Beneficial effects of the traditional medicine Igongsan and its constituent ergosterol on dextran sulfate sodium-induced colitis in mice

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Abstract. Ulcerative colitis (UC) is a type of inflammatory bowel disease and is considered a chronic gastrointestinal disorder. Igongsan (IGS) is a Korean herbal medicine, which has been used to treat digestive disorders. However, the ameliorative effect and molecular mechanisms of IGS in intestinal inflammation have not yet been studied in detail. The present study aimed to investigate the protective effects of IGS and its constituent, ergosterol, in a mouse model of dextran sulfate sodium (DSS)-induced colitis. Colitis was induced in mice by supplementing their drinking water with 5% (w/v) DSS for 7 days. The effects of IGS were then determined on DSS-induced clinical signs of colitis, including weight loss, colon shortening, diarrhea and obscure/gross bleeding. In addition, the effects of IGS were determined on the expression levels of inflammation-associated genes in the colon tissue of DSS-treated mice. The results of the present study demonstrated that mice treated with DSS exhibited marked clinical symptoms, including weight loss and reduced colon length. Treatment with IGS attenuated these symptoms and also suppressed the expression levels of tumor necrosis factor-α and interleukin-6, as well as the expression of cyclooxygenase-2 in the colon tissue of DSS-treated mice. IGS also reduced the activation of the transcription factor nuclear factor-κB p65 in the colon tissue of DSS-treated mice. In addition, ergosterol was shown to attenuate the DSS-induced clinical symptoms of colitis in mice. In conclusion, the present study provided experimental evidence that IGS may be a useful therapeutic drug for patients with UC.

Introduction

Ulcerative colitis (UC) is a common inflammatory intestinal disease, which belongs to a group of conditions known as inflammatory bowel diseases (IBD) (1). The pathogenesis of UC is believed to result from interactions between various genetic, immune and environmental factors (2). UC is associated with intestinal characteristics and symptoms, including weight loss, bloody diarrhea, fever and shortening of the colon (3). General features of tissue damage in UC include ulceration of the mucosa, blunting and loss of crypts, as well as infiltration of inflammatory cells (4). These features frequently result in epithelial dysplasia and DNA damage with microsatellite instability, which may result in cancer progression (5). Involvement of the entire colon for >10 years has been shown to predispose patients to colon cancer (5). In addition, it is well known that chronic inflammation of the colon contributes to colon carcinogenesis (6). To prevent the progression of cancer in patients with UC, an improved understanding of the pathogenesis of UC at the molecular and cellular level is required. UC is usually closely associated with intervals of acute exacerbation, and corticosteroid administration is effective for clinical remission (7). However, corticosteroids often have severe side effects, including hormonal disturbance, peptic ulcers, liver dysfunction and psychological issues (8). These adverse effects occasionally lead to discontinuation of corticosteroid treatment, which results in acute UC exacerbation (9). Therefore, alternative treatments for UC are required in order to help patients with UC avoid these clinical problems.

Recent studies have demonstrated that pro-inflammatory cytokines are associated with the initiation of colonic inflammation (10). Furthermore, it has been reported that mucosa from patients with UC exhibits increased expression levels of interleukin (IL)-6 (11,12). Mueller (13) previously detected...
high expression of tumor necrosis factor (TNF)-α in the blood, colonic tissue and stool samples of patients with UC (13). Therefore, in the field of UC treatment, there is a strong interest in developing agents that are able to block the generation or action of inflammatory cytokines.

Cyclooxygenase (COX) is a prostaglandin synthetase enzyme that is involved in the metabolism of arachidonic acid. There are two isoenzymes of COX: COX-1 and COX-2. COX-1 is expressed constitutively in the majority of tissues, particularly in the gastrointestinal tract, and is responsible for the production of prostaglandins that are associated with gastrointestinal integrity. Although COX-2 expression is low in the normal state, they are rapidly upregulated in response to various stimuli, including cytokines, growth factors, hormones and carcinogens. COX-2 is also responsible for producing prostaglandins associated with the mediation of inflammation (14). COX-2 expression has been shown to be increased in the mucosa of patients with UC (15,16).

Nuclear factor (NF)-κB is one of the most important transcription factors, which is responsible for the induction of genes that mediate innate and adaptive immunity. The role of NF-κB in UC has been investigated by numerous studies (17,18). Ma et al (19) demonstrated that increases in intestinal epithelial tight-junction permeability require the activation of NF-κB. In addition, increased DNA binding activity of NF-κB, which is associated with high levels of IL-1 and IL-6, is observed in patients with UC (20). These studies suggested that the activation of NF-κB has a critical role in the initiation of intestinal inflammation in UC. Therefore, inhibiting the activation of NF-κB has been suggested as an effective anti-inflammatory strategy for the treatment of UC (21).

Traditional herbal medicine has been the subject of increased interest for its potential in the treatment of inflammation. Iongsan (IGS) is a Korean herbal medicine that contains Ginseng Radix, Atractylodis Rhizoma Alba, Poria Sclerotium, Glycyrrhizae Radix et Rhizoma and Citri Unshius Pericarpium. IGS is widely used to treat digestive disorders, including abdominal pain, dyspepsia and diarrhea. The use of IGS is based on knowledge from Traditional Korean Medicine (22), with few scientific studies available regarding the effects of IGS on intestinal inflammation supporting its clinical applications.

In order to support the traditional use of IGS by providing experimental evidence, the present study examined the effects of IGS and its constituent, ergosterol, on dextran sulfate sodium (DSS)-induced colitis. The aims of the present study were to examine the effects of IGS and its constituent, ergosterol on inflammatory-associated gene expression in DSS-treated colon tissue.

Materials and methods

Reagents. DSS (mol wt, 36,000-50,000) was purchased from MP Biomedicals North America (Solon, OH, USA). Mouse TNF-α affinity purified polyclonal, goat IgG (cat no. AF-410-NA), mouse TNF-α biotinylated affinity purified polyclonal, goat IgG (cat no. BF410) and recombinant mouse TNF-α (cat no. 410-MT) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Purified rat anti-mouse IL-6 (cat no. 55440), biotin rat anti-mouse IL-6 (cat no. 554402) and recombinant mouse IL-6 (cat no. 554582) were purchased from BD Biosciences (San Diego, CA, USA). Avidin peroxidase (cat no. A3151), ergosterol (cat no. E6510), sulfasalazine (cat no. S0883) and 2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS; cat no. A9941) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (cat no. 23150-0350) was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). COX-2 (cat no. sc-1745), NF-κB/p65 (cat no. sc-7151) and histone H1 (cat no. sc-10806) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Peroxidase AffiniPure rabbit anti-goat IgG (H+L) (cat no. 305-035-003), peroxidase-conjugated AffiniPure goat anti-rabbit IgG (catalog no. 111-035-003) and peroxidase-conjugated AffiniPure goat anti-mouse IgG (catalog no. 115-035-062) antibodies were purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA).

Animals. Female BALB/c mice (four weeks old; body weight, 16-18 g) were purchased from the Daehan Experimental Animal Center (Eumsung, Korea). The mice (n=48) were maintained in the College of Pharmacy, Wonkwang University (Iksan, Korea), and 5-10 mice were housed per cage in a laminar air-flow room. The atmosphere was maintained at a temperature of 22±1°C and relative humidity of 55±10% with a 12 h light/dark cycle throughout the study. Animal experimental procedures were approved by the Animal Ethics Committee of Wonkwang University (approval number WKU14-05).

Preparation of IGS. IGS is a herbal medicine composed of five herbs: Ginseng Radix (root of Panax ginseng, Araliaceae), Atractylodis Rhizoma Alba (rhizome of Atractylodes macrocephala, Compositae), Poria Sclerotium (sclerotium of Poria cocos, Polyporaceae), Glycyrrhizae Radix et Rhizoma (root and rhizome of Glycyrrhiza uralensis, Leguminosae) and Citri Unshius Pericarpium (Pee of Citrus unshiu, Rutaceae). The composition of IGS is shown in Table I. The components of IGS used in the present study were purchased from Daehak Oriental Drugstore (Iksan, Korea), and their identity was confirmed by Professor Seung-Heon Hong (Department of Oriental Pharmacy, Wonkwang University, Jeonbuk, Korea). The extract was prepared by decocting the dried prescription with boiling distilled water (100 g/l). The extraction was decocted for 3 h, and was then filtered and lyophilized prior to being maintained at 4°C (yield, 7.26%). The samples were dissolved in distilled water and then filtered through a 0.22-µm syringe filter (Merck Millipore, Carrigtwohill, Ireland).

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount (g)</th>
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<tbody>
<tr>
<td>Ginseng Radix</td>
<td>20</td>
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<tr>
<td>Atractylodis Rhizoma Alba</td>
<td>20</td>
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<tr>
<td>Poria Sclerotium</td>
<td>20</td>
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<tr>
<td>Glycyrrhizae Radix et Rhizoma</td>
<td>20</td>
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<tr>
<td>Citri Unshius Pericarpium</td>
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Table I. Components of Igongsan.
Induction of colitis by DSS. Colitis was induced in mice as described in a previous study by our group (23). The mice were orally administered DSS dissolved in drinking water, which is a widely used experimental model of colitis. Briefly, acute colitis was induced in the mice by supplementing their drinking water with 5% (w/v) DSS for 7 days. The mice were checked daily for weight loss, stool consistency and the presence of gross bleeding. The mice were randomized into three groups (n=6): Mice receiving oral administration of IGS (100 mg/kg/day) or Ergosterol (20 mg/kg/day), mice treated with sulfasalazine (SFZ; 150 mg/kg/day) as a positive control, or mice treated with water as a negative control. IGS and SFZ were orally administered once a day for 7 days prior to DSS treatment. The mice were sacrificed by cervical dislocation and assessed after 7 days of treatment with DSS.

Disease activity index (DAI). The activity of intestinal disease was assessed through the following manifestations: Weight loss, diarrhea accompanied with blood and mucus, and shortening of the colon (3). In the present study, the DAI was obtained from the score of three major clinical symptoms: Weight loss, diarrhea and rectal bleeding, as described in a previous study by our group (23), based on a study by Murthy et al (24). Loss of body weight was calculated as the difference between initial and final weight. Diarrhea was defined by the absence of fecal pellet formation in the colon, and the presence of continuous fluid fecal material in the colon. The appearance of rectal bleeding was defined as diarrhea containing visible blood and gross rectal bleeding, and was scored as described for diarrhea. The DAI was calculated using the following formula: DAI = (weight loss score) + (diarrhea score) + (rectal bleeding score). The clinical parameters used here are comprehensive functional measures that are analogous to the subjective clinical symptoms observed in human UC (25). This method of scoring has been validated by repeated studies (24,26).

Cytokine assay. A cytokine assay was performed using a modified ELISA as described previously (27). Tissue segments excised from distal colon were prepared by homogenization and extraction by PRO-PREP Protein Extraction solution (Intron Biotechnology, Inc., Seongnam, Korea). Following protein quantification (DC™ Protein Assay kit; Bio‑Rad Laboratories, Inc., Hercules, CA, USA), equal protein concentrations of the tissue samples underwent ELISA. The ELISA was performed by coating 96-well plates with mouse monoclonal antibodies targeting TNF-α and IL-6 overnight at 4°C. Assay plates were sequentially exposed to biotinylated mouse TNF-α and IL-6 for 1 h, avidin peroxidase for 40 min and 2,2’-azinobis ABTS substrate solution containing 30% H₂O₂. The plates were read at a wavelength of 405 nm with the VersaMax™ ELISA...
Results

IGS alleviates clinical symptoms of DSS-induced colitis. It has previously been reported that DSS-induced colitis in mice has a similar phenotype to human acute and chronic UC (28). In the present study, the inhibitory effects of IGS on the intestines of mice with DSS-induced experimental colitis were evaluated (Fig. 1). The physiological symptoms of colitis (weight loss, colon shortening, diarrhea and obscure/gross bleeding) were observed following 7 days of 5% DSS treatment, and the DAIs of the mice were calculated. Mice treated with DSS exhibited significant weight loss (16.88±0.28 g) and colon shortening (6.23±0.50 cm) as compared with those in the mice with DSS-induced colitis (P<0.05) (Fig. 1A-C). In addition, the DAI was markedly decreased in the mice treated with IGS (4.25±0.33) as compared with that in the mice treated with DSS alone (5.22±0.25; P<0.05) (Fig. 1D). SFZ is a drug widely used to treat colitis, which was used as the positive control in the present study; results for the SFZ group were similar to those of the IGS group within the error range.

IGS inhibits increases in TNF-α and IL-6 levels in DSS-induced colitis. The effects of IGS on TNF-α and IL-6 levels in colon tissue from mice with experimental colitis were determined. At the end of the experiment, the mouse colons were homogenized and examined using ELISA. As shown in Fig. 2A and B, the levels of TNF-α and IL-6 were significantly increased in the colon tissue of DSS-treated mice (0.46±0.08; P<0.05) as compared with that in the control mice (0.25±0.02 and 0.6±0.04 ng/ml; P<0.05). However, administration of IGS reduced the DSS-induced increase in TNF-α and IL-6 levels (0.32±0.04 and 0.8±0.07 ng/ml, respectively; P<0.05). The rate of inhibition of TNF-α and IL-6 by IGS was 21.71 and 33.34%, respectively. In comparison, the rate of inhibition of TNF-α and IL-6 by SFZ was 25.01 and 36.11%, respectively.

IGS inhibits increases in COX-2 expression and PGE₂ levels in DSS-induced colitis. The effects of IGS on the protein expression levels of COX-2 were determined by western blot analysis. The expression levels of COX-2 were significantly increased in colon tissue of mice treated with DSS (0.88±0.10) as compared with those in the control mice (0.46±0.08; P<0.05). However, treatment with IGS reduced the expression levels of COX-2 in the DSS-treated mice (0.66±0.11; P<0.05) (Fig. 3A and B).

Statistical analysis. Values are expressed as the mean±standard error of the mean of at least three experiments. The data were examined by one-way analysis of variance with Tukey’s post hoc test. PASW Statistics software, version 18.0.0 (SPSS, Inc., Chicago, IL, USA) was used to conduct the statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Prostaglandin E₂ (PGE₂) assay. The concentration of PGE₂ in the colon tissue was measured by ELISA using a PGE₂ assay kit (Stressgen Biotechnologies Corporation, San Diego, CA USA), according to the manufacturer’s instructions. Duplicate aliquots of supernatant were measured for each sample.

Western blot analysis. The western blot analyses were performed as described previously (23). Briefly, the proteins were extracted from the distal colon tissue samples. The proteins were then separated using the Gradi-Gel 2 Gradient PAGE analysis kit (Elpis Biotech, Inc., DaeJeon, Korea) 7.5% gel for SDS-PAGE prior to being transferred onto nitrocellulose membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The membranes were then incubated with primary and secondary antibodies sequentially, and the protein bands were visualized using an Enhanced Chemiluminescence Detection system (GE Healthcare Bio-Sciences), according to the manufacturer’s instructions. Relative protein levels were evaluated using GAPDH or histone as the reference protein in each condition. The band intensities were measured using ImageJ software, version 1.48 (National Institutes of Health, Bethesda, MD, USA).

Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA).
COX-2 catalyzes the biosynthesis of PGE$_2$; therefore, the present study determined whether IGS exerted an effect on the levels of PGE$_2$ in colon tissue. As shown in Fig. 3C, PGE$_2$ levels were enhanced in response to DSS (1.46±0.10 pg/ml); however, this increase was significantly inhibited by IGS (1.16±0.10 pg/ml; P<0.05). The rate of inhibition of PGE$_2$ production by IGS (100 mg/kg/day) was 32.22%.

**IGS suppresses the activation of NF-κB p65 in DSS-induced colitis.** Activation of NF-κB p65 is involved in colitis (18); therefore, inhibition of NF-κB activation has been suggested as an anti-inflammatory therapeutic strategy for colitis. The present study investigated whether IGS regulated the activation of NF-κB p65 in colitis. As shown in Fig. 4, IGS significantly suppressed the DSS-induced activation of NF-κB p65 in colon tissue from mice with experimental colitis (DSS vs. DSS + Ergo, 1.32±0.10 vs. 1.07±0.11; P<0.05) (Fig. 5E and F).

**Discussion**

Traditional herbal medicines have been used effectively for centuries, based on traditional knowledge; however, the pharmacological mechanisms of action of these herbal prescriptions have yet to be fully elucidated. The present study aimed to provide experimental evidence regarding the use of IGS as a potential therapeutic drug for patients with UC. The present study demonstrated that IGS is able to inhibit inflammation...
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The results indicated that there is an important molecular mechanism by which IGS ameliorates intestinal inflammation.

UC is a typical inflammatory disease of the intestine, which belongs to a group of conditions known as IBD. However, despite numerous years of extensive research implicating immune dysfunction, genetic susceptibility and bacterial flora within the intestinal environment as possible factors associated with the development of the disease (29), its pathogenesis has remained to be elucidated. The signs and symptoms of UC include abdominal pain, weight loss and diarrhea accompanied with bleeding (30-32). Therapies for UC include glucocorticosteroids and SFZ (33,34); however, these treatments often have severe adverse effects. Therefore, the requirement for anti-inflammatory agents with fewer side effects is growing. Recently, traditional herbal medicine has received increased interest regarding the treatment of such disorders. Previous studies by our group have demonstrated that various herbal medicines have anti-colitis effects in mice (23,35). The present study investigated the effects of IGS on UC, using the DSS-induced mouse model of colitis. Treatment with IGS reduced DSS-induced weight loss and colon shortening. In addition, the DAI, which is a scale determined by measuring the levels of three major clinical symptoms (weight loss, diarrhea and rectal bleeding), was markedly inhibited in the group treated with IGS. The protective effect of IGS on colon shortening and DAI was similar to the protective effect of SFZ. These results therefore suggested that IGS may effectively inhibit the symptoms of DSS-induced colitis.

Figure 5. Effects of ergosterol on the clinical characteristics of DSS-induced colitis. Experimental colitis was induced in mice by supplementing their drinking water with 5% (w/v) DSS for 7 days. Ergosterol (20 mg/kg) was administered once a day for 7 days prior to intake of 5% DSS. (A) Body weight of the mice was measured. (B) Colon was harvested at day 7 after DSS treatment, and their length was measured. (C) Relative colon lengths are presented. (D) The DAI was calculated. (E) Nuclear extracts were isolated from the colon tissue and evaluated for NF-κB p65 expression. (F) Relative expression levels of NF-κB. SFZ (150 mg/kg) was used as a positive control. Values are expressed as the mean ± standard error of the mean (n=6) of duplicate determinations from three separate experiments. *P<0.05 vs. control; #P<0.05 vs. DSS alone. IGS, Igongsan; DSS, dextran sodium sulfate; SFZ, sulfasalazine; DAI, disease activity index; NF-κB; nuclear factor-κB.
Pro-inflammatory cytokines are closely associated with the initiation of the inflammatory response in colitis. It has previously been shown that TNF-α is strongly associated with the pathogenesis and progression of intestinal inflammatory disorders (36). It has also been reported that levels of IL-6 are markedly elevated in patients with UC, and that IL-6 has an integral role in its pathogenesis (37). Therefore, studies on novel biological therapies for UC have focused on blocking components of the inflammatory cascade, such as cytokines. COX-2 is also closely associated with numerous pathological processes, including UC. DSS elicits COX-2 expression to regulate the production of prostaglandins, which induce gastrointestinal fluid secretion in the inflammatory pathway (38). The present study demonstrated that the levels of TNF-α, IL-6 and COX-2 were increased in the colon tissue of DSS-treated mice as compared with those in the control mice, and treatment with IGS reduced these levels. In addition, the inhibitory effect of IGS on the increases in TNF-α, IL-6 and COX-2 levels in DSS-treated colon tissue was similar to the effect of SFZ. These results indicated that the anti-inflammatory effects of IGS may be attributed to the regulation of inflammatory mediators in DSS-induced colitis.

NF-κB is an important transcription factor, particularly for the activation of numerous inflammatory mediators, cytokines including IL-1β and IL-6, and COX-2. NF-κB p65 has been reported to be a critically important factor in chronic inflammatory diseases (39). Therefore, NF-κB is considered an ideal target for a molecular therapy of UC, and extensive efforts have been made to develop novel treatments targeting NF-κB (17,40,41). In the present study, it was observed that the activation of NF-κB p65 was significantly increased in the colon tissue of DSS-treated mice as compared with that in the control group, and this increase was attenuated by treatment with IGS. These results indicated that the mechanism of action of IGS is a novel mechanism involving the regulation of NF-κB p65 activation in DSS-induced colitis. Therefore, it may be hypothesized that IGS exerts its anti-inflammatory effect in UC by regulating the activation of NF-κB.

IGS is composed of five herbs, each of which have been reported to have various effects; in particular, ginsenoside Rb1, Rd and Re of Ginseng Radix (42-44), hesperidin of Unshius Pericarpium (45), and glycyrrhizic acid of Glycyrrhizae Radix et Rhizoma (46) have been shown to exhibit anti-colitis effects. Ergosterol is a compound present in Poria Sclerotium. It has previously been reported that ergosterol may be beneficial in the treatment of the allergic response by decreasing immunoglobulin E in mucosal-type mast cells (47). However, there is currently no information available regarding the effects of ergosterol on colitis. Therefore, an aim of the present study was to evaluate the possible modulating effects of ergosterol on colitis. The results of the present study demonstrated that ergosterol was able to attenuate clinical symptoms of DSS-induced colitis in mice.

In conclusion, the present study demonstrated that treatment with IGS significantly reduced the clinical symptoms and the levels of inflammatory mediators in a DSS-induced murine model of colitis. These results suggested that IGS may be a useful therapeutic candidate for colitis. However, further studies are required to reveal the precise mechanisms underlying the effects of IGS in intestinal inflammatory disorders.

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References


