Effect of *Eucommia ulmoides* Leaf Extract on Chronic Dextran Sodium Sulfate-Induced Colitis in Mice

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Received November 1, 2017; accepted March 18, 2018

Ulcerative colitis (UC) is a refractory disease that causes chronic inflammation or ulceration in the mucosa of the large intestine with multiple relapses. Although several drugs, including 5-aminosalicylic acid, steroids, immunosuppressants, and infliximab, are used for UC therapy, patients suffer from side effects of these drugs, and a new safer therapeutic agent is desired. *Eucommia ulmoides* Oliv. leaf extract (ELE) has an anti-inflammatory effect. Therefore, we examined the effect of ELE on UC using a chronic dextran sulfate sodium (DSS)-induced colitis model in mice. Chronic DSS-induced colitis was triggered by alternately repeating 5 days’ DSS and 7 days’ water administration in mice for 29 d. The severity of DSS-induced colitis was evaluated by daily body weight and bloody stool score, and colon length and myeloperoxidase (MPO) activity in colon tissue on day 29. ELE (3 or 9%) was administered in combination by feeding for 29 d, and the effect on colitis was evaluated. The mice given DSS exhibited chronic colitis symptoms with body weight loss, increased bloody stool score and MPO activity, and shortened colon length. Administration of 3 or 9% ELE suppressed the body weight loss, bloody stool score, colon shortening, and MPO activity in a dose-dependent manner. Histological analysis showed that the ELE-treated mice had less damages and leukocyte infiltration in the mucosal layer of the large intestine compared to DSS alone group. These results suggested that ELE has the potential to prevent the development of DSS-induced colitis and a therapeutic effect on UC in a safe manner.

Key words *Eucommia ulmoides* leaf extract (ELE); ulcerative colitis; inflammation

Ulcerative colitis (UC) is a subtype of inflammatory bowel disease (IBD), and causes chronic inflammation with erosions and ulcers in the mucosa of the large intestine. UC is characterized by a refractory nature with multiple relapses, and it reduces patients’ QOL. Although the pathogenesis of UC is not completely understood, immune disorders caused by complications of genetic and environmental factors (e.g., eating habits and intestinal bacterial flora) are considered to be responsible for the development and exacerbation of UC. Intake of a high-fat diet has been shown cause an elevation of inflammatory cytokines (e.g., tumor necrosis factor (TNF-α) and interleukin (IL)-1β) in dextran sulfate sodium (DSS)-induced colitis, a pathological model of UC, and is associated with the development of UC. In fact, the prevalence of UC is rising, particularly in countries where the intake of high-fat diets is increasing. 5-Aminosalicylic acid (5-ASA) products (oral agent, enema, suppository), steroids, immunosuppressants (azathioprine, 6-mercaptopurine), and infliximab comprise the main drug treatments for UC. However, these drugs cannot bring about a complete cure in all patients, but their side effects also considerably reduce patients’ QOL. For these reasons, the development of new preventive and therapeutic strategies is a problem to be resolved.

*Eucommia ulmoides* Oliv. bark is traditionally known as a medicinal herb and is used as an analeptic, analgesic, sedative, antihypertensive, and diuretic. Products derived from roasted leaves of *Eucommia ulmoides* Oliv. are widely used as ‘Tochu-cha’ for health maintenance. Geniposide acid contained in ‘Tochu-cha’ is known to improve hypertension and hyperlipidemia. Previous studies showed that geniposide, which is an iridoid glycosides purified from the fruit of *Gardenia jasminoides* Ellis, suppressed inflammatory cytokine (TNF-α and IL-1β) release and neutrophil infiltration (myeloperoxidase activity) in the colitis model. Additionally, *Eucommia ulmoides* Oliv. leaf extract (ELE) decreased TNF-α levels in rat plasma, which was increased by administration of a high-fat diet. Thus, it is expected that ELE may be a safe therapeutic and preventative agent for UC. However, as far as we know, there are no reports on the effect of ELE on UC. Therefore, we examined the effect of ELE on UC using a chronic DSS-induced model in mice.

MATERIALS AND METHODS

**Materials** ELE (Lot No. 120521; moisture content: 3.4%, bulk density: 52mL/20g, geniposide acid content: 6.582%) was provided by Kobayashi Pharmaceutical Company (Osaka, Japan). ELE was blended at 3 and 9% (w/w) to MF (Oriental Yeast Company, Tokyo, Japan). DSS (molecular weight, 5000; sulfur content, 15–20%) and hydrogen peroxide were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). O-Dianisidine dihydrochloride, hexadecyl trimethyl ammonium bromide (HTAB) and myeloperoxidase (MPO) from human leukocytes were purchased from Sigma-Aldrich (Saint Louis, MO, U.S.A.).

**Animals** Five-week-old wild-type male ICR mice were purchased from Japan SLC (Shizuoka, Japan). Environmental conditions were maintained at a temperature of 24±2°C, hu-
midity of 50±10%, and 7 a.m.–7 p.m./7 p.m.–7 a.m. light/dark cycle. We confirmed their health for 1 week after arrival and used for experiments with freely accessible drinking water with or without DSS and pelleted diet with or without ELE. This study was performed in accordance with Research Ethics Committee of Ehime University (Approval Number: No. 05-M025-1).

Development of Chronic DSS-Induced Colitis and Measurement of the Stool Score and Colon Length Chronic DSS colitis was induced by alternately repeating 5 days’ DSS and 7 days’ water administration for 29 d in total. DSS administered at 5% for the first 5 d and 3.5% for the second and third cycle of DSS administration of the study (Fig. 1). ELE (3 or 9%) was administered in combination with feeding for 29 d, and the effect on colitis was evaluated during the administration. The severity of chronic DSS-induced colitis was evaluated by daily body weight and blood stool score, and colon length and MPO activity in colon tissue on day 29. Bloody stool score was calculated in accordance with the previous study: (0, normal-colored stool; 1, brown stool; 2, reddish stool; 3, bloody stool). At the end of the protocol, animals were sacrificed by cervical dislocation. The colons were dissected, their length from the ileocecal junction to the anal verge was measured and used as a morphometric measure of the degree of inflammation.

Measurement of MPO Activity MPO activity in colon tissue was calculated in accordance with the method described previously, and used as indicator of leukocyte infiltration. On day 29, the colons were dissected, rinsed with cold 0.9% saline, and cut into small pieces. Then, tissue extracts were prepared by homogenization in 50 mM phosphate buffer with 0.5% (w/v) HTAB. Supernatants were collected after centrifugation (15000 rpm, 10 min, 4°C). MPO activity was determined in 5 mM phosphate buffer with 0.5 mM o-dianisidine, 0.00005% (w/v) hydrogen peroxide, and 1 mg of protein. MPO activity was obtained from the slope of the reaction curve. Specific activity was expressed as the number of molecules of hydrogen peroxide converted per 5 min per milligram of protein.

Histologic Analyses Histologic analyses were conducted using hematoxylin–eosin (HE) staining by a method described previously. The colons isolated from normal, DSS-treated and DSS+ELE-treated mice were dissected, rinsed with cold 0.9% saline, and fixed in Mildform. Then, colon samples were embedded in paraffin before being cut into sections (thickness, 4 µm). For histologic examination, sections were stained first with Mayer’s hematoxylin, and then with eosin solution. Samples were mounted with mounting medium and inspected with the aid of a BZ-9000 microscope (Keyence, Osaka, Japan). Histological disease score was calculated according to a previous study: (0, normal colonic mucosa; 1, loss of one-third of the crypts; 2, loss of two-thirds of the crypts; 3, the lamina propria is covered with a single layer of epithelium and mild inflammatory cell infiltration is present; 4, erosions and marked inflammatory cell infiltration are present). First, we randomly selected 8 fields (500 µm square) in each section, followed by scoring as above. The mean in each section was calculated by scoring the grades in 8 fields.

Statistical Analysis Data are the mean±standard error of the mean (S.E.M.) Student’s t-test (colon length and MPO activity) or Mann–Whitney U test (histological disease score) was used to evaluate differences between two groups. For more than three groups, bloody stool score was analyzed statistically by Steel’s test and weight, colon length and MPO activity were analyzed by one-way ANOVA with Dunnett’s test. Differences were considered significant at p<0.05.

RESULTS

Effect of ELE on Symptoms Caused by Chronic DSS-Induced Colitis Administration of DSS decreased body weight in the first relapse period (Day 17) and obviously increased bloody stool score in the all relapse period (Days 5, 17 and 29). Administration of 3% ELE had a tendency to suppress body weight reduction in the first relapse period, and after Day 12 3% ELE significantly increased body weight compared to the control group (DSS alone) (normal diet). Administra-
tion of 9% ELE obviously showed suppression of body weight reduction in the first relapse period and significantly increased it after Day 17. The effect of 9% ELE on body weight on Day 29 was greater than that of the 3% ELE-treated group (Fig. 2A). Bloody stool score increased by DSS administration was significantly improved by 3 or 9% ELE administration compared to the control group (DSS alone) on Day 17. On Day 29, ELE administration suppressed bloody stool score in a dose-dependent manner. There was significant difference between the group that received 9% ELE and the control group (DSS alone) (Fig. 2B).

**Fig. 3.** Effect of *Eucommia ulmoides* Oliv. Leaf Extract (ELE) on Shortening of Colon Length Caused by Chronic Dextran Sulfate Sodium (DSS)-Induced Colitis in Mice

Length of the colon was determined at the end of the experimental period on Day 29. Values indicate the mean ± S.E.M. *p* < 0.05 vs. normal control group and *p* < 0.01 vs. DSS group. ( ), number of mice.

**Fig. 4.** Effect of *Eucommia ulmoides* Oliv. Leaf Extract (ELE) on Ellevation of Myeloperoxidase (MPO) Activity Caused by Chronic Dextran Sulfate Sodium (DSS)-Induced Colitis in Mice

Colonic MPO activity was determined at the end of the experimental period on day 29. Values indicate the mean ± S.E.M. *p* < 0.05 vs. normal control group and *p* < 0.05 vs. DSS group. ( ), number of mice.

**Fig. 5.** Histochemical Images Showing Effect of *Eucommia ulmoides* Oliv. Leaves Extract (ELE) on the Level of Injury in the Mucosa Caused by Chronic Dextran Sulfate Sodium (DSS)-Induced Colitis in Mice

A); normal mouse. B); mouse treated with DSS alone. C); mouse treated with DSS +9% ELE. Histologic analyses were determined at the end of the experimental on Day 29.

Effect of ELE on Shortening of Colon Length Caused by Chronic DSS-Induced Colitis In previous reports, shorting of the length of the large intestine was observed after DSS administration. In the present study, significant shortening of the large intestine was also observed after DSS administration compared to the normal controls on Day 29, as shown in Fig. 3. The administration of ELE dose-dependently ameliorated colon shorting induced by DSS administration. A significant difference was found between the group that received 9% ELE and the control group (DSS alone) (Fig. 3).
Effect of ELE on Elevation of MPO Activity Caused by Chronic DSS-Induced Colitis

The MPO activity, an indicator of inflammatory cell infiltration, was significantly increased by DSS administration compared to the normal controls on Day 29, as shown in Fig. 4. Administration of 3% ELE had a strong tendency to decrease MPO activity. The administration of 9% ELE significantly decreased MPO activity to the level of normal group (Fig. 4).

Effect of ELE on the Level of Injury in the Colon Mucosa Caused by Chronic DSS-Induced Colitis

As shown in Fig. 5, on Day 29, crypt loss, epithelial destruction and leukocyte infiltration were observed in the mucosal layer of the large intestine in DSS-treated mice compared to normal control mice (Fig. 5B). These phenomena were improved by 9% ELE administration (Fig. 5C). The histological disease score for the distal segment of the colon was 0.0 in the normal control group, whereas scores in the DSS alone group (normal diet) (2.06±0.08, n=4) were significantly higher than that in control. As shown in Fig. 6, 9% ELE treatment significantly decreased the scores (1.29±0.17, n=3). In the DSS+9% ELE-treated group, crypt loss, epithelial destruction and leukocyte infiltration observed in the mucosal layer of the large intestine in DSS alone group were significantly improved (Fig. 6).

DISCUSSION

The present study is the first report that ELE administration markedly suppressed the symptoms induced by chronic DSS-induced colitis in mice, suggesting that ELE can be effective for the treatment of UC. As a chronic experimental model of colitis with multiple relapses, mild DSS-induced colitis with both active and remission phase (data not shown) was induced in mice using our experimental protocol, with alternative administration of DSS and water. This model was considered to be suitable to examine the anti-colitis effect of ELE. Combined administration of 3 or 9% ELE with DDS suppressed both body weight reduction and increased bloody stool score resulting from DSS administration. These findings suggested that ELE is able to ameliorate the clinical symptoms of chronic colitis. In addition, ELE improved shortening of the colon, which provides a morphometric measure of the colitis inhibitory effect. Furthermore, ELE cancelled the elevation of MPO activity, an indicator of inflammatory cell infiltration, in colonic tissues of the chronic colitis to the normal levels. Additionally, these findings were supported by the present histological observation that the ELE-treated mice had less damages and leukocyte infiltration in the mucosal layer of the large intestine, compared to control group (DSS alone). From these results, it appears that ELE exerted anti-inflammatory effects in the chronic DSS-induced-colitis.

Many studies have shown that DSS-induced colitis involves elevated level of inflammatory cytokines (e.g., TNF-α and IL-1β), and reactive oxygen species (ROS) production. On the other hand, ELE decreased TNF-α levels in rat plasma increased by administration of a high-fat diet. Xu et al. demonstrated that geniposide, which is an iridoid glycosides purified from the fruit of Gardenia jasminoides Ellis, suppressed inflammatory cytokine (TNF-α and IL-1β) release and neutrophil infiltration (MPO activity) in the colon, and also ameliorated lipopolysaccharides-induced endothelial barrier dysfunction in Caco-2 cells. Jin et al. reported that geniposide acid has an anti-inflammatory action to decrease the plasma level of TNF-α and IL-1β protein in adjuvant-induced arthritis rats. Moreover, ELE has been reported to have radical scavenging action in vitro, and to elevate superoxide dismutase activity and catalase activity in erythrocytes in diabetes model mice. From these findings and the present results, the underlying mechanism of the anti-colitis effect of ELE may involve regulation of cytokines and ROS.

Cell death and up-regulated caspase 3-positive cells in the intestinal mucosa has also been reported to be involved in the development of DSS-induced colitis. ELE has been shown to suppress H2O2-induced rat osteoblastic MC3T3-E1 cell apoptosis by inhibiting the expression of caspases 3, 6, 7, and 9. Therefore, it is likely that ELE administration may suppress cell death of intestinal mucosal cells as a potential mechanism for its anti-colitis effect.

Furthermore, ELE is known to decrease cholesterol and triglyceride levels. Fujikawa et al. reported that the anti-obesity effect of ELE in high-fat diet conditions may include several mechanisms: enhancement of metabolic function across several organs; diminishment of ATP production in white adipose tissue; acceleration of β-oxidation in the liver; and increasing the use of ketone bodies/glucose in skeletal muscle. A more recent study revealed that asperuloside contained in ELE has anti-obesity and anti-inflammatory actions. ELE and asperuloside increased plasma adiponectin levels and decreased plasma TNF-α levels, compared with administration of only a high-fat diet in rats. In fact, the intake of a high-fat diet exacerbates DSS-induced colitis, and uptake of a high-fat diet is considered to be a risk factor for UC. Although we did not use a high-fat diet in the present study, it seems likely that the anti-obesity and anti-inflammatory effect of ELE become important for suppressing UC in clinical practice where ingestion of a high-fat diet is assumed. From these reports, it is very likely that geniposide acid and asperuloside, which are main component contained in ELE, have pharmacological effects including anti-inflammatory and anti-colitis actions.
in DSS-induced colitis mice. However, further study will be needed to examine detailed mechanisms of anti-inflammatory and/or anti-colitis effect of ELE.

In clinical guideline for UC patients, 5-ASA and steroidal anti-inflammatory drug are mainly recommended to treat it, but these medications have clinical problems to induce several side effects including glucose and/or lipid metabolism disorder, insomnia, and weight gain with an increased appetite. On the other hand, our previous study have demonstrated that ELE treatment decreased a blood glucose levels in type 2 diabetes model rat(8) and visceral fat in high-fat diet rat (not yet published) with no side effects. These results seem to show a safety aspect of ELE.

In conclusion, the present results suggest the ELE is able to prevent UC development and has a therapeutic effect on UC in a safe manner.

Acknowledgments We thank Mr. Takeshi Kiyoi at the Division of Analytical Bio-Medicine, the Advanced Research Support Center (ADRES), Ehime University for his technical assistance to carry out histological analysis. This work was supported by Japanese Society of Eucommia.

Conflict of Interest The authors declare no conflict of interest.

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