The Therapeutic Effect of Chelidonic Acid on Ulcerative Colitis

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Chelidonic acid (CA), a constituent of Chelidonium majus L., has many pharmacological effects, including mild analgesic and antimicrobial effects. However, the effects of CA on intestinal inflammation and the molecular mechanisms responsible are poorly understood. The aim of this study was to investigate the protective effects of CA against dextran sulfate sodium (DSS)-induced ulcerative colitis (UC). Mice treated with DSS displayed obvious clinic signs, such as, body weight loss and a shortening of colon length, but the administration of CA attenuated both of these signs. Additionally, CA was found to regulate levels of interleukin-6 and tumor necrosis factor-alpha (TNF-α) in serum. In colonic tissues, prostaglandin E2 (PGE2) production levels and cyclooxygenase-2 (COX-2) and hypoxia induced factor-1 expression levels were increased by DSS, but CA attenuated increases in COX-2 and HIF-1α levels. These results provide novel insights into the pharmacological actions of CA and its potential use for the treatment of intestinal inflammation.

Key words chelidonic acid; intestinal inflammation; dextran sulfate sodium; ulcerative colitis; cyclooxygenase-2; hypoxia induced factor-1α

Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD), and is a chronic inflammatory disorder characterized by uncontrolled inflammation of the intestine, especially of the colon and rectum. The mechanisms of the pathogenesis of UC are not completely understood, but environmental factors, heredity, immune system dysfunction, and other as yet unidentified factors are believed to be responsible. UC usually presents with abdominal pain, vomiting, diarrhea, rectal bleeding, and weight loss. From a histopathological perspective, UC manifests as inflammation, ulceration, shortening of the colon, and the infiltration of inflammatory cells into the lesion.

Cytokines are key signals in the intestinal immune system, and immune cells, such as, macrophages, dendritic cells, T cells, and intestinal epithelial cells are known to secrete various cytokines that regulate inflammatory response in UC. Furthermore, elevated levels of cytokines, such as, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin-1 (IL-1), IL-6, IL-8, and IL-12, have been reported in mucosal tissue samples from patients with IBD. In addition, it has been reported that serum IL-6 and TNF-α levels are elevated in patients with an inflammatory bowel condition, and it has been proposed that they play an integral role in the pathogenesis of the disease. Hence, there is a strong interest in agents that can block the generation or actions of inflammatory cytokines.

Cyclooxygenases (COX) have been implicated in a number of physiological events, including the progression of inflammation, immunomodulation, and the transmission of pain. In particular, COX-2 is an inducible enzyme, which is present at low concentration in healthy tissues, but up-regulated in response to tissue damage during inflammation. COX-2 is responsible for the production of prostaglandins (PGs) associated with the mediation of inflammation. Furthermore, it has been reported that the expression levels of COX-2 and PGE2 are elevated in the inflamed mucosal tissues of patients with UC.

Hypoxia inducible factor-1 (HIF-1) is a transcription factor, and is produced or activated in response to hypoxia. Moreover, inflamed tissues are hypoxic relative to normal healthy tissues, and growing evidence indicates that HIF-1 is involved in the inflammatory process by regulating angiogenesis. Furthermore, HIF-1 is a pivotal transcription factor that links inflammatory pathways, and HIF-1α expression levels have been reported to be elevated in the colonic tissues of patients with colitis.

Chelidonic acid (CA) is a constituent of Chelidonium majus L., a plant that grows in east Asian countries, and is used as a herbal medicine in Korea. CA has several known therapeutic effects, which include mild analgesic, antimicrobial, and oncostatic effects, and it acts as a sedative in the central nervous system. However, the anti-inflammatory effects of CA and the mechanism responsible for its effect on intestinal inflammation have not been determined.

Dextran sulfate sodium (DSS)-induced colitis is characterized by the mucosal infiltration of inflammatory cells, epithelial injury, and ulceration. It has also been reported that DSS-induced colitis in mice has a phenotype similar to acute and chronic UC in man. In order to provide experimental evidence that CA might be a useful therapeutic in UC, we examined its effects on DSS-induced colitis in a murine model. The specific aims of the study were: I) to determine the effects of CA on clinical signs (weight loss, colon length, diarrhea, and occult/gross bleeding), and II) to investigate the effect of CA on inflammatory-related gene expression in DSS-treated colon tissues.

MATERIALS AND METHODS

Animals and Reagents Female BALB/c mice (6 weeks old) were obtained from the Da-Mool Science (Taejeon, South Korea). Mice were acclimatized in a specific pathogen-free

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environment under controlled conditions (22±2°C under a 12 h light/dark cycle) for at least one week. All animal studies were carried out in accordance with the regulation issued by the Institutional Review Board of Wonkwang University (confirmation number: WKU11-10). DSS (mol wt 36000–50000) was purchased from MP Biomedicals (Solon, OH, U.S.A.). CA was purchased from Fluka (Buchs, Switzerland). Purified anti-mouse IL-6, recombinant mouse (rm) IL-6, and biotinylated anti-mouse IL-6 were obtained from BD-Pharmingen (San Diego, CA, U.S.A.). Specific antibodies (Abs) against COX-2, HIF-1α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other chemical reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

**Induction of Colitis by DSS**  
Acute colitis was induced in mice by adding 5% (w/v) DSS to drinking water for seven days. Mice were checked daily for body weight, stool consistency and the presence of gross bleeding. Animals were randomized to four groups: animals in the control group were fed a normal commercial diet and no DSS, animals in the DSS group were fed a commercial diet and DSS was added to drinking water, animals in the CA group were administrated CA (20 mg/kg) in diet and DSS in drinking water, and animals in the sulfasalazine (SFZ) group were administrated SFZ (100 mg/kg; a positive control) and DSS in water. CA and SFZ were diluted with purified water and orally administered once daily during the experiment. Animals were sacrificed and assessed after 7 d of DSS treatment.

**Disease Activity Index (DAI)**  
Intestinal disease activity was assessed based on weight loss, the presence of diarrhea accompanied by blood and mucus, and colonic shortening.18) DAI were calculated by scoring weight loss, diarrhea, and rectal bleeding, as described by Murthy et al.19) Weight loss was defined as the difference between initial and final weights, and diarrhea as the absence of fecal pellet formation and the presence of continuous fluid fecal material in the colon. Rectal bleeding was assessed based on the presence of diarrhea containing visible blood and on the presence of gross rectal bleeding, and was scored as diarrhea.

DAI values were calculated using the following formula:  
\[ \text{DAI} = (\text{weight loss score}) + (\text{diarrhea score}) + (\text{rectal bleeding score}) \].  
The clinical parameters used in the present study were chosen to represent the subjective clinical symptoms observed in human ulcerative colitis.

**Cytokine Assays**  
Levels of IL-6 and TNF-α in serum and colon tissue were measured using an enzyme-linked immunosorbent assay (ELISA), as previously described.20) Briefly, 96-well plates were coated with 100 µL of anti-mouse monoclonal antibodies (1.0 mg/mL and pH 7.4 in phosphate buffered saline (PBS)) and incubated overnight at 4°C. After additional...
washes, 100 µL of sample or an IL-6 or TNF-α standard were added and incubated at room temperature for 2h. Plates were then washed and 0.2 µg/mL of biotinylated anti-mouse antibody was added and incubated at room temperature for 2h. After washing plates, avidin-peroxidase was added, and plates were incubated for 30 min at 37°C. The plates were then re-washed and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. Standard curves were prepared using serial dilutions of recombinant antibodies. Protein concentrations were measured using bicinechonic acid (BCA) protein assay reagent (Sigma).

**PGE₂ Assay** PGE₂ levels were quantified using immunoassay kits according to the manufacturer’s instructions (Stressgen Biotechnologies, Ann Arbor, MI, U.S.A.).

**Western Blot Analysis** Distal colons were homogenized in lysis buffer (nNIRON Biotech, South Korea), and centrifuged at 13000 rpm for 5 min. The supernatants were transferred to fresh tubes and protein concentrations were determined using BCA protein assay reagent (Sigma). Lysates (50 µg of protein) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), which were then blocked with 5% skim milk in PBS-Tween-20 (PBST) for 1 h at room temperature. They were then incubated overnight with primary Abs against COX-2 and HIF-1α, and washed 3 times with PBST. Blots were incubated with secondary Abs for 1 h at room temperature, and antibody-specific proteins were visualized using an enhanced chemiluminescence detection system (Amersham & Biosciences, U.K.). Protein densities were quantified by densitometry.

**Statistical Analysis** The results are presented as means±S.E.M.s of at least three independent experiments. Results were analyzed using the independent t-test and by analysis of variance (ANOVA) with Tukey’s post hoc test. p values of <0.05 were considered significant.

**RESULTS**

**The Effects of CA on Clinical Signs of DSS-Induced Colitis** Clinical signs (weight loss, colon length, diarrhea, and occult/gross bleeding) were monitored after 7 d of treatment with 5% DSS, and DAI scores were calculated. As shown in Figs. 1A and B, the DSS treatment group showed body weight loss and colon shortening as compared with control group, and percentages of body weight loss and colon shortening of 21.01±2.8% and 27.11±3.1%, respectively. No side effects were observed in CA treated group. However, the CA treatment group showed a significant reduction in body weight loss and colon shortening by DSS. Relative colon lengths are shown in Fig. 1C. Another common feature of the DSS-induced model of colitis is an increase in DAI. DAI scores were lower in the CA group than in the control group (0.030±0.004, 0.364±0.060 ng/mL, respectively). However, CA significantly reduced increases in both IL-6 and TNF-α (0.027±0.003 ng/mL, 0.291±0.048 ng/mL, respectively).

**The Effect of CA on PGE₂ Production and COX-2 Expression in Colon Tissue** Western blot analysis was conducted to evaluate the effects of CA on COX-2 expression in colon tissues. As shown in Fig. 3A, DSS markedly induced COX-2 expression in colon tissue versus controls, but this increase was significantly reduced by CA and SFZ administration. Relative expression levels of COX-2 are presented in Fig. 3B.

COX-2 catalyzes PGE₂ biosynthesis, and therefore, we examined whether CA affects PGE₂ levels. The results obtained showed that PGE₂ levels were enhanced by DSS, and that this increase was significantly inhibited by CA and by SFZ (Fig. 3B).

**The Effect of CA on HIF-1α in Colon Tissue** HIF-1 is involved in the inflammatory process, and has been reported to be highly activated in DSS-induced colon tissue. In the present study, Western blot analysis was performed to assess the effects of CA on the expression levels of HIF-1α in colon tissue. As shown in Fig. 4A, DSS increased HIF-1α expression in colon tissue versus controls, but CA and SFZ both reduced this increase. The relative expression levels of HIF-1α are presented in Fig. 4B.
DISCUSSION

CA is known to have various beneficial effects on human health, which include analgesic and antimicrobial effects. In addition, CA regulates neurotransmitters by restricting glutamate decarboxylase in rat brain. However, no information is available on whether CA regulates intestinal inflammation. The present study shows for the first time that CA does inhibit the inflammatory response and colon injury provoked by DSS in a murine model. In addition, our findings suggest the molecular mechanism by which CA ameliorates intestinal inflammation.

UC is an idiopathic disease characterized by the development of intestinal inflammation. Approximately 10 individuals/100000 of the population/year are diagnosed with UC. The pathogenesis of UC remains uncertain, but it is likely to be dependent on the interaction between local immune reactions and environmental factors in genetically susceptible individuals. Most UC therapies are based on glucocorticosteroids, sulfasalazine, or others, but they have serious side effects. Consequently, anti-inflammatory agents with fewer side effects are needed.

To date, more than 20 animal models of colitis have been established. Among these, DSS-induced colitis is one of the most widely used experimental models. In this study, we focused on how CA affects clinical signs, that is, weight loss, colon length, diarrhea, and occult/gross bleeding. Our results show that DSS induces an acute form of colitis characterized by stool consistency, ulceration, blood in feces, and body weight loss. However, CA administration reduced the weight loss and colonic shortening caused by DSS. In addition, DAI scores were remarkably lower in the CA group and in the SFZ group than in the DSS group. These results suggest that CA effectively ameliorates the severity of colitis symptoms induced by DSS.

It has been shown that inflammatory mediators like IL-6, TNF-α, and IFN-γ play critical roles in the pathogenesis of murine colitis. Immune cells like T lymphocytes and macrophages participate in the inflammatory response of IBD and secrete cytokines and chemokines that facilitate and amplify cell interactions. Furthermore, the activations of these effector cells change the balance between pro- and anti-inflammatory cytokines, characterized by the excessive productions of pro-inflammatory cytokines like IL-6 and TNF-α, and it is known that IL-6 and TNF-α gene expression is elevated in the rectal mucosa of UC patients. In this study, we studied whether CA reduces DSS-induced IL-6 and TNF-α production in serum. Our results suggest that CA exerts an effect on anti-
inflammatory response by suppressing IL-6 and TNF-α levels. Furthermore, inhibitory effects on IL-6 level were more obvious in the CA group than in the SFZ group. Although we found that CA reduced TNF-α and IL-6 levels in serum, we did not examine the effects of CA on other cytokines. Thus, further study is needed to determine comprehensively the effects of CA on other cytokines induced by DSS.

COX-2 is a major mediator of inflammatory reactions, and is also strongly induced in activated macrophages. COX-2 carries out crucial functions in certain physiological processes, and is an inducible enzyme that only present at low concentrations in healthy tissues. During inflammation, COX-2 levels are upregulated, and several recent studies have demonstrated that PGs, which are metabolites of COX-2, contribute to the pain and swelling associated with inflammation. In addition, it has been reported that levels of PGE2 are elevated in the intestines of IBD patients. Because PGE2 and COX-2 are considered important mediators in UC, we evaluated the effect of CA on PGE2 and COX-2 level in the colon tissues of DSS treated mice. We observed that DSS increased COX-2 and PGE2 expression, but CA suppressed both PGE2 production and COX-2 expression, as effective as SFZ. However, the PGE2 level was not much suppressed in comparison with COX-2 mRNA level. Although COX-2 converts arachidonic acid to prostaglandins, prostaglandin syntheses (PGESs) also affects to complete PGE2. Multidrug resistance protein 4 (MRP-4) is also responsible for transportation of PGE out of cell. The difference of inhibitory effect of CA on PGE2 and COX-2 might be due to the interaction of CA with other factors such as PGESs or MRP-4.

Inflammatory mucosal lesions observed in colitic mice were highly hypoxic, similar to those observed in large tumors. Hypoxia signaling pathways are involved in the inflammatory process, and HIF-1α serves as a marker of inflammatory disease. HIF-1α, a hypoxia-mediated gene regulating transcription factor, is accumulated in the case of toll-like receptors (TLR)-mediated inflammatory reactions. It has been reported that HIF-1α is overexpressed in colitis affected human and murine colon tissues. In agreement with previous observations, HIF-1α was found to be elevated in the DSS group, but CA was found to reduce this increase, not to the same extent as SFZ. These results indicate that the inflammation observed in our DSS model is the result of both COX-2 and HIF-1α mediated response and that the inhibition of inflammatory response by CA is effective against both inflammatory molecules. In DSS-induced colitis model, not only IL-6, TNF-α, COX-2, and HIF-1α but also inflammation-related factors including caspases or mitogen-activated protein kinases affect clinical signs. Though the recovery of clinical signs did not reach to the control as IL-6, TNF-α, COX-2, and HIF-1α attenuated, considering those factors, further investigation of CA effects on colitis should be executed to clarify the discrepancy.

It is generally believed that DSS administration is directly toxic to gut epithelial cells of the basal crypts so that disrupts mucosal barrier which protects intestines. Once intestinal epithelial barrier is exposed to luminal bacteria products like lipopolysaccharide, TLRs recognize the stimulations which trigger colon mucosal immune system responses and induce inflammation. TLR4 downstream pathway leads to the activation of transcription factors (such as nuclear factor-κB) and therefore results in the expression of COX-2 and the production of pro-inflammatory cytokines. Therefore, TLR4 is a possible target for treatment of UC. In this study, although we suggest a possible mechanism underlying the regulation of IL-6, TNF-α, and COX-2, and HIF-1α by CA, further investigation is warranted to confirm the finding from present study.

In summary, the anti-inflammatory effects of CA were found to be as effective as those of SFZ, a well known anti-inflammatory agent used to treat IBD. Our findings suggest that CA inhibits inflammation by regulating inflammatory mediators, that is, COX-2 and HIF-1α. Furthermore, they indicate that CA might be a useful therapeutic for the treatment of inflammatory diseases, and that herbs containing chelidonic acid, like celandine (Chelidonium majus), could be used as a dietary supplement.

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