivalent to doses of 0.7 and 1.5 g/kg body weight, respectively. Total food intake was similar among the different diet groups (data not shown).

The mice were treated according to 4 different protocols for 2% OGT administration, as depicted in Fig. 2. OGT-administered mice from day 0 to day 3 exhibited the same degree of survival as mice administered indomethacin from day 0 to day 7. Furthermore, the lethality rate in mice that were

pre-treated with OGT for 3 d before indomethacin-injection was partially reduced (30% *versus* 70% of control day 9). Conversely, OGT given to indomethacin-injected mice from day 3 to 7 failed to reduce lethality. These results suggest that the protective effect of OGT is due to the prevention and/or counteraction of the initial indomethacin-induced damage. Therefore, in the following experiments we focused the investigation on the early (48 h or 54 h) phase of enteropathy.

OGT Prevents Enteropathy and Bleeding Histological results are shown in Figs. 3 and 4. Indomethacin injection induced numerous ulcerations that appeared in a punctuate pattern; they were deep and reached the muscularis propria and underlying tissues (Fig. 3A). Almost all remaining villi were injured at the top of surface. Some mice had perforations or adhesions of the small intestine. OGT improved the intestinal lesions induced by indomethacin (Fig. 3B); a significant reduction was observed in the number and area of ulcerations and number of lesions scored histologically as grade 2 and 5 (Fig. 4). Perforations or coalescence of small intestine obtained from OGT-administered mice were decreased, both in size and number, compared to those of control mice. More-

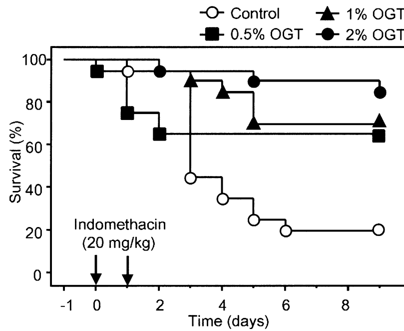

Fig. 1. Effect of OGT on Indomethacin-Mediated Lethality

Animal survival was assessed after 2 injections of indomethacin (20 mg/kg), 24 h apart, as described in Materials and Methods. OGT was administered by admixture in the diet during the examination period from the first injection of indomethacin ($n=20$). OGT was administered in the food because it is an orally active drug, and intragastric administration has been found to be highly stressful, especially when mice are subjected to the first indomethacin injection. Survival curves were drawn using the Kaplan–Meier method. Statistically significant differences between the 2% OGT group and the control group identified by log-rank test ($p<0.0001$).

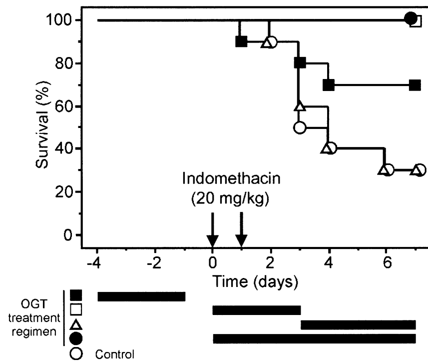

Fig. 2. Survival in Groups of Mice under Various OGT Administration Protocols.

Mice were treated with varying regimens (pretreatment, co-treatment, and post-treatment) of OGT as shown. Two doses of indomethacin (20 mg/kg) were injected 24 h apart as described in Materials and Methods ($n=10$). Survival curves were drawn using the Kaplan–Meier method.

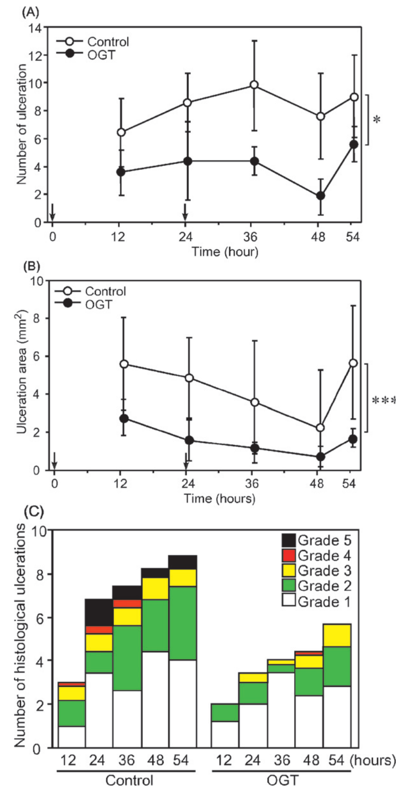

Fig. 4. Effect of OGT on Indomethacin-Induced Intestinal Lesions

Indomethacin was injected to mice at 0 and 24 h (arrows in graphs). Mice were killed 12, 24, 36, 48, or 54 h after the first indomethacin-injection. Morphological changes at hour 54 were almost the same as at hour 60 after the first indomethacin-injection in Fig. 3. The number (A) and areas (B) of ulceration in formalin-fixed tissues were calculated macroscopically. OGT significantly reduced the number and the sum of ulceration areas ($p<0.05$, 0.0005 respectively). The mean$\pm$S.E. are presented. (C) The injury was calculated. The scores were determined as described in Materials and Methods. Histologically visible ulcers between the pylorus and ileocecum were counted. Data for each grade shows the respective mean values for each group ($n=5$). Two-way ANOVA with treatment and time as factors followed by Scheffe's *post hoc* analysis were used.

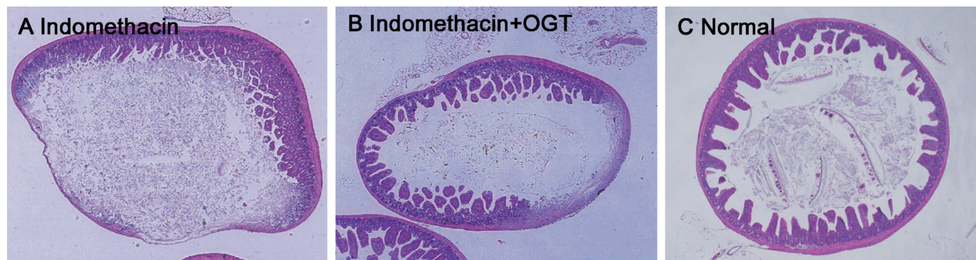

Fig. 3. Typical Microscopic Observations of Histological Sections of the Small Intestine

Tissues were obtained 60 h after the first indomethacin injection. H&E-stained section of small intestine from a mouse treated with indomethacin and without (A) or with (B) OGT (10× magnification). (A) Control mice had atypical large and deep ulcerations. (B) In the OGT-treated mice, tissue damage was milder and the muscularis propria was thicker than that in control mice. (C) Small intestine of a naive mouse which did not have any noticeable lesions.

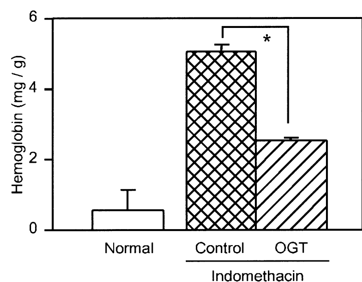

Fig. 5. Effect of OGT on Fecal Hemoglobin in Cecum

Whole stools in cecum were obtained from mice 48 h after the first indomethacin injection. As a negative control, hemoglobin concentrations in stools from naive mice (normal group in the figure) were quantified. Data are expressed as mean±S.E. (n=6). Statistical analysis was performed using the Mann–Whitney U test. * Significant difference from control, p<0.05.

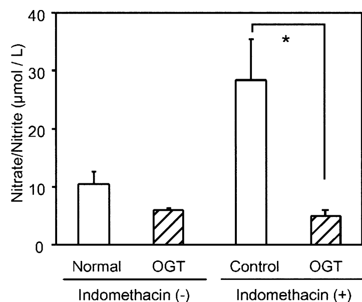

Fig. 6. Effect of OGT on Serum NOx Concentrations

Serum samples were obtained from mice 48 h after the first indomethacin-injection. Indomethacin-untreated serum samples were obtained from mice that were re-fed for 48 h after a 24-h fast. Data are expressed as mean±S.E. (n=6). Statistical analysis was performed using the Mann–Whitney U test. * Significant difference from control, p<0.05.

over fecal hemoglobin concentrations showed approximately 9-fold elevation by indomethacin-treatment and this elevation was diminished by OGT (Fig. 5). Likewise, indomethacin increased serum NOx concentration 3-fold above the basal level and OGT significantly abrogated the increase in NOx (Fig. 6).

OGT Increases Small Intestinal Mucosal PGE2 PGE2 is known as a regulator of gastrointestinal homeostasis.[1] PGE2 levels in mucosa and muscularis propria at 24 h after the second indomethacin injection were significantly reduced as compared with naive mice (Table 1). Figure 7 summarizes the sequential changes of intestinal PGE2 levels in mice treated with indomethacin. OGT significantly prevented the decrease of mucosal PGE2 levels induced by indomethacin.

To clarify whether OGT directly increased PGE2 levels or merely inhibited the PGE2 reduction, OGT was administered to mice that were not injected with indomethacin. OGT by itself increased mucosal PGE2 levels (Fig. 8) and produced no histological changes of the small intestine, such as villous atrophy, crypt hyperplasia, lesions, or cellular infiltrates (data not shown).

OGT Increased COX-2 Expressing Cells in the Lamina Propria Since PGE2 is generated by COX-1 and COX-2, we examined the influence of COX inhibitors on mucosal PGE2 production *ex vivo*. In the study described in the previous paragraph, we measured PGE2 levels *ex vivo* using the ongoing reaction process in which endogenous arachidonic acid is converted to PGE2 by COX enzymes in the tissue. By adding COX inhibitors to this reaction process, we investigated which COX enzyme was affected by OGT. As shown in Fig. 8, OGT increased mucosal PGE2 levels, and when the COX-1/COX-2 inhibitor indomethacin was added to the reaction, the production of mucosal PGE2 in OGT-treated mice decreased (Fig. 9). Furthermore, addition of the COX-2 se-

Table 1. Change of PGE2 Levels in Small Intestine Induced by 2 Injections of Indomethacin (20 mg/kg)

Treatment	Mucosa (ng/100 mg tissue)	Muscularis propria (ng/100 mg tissue)
None (n=5)	35.5±7.3	117.9±31.4
Indomethacin (n=5)	4.23±0.96#	14.9±2.0#

Indomethacin was injected using the same protocol as in Fig. 1 and small intestines were obtained 24 h after the second indomethacin injection. # p<0.05 *vs.* non-treated group.

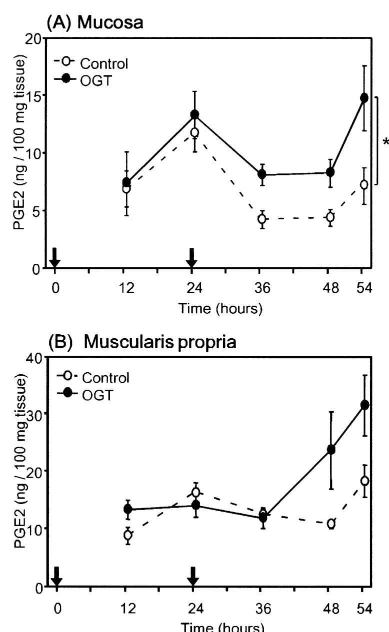

Fig. 7. Effect of OGT on PGE2 Levels in the Intestinal Mucosa (A) and Muscularis Propria (B) in Indomethacin-Injected Mice

Indomethacin was injected at hour 0 and 24 (arrows in graphs). Mice were killed 12, 24, 36, 48, or 54 h after the first indomethacin-injection. Mucosal PGE2 levels in the OGT-administered group differed significantly from the control group (** p<0.005). n=5 for each group except for the control group at 54 h (n=4). Data represent mean ±S.E. Two-way ANOVA with treatment and time as factors followed by Scheffe's *post hoc* analysis were used.

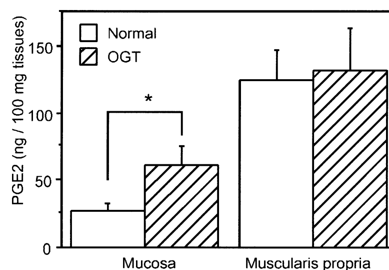

Fig. 8. Effect of OGT on PGE2 Levels in the Intestinal Mucosa of Mice Not Treated with Indomethacin

Intestinal tissue samples taken from mice were fasted for 24 h and then re-fed the OGT-containing or normal diet for 48 h without indomethacin-injection. PGE2 levels were significantly increased by OGT alone (* p<0.05 *vs.* control). Data represent mean ±S.E. (n=5) from 1 of 3 replications. Statistical analysis was performed using the Mann–Whitney U test.

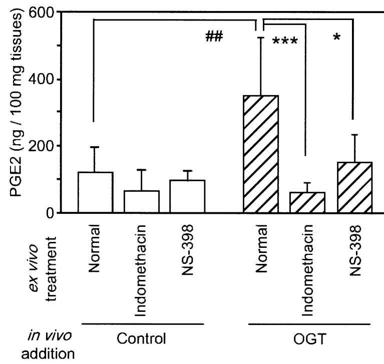

Fig. 9. Influence of COX Inhibition on PGE2 Production in Tissue Homogenates of the Intestinal Mucosa

Mice were fasted for 24 h and then re-fed the OGT-containing or normal diet for 48 h without indomethacin-injection. PGE2 levels were significantly increased by OGT but when indomethacin or NS-398 was mixed with the tissue homogenate, it was inhibited significantly. Data represent mean±S.E. ($n=6$). Two-way ANOVA followed by Scheffe's *post hoc* analysis were used. $^{\#\#}p<0.005$ *vs*. the reaction mixture without COX inhibitor obtained from normal mice, $^{*}p<0.05$, $^{***}<0.0005$ *vs*. the reaction mixture without COX inhibitor obtained from OGT-treated mice.

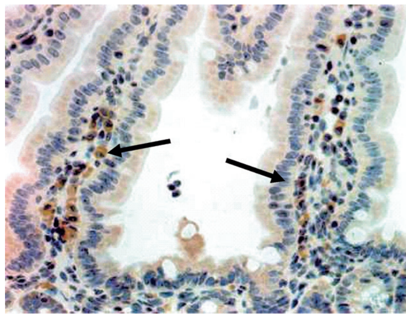

Fig. 10. Localization of COX-2 Expressing Cells

Positive cells were stained brown immunohistochemically. COX-2 expressing cells (arrow) of the small intestine obtained from naive mice were mainly seen in lamina propria. In accordance with previous studies[22,53] COX-1 expressing cells were found throughout the small intestine, rarely in the villus, diffusely in the lamina propria, and ubiquitously in intestinal smooth muscle (data not shown). Original magnification; 100×.

lective inhibitor NS-398 to the reaction also reduced the production of mucosal PGE2 in OGT-treated mice (Fig. 9). This data suggest that the increase in PGE2 in OGT-treated tissue is due to COX-2.

To further clarify the influence of COX-2, COX-2 expressing cells were stained immunohistochemically. COX-2 expressing cells were detected in the lamina propria (Fig. 10). The number of mucosal COX-2 expressing cells was decreased by indomethacin, and OGT significantly abrogated the decrease in the number of COX-2 expressing cells at 12 h after the indomethacin injection (Fig. 11).

OGT Modulates Immunological Properties in Lamina Propria Cells Lastly, we tested whether OGT influences PGE2 and cytokine production by LPMC (Fig. 12). Lamina propria is a major site of mucosal PGE2 production, which has recently been reported to lead to production of IL-10, a key cytokine involved in mucosal tolerance.[5,6,22,24] In agreement with previous studies,[5,24] we observed that LPMC produced PGE2 and IL-10 spontaneously (Fig. 12A-a and c). *In vivo* addition of OGT increased those mediators whereas it decreased IL-1β (Fig. 12A-b). A similar effect of OGT was observed in LPMC that were prepared from small intestine injured by indomethacin-treatment (Fig. 12B).

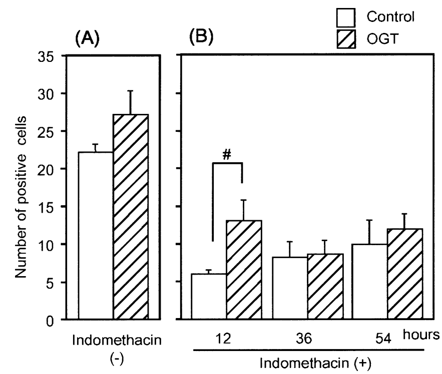

Fig. 11. OGT Prevented the Degradation of COX-2 Expressing Cells in the Lamina Propria by Indomethacin

Indomethacin was injected to mice at hour 0 and 24. Tissues from mice killed 12, 36, or 54 h after the first indomethacin-injection were used for immunostaining of COX-2. COX-2 expressing cells were counted in 5 random high-power fields per mouse (200× magnification). Indomethacin treatment decreased the number of COX-2 expressing cells in LPMC. OGT administration significantly inhibited the decrease in the number of COX-2 expressing cells as compared with the control group at 12 h after indomethacin treatment. Data represent mean±S.E. ($n=5$). Statistical analysis was performed using the Mann–Whitney U test. $^{\#}p<0.05$ *vs*. control mice at 12 h after indomethacin treatment.

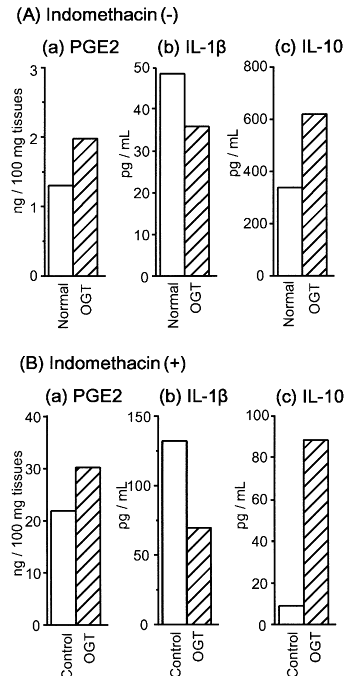

Fig. 12. OGT Modulated Immunological Properties in LPMC

(A) Mice were fasted for 24 h and then re-fed the OGT-containing or normal diet for 48 h without indomethacin-injection. Percoll-fractionated LPMC were cultured for 24 h without OGT or any stimuli. Cells were collected from 3 mice, and the data represent 1 of 2 replications. (B) Small intestines were obtained from mice 48 h after the first injection of indomethacin. Semi-purified LPMC (the filtrate from a glass wool column without Percoll fractionation) were collected from 3 mice and cultured for 24 h without OGT or any stimuli. Data represent 1 of 2 replications. Differences in the levels of mediators between (A) and (B) were due to differences in cell preparation procedures.

DISCUSSION

In this study we have investigated the effect of OGT on NSAID-induced enterophathy. We found that OGT not only prevented the enteropathy but also reduced the lethality of indomethacin treatment in mice. Administration of indomethacin subcutaneously at 20 mg/kg (once daily for 2 d) provoked severe hemorrhagic lesions and decreased PGE2

levels in the small intestine, and 80% of the mice died within 9 d (Fig. 1). Many agents have been found to have protective effects in the indomethacin-induced enteropathy model which results in relatively milder injury and little lethality.[26—32] So far, only human glucagon-like peptide 2 has been reported to be protective against indomethacin-induced lethality.[21)]

We also investigated the effect of OGT on intestinal PGE2 levels. OGT significantly increased the mucosal PGE2 levels and PGE2 production in LPMC both in the presence and absence of indomethacin-injection (Figs. 7, 8, 12). These results may explain the multiple actions of OGT, namely: 1) its effect on survival rate with pre- and co-treatment but not with post-treatment (Fig. 2); 2) its ability to prevent legions from worsening over time (Fig. 4C), and; 3) its ability to restore PGE2 levels after initial depletion by indomethacin (Fig. 7A). Even though the elevation of PGE2 levels induced by OGT was moderate, even a slight difference in mucosal PGE2 levels can produce profound differences in mucosal injury, as reported by Bertrand *et al.*[33] In their report, indomethacin at a dose of 3 mg/kg did not induce ulceration, although it decreased PGE2 levels (approximately 14 ng/100 mg tissue in treated mice *versus* 34 ng/100 mg tissue in naive mice). However, indomethacin at higher doses (≧10 mg/kg) induced a proportional decrease and increase, respectively, in intestinal PGE2 levels (less than 11 ng/100 mg tissue) and damage scores. Since the PGE2 levels reported by Bertrand *et al.* were similar to those in the present study, it is reasonable to assume that the 2-fold elevation of PGE2 by OGT contributes to protection against mucosal injury. However, due to the differences in animal species, treatment protocols, and the methods of measuring PGE2 between their study and ours, further investigation is necessary to verify this point. Furthermore, in the present study OGT also increased the production of PGE2 and IL-10 by LPMC, and decreased that of IL-1β (Fig. 12). IL-10 is a well known anti-inflammatory and immunoregulatory cytokine, and IL-10-deficient mice develop chronic IBD.[15] According to Newberry *et al.*,[5,24] PGE2 orients the T cell-mediated response towards immunosupression by several possible mechanisms, including inhibition of pro-inflammatory cytokines and downregulation of T cell activation. OGT therefore may immunomodulate the T cell-mediated response *via* PGE2.

In the present we speculated that the increase in mucosal PGE2 by OGT was caused by induction of COX-2-expressing cells in the lamina propria. There are several possible explanations by which OGT could increase COX-2 expressing cells in lamina propria. First, OGT may directly inhibit apoptosis induced by indomethacin. It is well known that indomethacin induces apoptosis and suppression of cell growth in several cell types,[34,35] and we previously showed that OGT can inhibit dexamethasone-induced thymocyte apoptosis.[36] Secondly, OGT may induce the migration of COX-2 expressing cells into the lamina propria. Even without indomethacin injection, OGT was able to increase mucosal PGE2 levels (Fig. 8) and tended to increase COX-2 expressing cells in the lamina propria (Fig. 11). Luminal contents, including food mixtures and bacteria, have been known to affect the distribution of lamina proprial cells *via* the interaction with lamina proprial dendritic cells which can recognize luminal antigen directly, or indirectly after the uptake and presentation by M cells.[37,38] Thus, OGT and its metabolites may be recognized by dendritic cells and affect the distribution of lamina proprial cells directly. Alternatively, because OGT and its ingredients can influence bacterial growth and production of enterotoxin,[39,40] the OGT-induced change in intestinal bacteria, might cause the migration of COX-2 expressing into the lamina propria. Finally, OGT could conceivably directly or indirectly induce COX-2 mRNA and/or protein. Further detailed investigations are needed to clarify the mechanism of action of OGT.

In a previous study, we demonstrated that OGT potently suppresses the formation of aberrant crypt foci in azoxymethane-treated mice,[20] and berberine, one of the major ingredients of OGT, has been shown to inhibit COX-2 activity[41] and repress transcription of COX-2 in colon cancer cells.[42] COX-2 plays a critical role in colon tumorigenesis and COX-2 inhibitors have been shown to have a potent protective effect on clinical and experimental colon cancer. Furthermore, suppression of PGE2 production by OGT has also been reported in the TNBS-induced colitis model.[19] These findings had initially led to the assumption that OGT may have NSAID-like properties. However, in the present study we found that OGT pretreatment does not exacerbate NSAID-induced enteropathy but effectively inhibits NSAID-induced severe intestinal injury (Figs. 3, 4) and enhances mucosal PGE2 production (Figs. 7A, 8, 12). This discrepancy may be reconciled by the following explanations; COX-2 expression and PGE2 production in colon tumorigenesis and inflammation predominantly occur in epithelial-type cells under malignant alteration (in colorectal adenomatous polyps and carcinomas) or severe inflammation (in the TNBS-induce colitis model). Under those conditions, OGT and its ingredients made contact with epithelial-type cells directly and inhibited COX-2 activity. Conversely, the OGT-induced increase in COX-2 and PGE2 found in the present study is presumed to occur in LPMC located in the lamina propria, where a direct interaction with intraluminal drugs is prevented by a layer of epithelial cells. Under normal conditions, the lamina propria is a major site of PGE2 production in the intestinal mucosa.[24] In the present study, isolated LPMC that was removed almost of all the epithelial cells produced PGE2. The difference between cell types can resultin different responses to OGT. Furthermore, the different ingredients in OGT may show differential ability to reach and act on these respective cell types, one of which is located at the luminal surface and the other inside in the lamina propria. It is therefore possible that OGT has opposing effects on PGE2 production depending on the immunological condition of the intestinal mucosa in which the several type of cells (epithelial-type cell and LPMC) play a dominant role in PGE2 production.

In addition to regulating PGE2 levels, OGT has other beneficial effects for the prevention of enteropathy. Firstly, OGT and one of its main ingredients, berberine, exhibit antimicrobial activity.[39,40] Bacterial translocation plays a major pathogenic role in the development of enteropathy.[43,44] Secondly, it was found that OGT prevented serum NOx elevation induced by indomethacin (Fig. 6), and in a previous study, it was found that OGT strongly inhibits inducible nitric oxide synthase (iNOS) induction in lipopolysaccharide-stimulated macrophages.[45] NOx production is a contributing factor in the pathogenesis of enteropathy.[31,32,46,47] It has been reported

that the iNOS is closely related to the development of intestinal microvascular injury induced by indomethacin.[47] OGT influences microcirculatory blood flow, which is one of the mechanisms responsible for the efficacy of OGT.[48] Finally, there are many reports that OGT has anti-inflammatory effects,[49—52] such as the ability to inhibit rat paw oedema.

Efforts to identify the active ingredients of OGT and the properties of each ingredient are now under way. Mice received diet containing OGT-constituent herbs at a concentration of 2% Oren (Coptidis rhizoma), Obaku (Phellodendri cortex), and Ogon (Scutellariae radix) showed improved survival rates in this model (90, 70 and 60% survival, respectively *versus* 40% survival in the control). More than 40% of the mice that received 0.07 % berberine, the major ingredient of Oren and Obaku, survived (0% in indomethacin-injected control mice). Further studies are needed to elucidate the effects of these herbs and their ingredients on PGE2 production in the intestinal mucosa.

In conclusion, we have shown that OGT can significantly attenuate the lethality, intestinal lesions, and mucosal PGE2 reduction induced by indomethacin. We have also shown that treatment with OGT increases mucosal PGE2 and PGE2 production from LPMC. Our findings suggest that OGT may have therapeutic value not only for the reduction of adverse reactions to NSAIDs but also for the treatment of human intestinal disorders like as IBD.

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