Perilla frutescens extract ameliorates DSS-induced colitis by suppressing proinflammatory cytokines and inducing anti-inflammatory cytokines

Hayato Urushima,1 Junichi Nishimura,2 Tsunekazu Mizushima,2 Noriyuki Hayashi,1 Kazuhisa Maeda,1 and Toshinori Ito1,2

1Department of Complementary & Alternative Medicine, Osaka University Graduate School of Medicine, Suita City, Osaka, Japan; and 2Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Suita City, Osaka, Japan

Submitted 7 August 2014; accepted in final form 26 October 2014

Urushima H, Nishimura J, Mizushima T, Hayashi N, Maeda K, Ito T. Perilla frutescens extract ameliorates DSS-induced colitis by suppressing proinflammatory cytokines and inducing anti-inflammatory cytokines. Am J Physiol Gastrointest Liver Physiol 308: G32–G41, 2015. First published October 30, 2014; doi:10.1152/ajpgi.00294.2014.—Anti-inflammatory effects have been reported in Perilla frutescens leaf extract (PE), which is a plant of the genus belonging to the Lamiaceae family. We examined the effect of PE on dextran sulfate sodium (DSS)-induced colitis. Preliminarily, PE was safely administered for 7 wk without any adverse effects. In the preventive protocol, mice were fed 1.5% DSS solution dissolved in distilled water (control group) or 0.54% PE solution (PE group) ad libitum for 7 days. In the therapeutic protocol, distilled water or 0.54% PE solution was given for 10 days just after administration of 1.5% DSS for 5 days. PE intake significantly improved body weight loss. The serum cytokine profile demonstrated that TNF-α, IL-17A, and IL-10 were significantly lower in the PE group than in the control group. In the therapeutic protocol, mice in the PE group showed significantly higher body weight and lower histological colitis scores compared with mice in the control group on day 15. The serum cytokine profile demonstrated that TGF-β was significantly higher in the PE group than in the control group. In distal colon mRNA expression, TNF-α, and IL-17A were significantly downregulated. In vitro analyses of biologically active ingredients, such as luteolin, apigenin, and rosmarinic acid, in PE were performed. Luteolin suppressed production of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-17A. Apigenin also suppressed secretion of IL-17A and increased the anti-inflammatory cytokine IL-10. Rosmarinic acid increased the regulatory T cell population. We conclude that PE might be useful in treatment and prevention of DSS-induced colitis.

interleukin-17A; Perilla frutescens; T helper 17; tumor necrosis factor-α; regulatory T cells

INFLAMMATORY BOWEL DISEASE (IBD), such as Crohn’s disease and ulcerative colitis, is an intractable and chronic disease characterized by repeated exacerbation and remission of symptoms. Because of the involvement of multifactorial interactions between genetic factors and environmental triggers, the pathogenesis of IBD has not yet been elucidated (24). However, inappropriate and ongoing activation of the mucosal immune system caused by breakdown of immunological tolerance to exogenous antigens or luminal flora are believed to be one of the causes of IBD, which lead to excessive secretion of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α (27, 33, 37).

In recent years, the efficacy of anti-TNF-α antibody therapy on IBD was reported (11, 49). However, there are some adverse events due to anti-TNF-α therapy, such as infusion reaction, opportunistic infection (6, 22), and attenuation of effects caused by appearance of neutral antibodies (5, 11, 42). In addition, several issues, such as the safety of long-term use of anti-TNF-α antibody and economic burden, also remain unresolved.

Recently, it was reported that several functional foods, such as phytochemicals, probiotics, dietary fiber, and omega-3 fatty acid, influenced cytokine secretion by immune cells. These foods are known as complementary medicines for IBD (15). Especially, it is important to confirm the safety of these functional foods when used in some kinds of the immunocompromized hosts, such as IBD patients. In Japan, the leaves of Perilla frutescens are traditionally used as garnish on raw fish for detoxification. These leaves are also used medicinally as a sedative (13) and for indigestion (25). Dried Perilla leaves, “soyou,” are also used as one of the components of kampo medicine, such as “saibokuto,” “hange-kobokuto,” and “kosonan,” for treatment of cough and stress symptoms. P. frutescens leaves extract (PE), extracted into hot water, has shown anti-inflammatory activity by inhibiting TNF-α production (46, 47). It has been reported that PE has also shown efficacy for suppression of production of anti-dinitrophenyl IgE, production of Th2-type cytokines, such as IL-4, systemic allergic reaction induced by compound 48/80, and IgA nephropathy (18, 19, 29, 43).

In terms of the immunological aspects in IBD pathogenesis, we have recently shown that a new sphingosine 1-phosphate receptor agonist, W-061, ameliorated colitis induced by suppression of homing of T helper 17 (Th17) into lamina propria with dextran sulfate sodium (DSS) in wild-type mice, which are immunologically competent (41). In this experiment, we confirmed that Th17 and regulatory T cells (Tregs) in lamina propria had some important roles for the pathogenesis of colitis.

We hypothesized PE may ameliorate DSS-induced colitis through its anti-inflammatory properties. Therefore, in the present study, we investigated the efficacy of PE on two different protocols, preventive and therapeutic experiments, in a DSS-induced colitis model focusing the balance between Th17 and Treg.

MATERIALS AND METHODS

Safety of long-term PE intake. Five-week-old C57BL/6 female mice were given distilled water or 0.54% PE (Amino UP Chemical, Hokkaido, Japan) for 7 wk. Thereafter, mice were killed, and serum
Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride, and total cholesterol (all purchased from Wako Chemical, Tokyo, Japan) were analyzed.

Lymphocytes were isolated from the lamina propria, and the population of Tregs was analyzed by flow cytometry. Moreover, we evaluated serum cytokine levels of IL-10 and TGF-β, which were mainly secreted by Tregs using an ELISA kit (eBioscience, San Diego, CA).

Prevention experiment. Eight-week-old C57BL/6 female mice were divided into two groups (n = 6). On day 1, mice were fed 1.5% DSS (MP Biomedicals) solution dissolved in distilled water (control group) or PE (PE group) ad libitum for 7 days. For each mouse, we measured food and drink intake and body weight. On day 8, mice were killed.

Therapeutic experiment. First, 8-wk-old C57BL/6 female mice were given 1.5% DSS solution for 5 days. After induced inflammation, the intake of DSS was stopped, and mice were divided into two groups. The control group was given distilled water, and the PE group received 0.54% PE from day 6 to day 15. We evaluated the histological score, rate of Foxp3+ cells gated by CD4 cells, and mRNA expression of several cytokines in the distal colon on day 10. We also measured the levels of serum cytokines on day 15.

Histological score. The distal colon tissues were fixed in buffered 4% paraformaldehyde. Five-micrometer paraffin-embedded colon

**Fig. 1. Safety evaluation and immunological aspects of long-term Perilla frutescens leaf extract (PE) intake.** Mice were given distilled water or 0.54% PE for 7 wk. A: there was no significant difference in the percent body weight gain between the control and PE groups after 7 wk of treatment. B: the level of aspartate aminotransferase (AST) was significantly lower in the PE group (P < 0.05). There were no differences in the levels of serum alanine aminotransferase (ALT), triglyceride (TG), and total cholesterol (T-chol) between the two groups. PE is also likely to induce CD4+ Foxp3+ regulatory T cells (Tregs) after treatment for 7 wk. In addition, compared with the control group, the PE group had a tendency to show increasing serum IL-10. Results are expressed as means ± SD. *P < 0.05.
sections were stained with hematoxylin and eosin. The degree of colitis was measured using the modified scoring system of Iba et al. (16). Inflammatory colitis was scored from zero to three for lesions on the basis of loss of epithelium, length of crypts, depletion of goblet cells, and infiltration of leukocytes. The total histological score ranged from 0 to 12, which represented the summed scores of histological changes.

Isolation of lymphocyte. Lymphocytes were isolated from spleen and lamina propria using a modification of the method reported by Atarashi et al. (3). Briefly, for isolation from lamina propria lymphocytes, intestines were opened longitudinally, washed with ice-cold PBS to remove fecal content, and shaken in HBSS (Wako Chemical) supplemented with 2% FBS and 5 mM EDTA for 20 min at 37°C. Intestines were then cut into small pieces and shaken with RPMI 1640 containing 2% FBS and 1 mg/ml collagenase type II for 25 min at 37°C. Digested tissues were resuspended with 8 ml of 40% Percoll (GE Healthcare) and overlaid on 4 ml of 75% Percoll. Gradient separation was performed by centrifugation at 760 g for 20 min at 37°C. Lymphocytes were collected at the interface of the Percoll gradient, washed, and suspended in RPMI 1640 containing 2% FBS.

Th17 and Treg population of lamina propria on day 10. On day 10 of the therapeutic protocol, lymphocytes were isolated from colonic lamina propria and spleen. Next, cells were stained with antimouse specific antibodies, Foxp3-FITC, IL-17A-phycoerythrin, and CD4-PE Cy5 (BD Biosciences, San Jose, CA). After staining, a fluorescence-activated cell sorter (FACS Calibur flow cytometer; Becton-Dickinson, Mountain View, CA) was used to analyze cells.

Quantitative real-time PCR analysis. Total RNA was isolated from the distal colon, and first-strand cDNA was synthesized using a ReverTra Ace-H9251 first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). A Light

Table 1. Serum cytokines on day 8 in the preventive protocol

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control Group</th>
<th>PE Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>34.7 ± 3.6**</td>
<td>23.0 ± 2.8**</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IL-1β</td>
<td>14.4 ± 1.1</td>
<td>13.7 ± 0.3</td>
<td>0.21</td>
</tr>
<tr>
<td>IL-6</td>
<td>201.9 ± 107.9</td>
<td>152.6 ± 92.6</td>
<td>0.23</td>
</tr>
<tr>
<td>IL-17A</td>
<td>659.6 ± 126.0*</td>
<td>374.0 ± 262.0*</td>
<td>0.037</td>
</tr>
<tr>
<td>IL-10</td>
<td>462.4 ± 122.8**</td>
<td>286.3 ± 47.7**</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TGF-β</td>
<td>29.5 ± 29.6</td>
<td>30.7 ± 16.4</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD; units are pg/ml. PE, Perilla frutescens leaf extract. *P < 0.05 and **P < 0.01.
Cycler system (Roche Diagnostics, Tokyo, Japan) was used to perform PCR amplification. Primer sequences were as follows: B-actin: forward 5'-TACCACAGCATTGATGG-3', reverse 5'-TTTGATGGCAGGACCTTTT-3'; TNF-α: forward 5'-CAGGTGAGCCTTGTGATGC-3'; IL-6: forward ACAACGACGACCTTACTGTG-3', reverse 5'-ATGCTGCTGCTTACTGTG-3'; IL-10: forward 5'-ATGCTGCTGCTTACTGTG-3', reverse 5'-ATGCTGCTGCTTACTGTG-3'; IL-17A: forward 5'-AGGACGCGCAAACATGA-3', reverse 5'-GCAACAGCATCAGAGACACAGAT-3'; TGF-β: forward 5'-GCAACAGCATCAGAGACACAGAT-3', reverse 5'-GCCCGTACGAGTTCG-3'; Foxp3: forward 5'-AGGAAGCTGGGAGCTATGC-3', reverse 5'-GGTGCTACGAGTTCG-3'. SYBR green dye (Light Cycler DNA Master SYBR green I; Roche Diagnostics) was used for detection of cDNA. Relative quantification of the signals was performed by normalizing their signals relative to the β-actin signals.

In vitro study. From 8-wk-old wild-type C57/BL6 female mice, mononuclear cells were obtained by gently pressing the spleen and mesenteric lymph nodes, filtering through 70-μm nylon meshes, and suspending in HBSS supplemented with 2% fetal bovine serum (FBS). Cells from lymphocytes were treated with red blood cell lysis buffer (eBioscience) before suspension. Isolated mononuclear cells (5.0 × 10⁶/well) were stimulated with 1 ng/ml lipopolysaccharide (LPS) in Dulbecco’s modified Eagle’s medium containing 10% FCS with or without a PE component [luteolin (LU): 10 μM, 25 μM; apigenin (AG): 5 μM, 50 μM; rosmarinic acid (RA): 5 μM, 25 μM; Wako Chemical]. After staining, cells were analyzed by flow cytometry.

Analysis of serum and supernatant cytokines. Cytokines in the serum and the supernatant were examined using a commercial ELISA kit according to the manufacturer’s instructions [IL-1β, IL-10, and TGF-β (eBioscience); IL-6, IL-17A, and TNF-α (Biolegend)].

Statistical analysis. Student’s t-test was used to examine differences between the control and PE groups. Differences were consid-

---

**Fig. 3. Therapeutic protocol.**

- **A:** After induction of colitis following administration of 1.5% dextran sulfate sodium (DSS) for 5 days, recovery of weight is earlier and weight is significantly higher in the PE group than in the control group on day 15 (108.4 ± 7 vs. 97.2 ± 7%, respectively, \( P = 0.032 \)).
- **B:** Colon length is longer in the PE group than in the control group (7.1 ± 0.5 vs. 6.3 ± 0.6 cm, respectively, \( P = 0.035 \)).
- **C:** On day 10, microscopic examination shows that goblet cells and villi are regenerated in the PE group (stained by hematoxylin and eosin).
- **D:** The histological score is lower in the PE group than in the control group (\( P < 0.05 \)). Results are expressed as means ± SD.
Likely to induce CD4+Foxp3+ Treg and decreased CD4+IL-17A+ Th17 cells in lamina propria at the recovery stage from colitis. However, there were no differences in the population of Treg and Th17 in spleen (B). Results are expressed as means ± SD. *P < 0.05 and **P < 0.01.

Preventive protocol. On day 8, the degree of weight loss was significantly smaller (94.0 ± 6.2 vs. 86.5 ± 6.1%, respectively, P < 0.05), and colon length was significantly longer in the PE group than in the control group (6.3 ± 0.4 vs. 5.2 ± 0.3 cm, respectively, P < 0.01) (Fig. 2A-C). Histologically, the colons of the PE group had less damaged epithelium and less infiltration of inflammatory cells than the control group (Fig. 2D), which resulted in significantly better histological scores in the PE group than in the control group (7.3 ± 1.6 vs. 10.3 ± 0.5, respectively, P < 0.01) (Fig. 2E). Serum levels of TNF-α, IL-17A, and IL-10 were significantly lower in the PE group than in the control group (TNF-α: 23.0 ± 2.8 vs. 34.7 ± 3.6 pg/ml, respectively, P < 0.01; IL-17A: 334.0 ± 262 vs. 659.6 ± 126 pg/ml, respectively, P < 0.05; IL-10: 286.30 ± 48 vs. 462.4 ± 123 pg/ml, respectively, P < 0.01). However, there were no differences in the levels of IL-1β, IL-6, and TGF-β between the two groups (Table 1).

Therapeutic protocol. Body weight was significantly higher in the PE group than in the control group (108.4 ± 7.2 vs. 97.2 ± 7.2%, respectively, P < 0.05) (Fig. 3A). On day 15, the colon was significantly longer in the PE group than in the control group (7.1 ± 0.5 vs. 6.3 ± 0.6 cm, respectively, P < 0.05) (Fig. 3B). Histologically, the epithelial architecture of the PE group was well preserved on day 10 (Fig. 3C). Consequently, the histological scores were significantly lower in the PE group than in the control group on day 10 (3.5 ± 1.9 vs. 7.3 ± 2.1, respectively, P < 0.05) (Fig. 3D).

Table 2. Serum cytokines on day 15 in the therapeutic protocol

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control Group</th>
<th>PE Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>10.5 ± 1.6</td>
<td>11.2 ± 1.1</td>
<td>0.44</td>
</tr>
<tr>
<td>IL-6</td>
<td>95.3 ± 31.1</td>
<td>106.6 ± 64.6</td>
<td>0.61</td>
</tr>
<tr>
<td>IL-17A</td>
<td>121.2 ± 47.0</td>
<td>107.6 ± 36.4</td>
<td>0.59</td>
</tr>
<tr>
<td>IL-10</td>
<td>62.9 ± 19.6</td>
<td>45.8 ± 13.6</td>
<td>0.11</td>
</tr>
<tr>
<td>TGF-β</td>
<td>64.7 ± 6.7**</td>
<td>81.0 ± 10.8**</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD. Units are pg/ml. **P < 0.01.
In the PE group, mRNA expressions of TNF-α and IL-17A in the distal colon were significantly suppressed relative to those in the control group on day 10 (TNF-α: 0.39 ± 0.2 vs. 0.89 ± 0.5 arbitrary units, respectively, \( P < 0.05 \); IL-17A: 0.13 ± 0.05 vs. 0.37 ± 0.3 arbitrary units, respectively, \( P < 0.05 \)). In addition, there was a tendency to suppress IL-6 mRNA expression in the PE group on day 10 (\( P = 0.08 \)) (Fig. 4).

On day 10, CD4\(^+\) Foxp3\(^+\) T cells in lamina propria were significantly higher in the PE group than in the control group.

---

**Fig. 6. Influence of each ingredient for mononuclear cells.** Mononuclear cells were isolated from wild-type mice, stimulated by lipopolysaccharide (LPS), and cocultured with each ingredient for 48 h. Cytokines in supernatant were examined using ELISA. Each value is the mean ± SD (\( n = 3 \) experiments). Statistically significant differences from the treatment with LPS only were analyzed by \( t \)-test and are each indicated by asterisks (*\( P < 0.05 \) and **\( P < 0.01 \)).
Fig. 7. Treatment with luteolin and apigenin decreased CD4^+ IL-17^+ T cells. A and B: isolated splenic mononuclear cells from wild-type mice were stimulated by LPS with or without luteolin or apigenin for 48 h and analyzed by flow cytometry. Statistically significant differences from the treatment with LPS only were analyzed by t-test and are each indicated by asterisks (*P < 0.05 and **P < 0.01). C: rosmarinic acid had no influence for CD4^+ IL-17^+ T cells. D: representative data of luteolin treatment.

Fig. 8. Treg cells were increased by treatment with rosmarinic acid. Mononuclear cells were isolated from mesenteric lymph nodes of wild-type mice, stimulated by LPS with or without rosmarinic acid for 48 h, and analyzed by flow cytometry (n = 3). A: representative data of TGF-β^+ CD4^+ T cells. B and C: TGF-β^+ CD4^+ T cells and Foxp3^+ CD4^+ T cells are increased by rosmarinic acid in a dose-dependent manner. Statistically significant differences from the treatment with LPS only as control were analyzed by t-test and are each indicated by asterisks (*P < 0.05 and **P < 0.01). D: mRNA was isolated from stimulated lymphocytes, and expression of Foxp3 was examined by qPCR. By treatment with 50 μM rosmarinic acid, expression of Foxp3 mRNA is significantly increased relative to that from treatment with LPS only. E: there was no influence for Foxp3^+ CD4^+ T cells by treatment with apigenin. F: treatment with 25 μM luteolin significantly suppressed Foxp3^+ CD4^+ T cell differentiation.
induction of CD4+ T cells in lamina propria were significantly lower in the PE group than in the control group (7.3 ± 0.6 vs. 9.3 ± 1.0%, respectively, P < 0.01) (Fig. 5A). However, there were no differences in splenic CD4+ Foxp3+ T cells and CD4+ IL-17A+ T cells between the control and PE group (Fig. 5B). Serum levels of TGF-β were significantly higher in the PE group than in the control group on day 15 (81.0 ± 10.8 vs. 64.7 ± 6.7 pg/ml, respectively, P < 0.01). For the other serum cytokines, there were no differences between the two groups (Table 2).

In vitro analysis of biologically active ingredients in PE. As shown in Fig. 6, TNF-α, IL-1β, IL-6, and IL-17A in the culture supernatants under LPS stimulation were significantly suppressed by addition of 25 μM LU. The levels of both TNF-α and IL-6 were not affected by 50 μM AG, but IL-17A and IL-18 were significantly suppressed by 50 μM AG. Production of IL-10 was significantly increased by 50 μM AG.

Treated with 25 μM RA, secretion of IL-1β was significantly suppressed. However, TNF-α, IL-6, and IL-17A were not affected by RA treatment. After observing inhibition of IL-17A in supernatants by addition of LU and AG, we used flow cytometry to examine the influence of these ingredients on induction of CD4+ IL-17+ T cells. CD4+ IL-17A+ T cells, which were significantly induced by LPS stimulation, were significantly suppressed by LU and AG (Fig. 7, A and B). There was no influence for CD4+ IL-17A+ T cells by RA treatment (Fig. 7C). These results were compatible with those of the ELISA assay and suggested that LU and AG might suppress induction of CD4+ IL-17A+ T cells in an inflammation state.

Because of the increases in IL-10 and TGF-β in culture supernatants by RA, we hypothesized that RA may increase Treg, so we investigated the influence of RA on the differentiation of Tregs by flow cytometry. CD4+ TGF-β+ T cells and CD4+ Foxp3+ T cells were significantly increased by addition of RA (Fig. 8, B and C). Moreover, the expression of Foxp3 in lymphocytes was also significantly enhanced (Fig. 8D). There was no influence for CD4+ Foxp3+ T cells by AG treatment (Fig. 8E), and CD4+ Foxp3+ T cells were significantly suppressed by 25 μM LU treatment (Fig. 8F).

**DISCUSSION**

We showed that PE had some preventive and restorative efficacy for DSS-induced colitis via suppression of proinflammatory cytokines and induction of anti-inflammatory cytokines. It has been reported that several bioactive compounds are present in *P. frutescens* leaves (17). Some volatile components, such as perillaldehyde and perilla ketone, which induce skin allergic reactions (48), were removed during the manufacturing process for the PE used in this study. In *P. frutescens*, LU, AG, and RA are representative bioactive ingredients. This PE contains LU (10.5 μM), AG (13.7 μM), and RA (207.2 μM).

These ingredients have been reported to decrease secretion of TNF-α and IL-6 from macrophages by inhibition of the NF-κB pathway (20, 26, 34, 36, 40). In addition, we previously reported in the treatment of DSS for 5 days that Th17 cells significantly increased as early as on day 6 and then decreased on day 10, whereas Th1 cells significantly increased on day 10 with a delay in lamina propria (41). Therefore, we examined the influence of each ingredient on cytokine secretion of splenic mononuclear cells, which consist of macrophages, dendritic cells, and lymphocytes. PE suppressed TNF-α mRNA expression in distal colon, and the ingredients inhibited the production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in vitro. In addition to these proinflammatory cytokines, IL-17A, which is secreted by Th17 cells, is also a crucial cytokine in IBD (9, 12, 14, 45). In this study, PE significantly decreased secretion of serum IL-17A and expression of colonic IL-17A mRNA. Both LU and AG suppressed Th17 activity and secretion of IL-17A. Moreover, IL-1β seemed to have an important role in differentiation of human Th17 (1, 7). LU, AG, and RA inhibited secretion of IL-1β, which suggested that these ingredients may suppress differentiation of Th17 in the human gut. Taken together, these results suggested that these ingredients contained in PE suppressed proinflammatory cytokines from mononuclear cells, which led to a decrease in DSS-induced colitis.

In contrast, immunoregulatory cells, especially CD4+ Foxp3+ Tregs, are known to be involved in the maintenance of immunological self-tolerance and immune homeostasis, which has an important role in regulation of IBD pathogenesis (23, 50). Tregs secrete anti-inflammatory cytokines, such as IL-10 and TGF-β. IL-10-deficient mice spontaneously develop IBD-like colitis (31, 44). In addition, the use of anti-TGF-β or anti-IL-10 antibodies exacerbated colitis (2, 38, 39). In the present study, however, we used immunocompetent C57BL6 mice because...
the analysis of Tregs cannot be evaluated in IL-10-deficient mice. At first, a tendency of Treg induction was observed in the long-term administration of PE. We also confirmed that PE increased CD4+ Foxp3+ Tregs in the lamina propria on day 10 of the therapeutic protocol, which suggested that PE not only suppresses proinflammatory cytokines but also induces Tregs, which then alleviates colitis. RA increased production of IL-10 and TGF-β and expression of Foxp3 mRNA in lymphocytes. Moreover, we found that RA increased the incidence of Foxp3+ cells and TGF-β+ cells in the CD4+ T cell population. These results suggested that RA at least might promote the differentiation of naïve T cells into Tregs in the inflammatory site. AG also increased production of IL-10, whereas there was no effect on CD4+ Foxp3+ T cells (Fig. 8E). Moreover, AG did not increase expression of Foxp3 mRNA in lymphocytes (Fig. 8D). In addition to Tregs, several cell types, such as Tr1 and M2 macrophages, also secrete IL-10 (32), which suggested that AG might increase IL-10 through these cells.

It has been reported that IL-6 regulates the balance between Tregs and Th17 (4, 30). Namely, IL-6 induces Th17 differentiation from naïve T cells together with TGF-β. On the other hand, anti-IL-6 blockade induces Treg differentiation in the presence of TGF-β (45). LU but not AG or RA suppressed production of IL-6 from mononuclear cells, which suggested that LU suppressed differentiation of Th17 and promoted induction of Tregs indirectly by reduction of IL-6 at inflammatory sites. LU and AG ameliorated DSS-induced colitis when used singly (10, 35). However, it has also been reported that LU worsened DSS-induced colitis by blocking NF-kB-dependent protective molecules in enterocytes (21). In our study, LU suppressed the secretion of IL-10 from mononuclear cells and the differentiation into Treg. There is still a question as to whether LU has some efficacy for chronic use when used singly.

PE significantly increased Treg and decreased Th17 in lamina propria on day 10 in a therapeutic protocol. However, the efficacy was not observed in spleen. LU, AG, and RA interacts with the transport system in intestinal cells (8), suggesting that these ingredients might work locally at the colon. Besides LU, AG, and RA, some bioactive substances, such as scutellaran and caffeic acid in PE (28), may contribute to suppression of colitis. Further investigation will be needed. In this study, we examined the influence of each ingredient for mononuclear cells by in vitro study only. It may be speculative whether the mixed or single use of these components works in vivo or not. Further investigation is needed about whether each component influences for helper T cell differentiation directly or indirectly via macrophage and dendritic cells.

In conclusion, the individual compounds found in PE may additionally or synergistically work to regulate colitis due to not only suppression of proinflammatory cytokines and but also promotion of anti-inflammatory cytokines at certain stages of disease (Fig. 9), which suggests the efficacy of PE for prevention and in the mucosal wound-healing stage in IBD.

ACKNOWLEDGMENTS

We owe our deepest gratitude to Takehiro Miura and Takahiro Maeda, Amino Up Chemical, for tremendous support.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


