응용약물학회지, The Journal of Applied Pharmacology 5, 165-173(1997)

Beneficial Effect of DA-9601, an Extract of Artemisiae Herba, on Animals Models of Inflammatory Bowel Disease

Byoung Ok AHN, Byong Kweon RYU, Jun Il KO, Tae Young OH, Soon Hoe KIM*, Won Bae KIM, Junnick YANG, Eun Bang LEE[1] and Ki Baik HAHM[2]

Research Laboratories, Dong-A Pharm. Co. Ltd., Kyunggido 449-900, Korea
[1]Natural Products Institute, Seoul National University, Seoul 110-460, Korea
[2]Department of Gastroenterology, College of Medicine, Ajou University, Kyunggido 442-749, Korea

(Received May 12, 1997; accepted June 1, 1997)

Abstract – This study was conducted to investigate the effect of DA-9601, an extract of Artemisiae Herba, which is known to possess mucoprotective action either by free radical scavenging effect or increase of mucus secretion, against animal models of inflammatory bowel disease (IBD) induced by trinitrobenzene sulfonic acid (TNBS) or other noxious agents. Experimental colitis was induced by intracolonic administration of TNBS in 50% ethanol, or 1 ml of 7% acetic acid solution (AA), by subcutaneous injection of indomethacin (INDO) in rats, or by supplementing drinking water with 5% dextran sodium sulfate (DSS) in albino mice. DA-9601 was treated orally for 4 to 7 days. Animals were euthanized 1 day after the last treatment for morphological and biochemical analysises. All the noxious agents including TNBS, AA, INDO and DSS elicited severe colitis. The animals treated with DA-9601 showed a consistent, dose-related reduction in the severity of colitis, grossly and histologically. The reduction was significant ($p<0.05$) after administration of DA-9601 at dose range of 10 mg/kg or above. In TNBS-induced colitis, the rats receiving DA-9601 showed significantly decreased mucosal myeloperoxidase (MPO) and thiobarbituric acid-reactive substances (TBA-RS), when compared to control and mesalazine groups. Mucosal proinflammatory cytokine levels were also decreased after DA-9601 treatment. In conclusion, DA-9601 ameliorated macroscopic and histologic scores in experimental colitis either through decreasing oxidative stress or by attenuating cytokines involved in inflammation. DA-9601 could be a promising drug for the therapy of IBD.

Keywords □ DA-9601, Artemisiae Herba, inflammatory bowel disease, colitis

The aerial parts of Artemisiae Herba has been used in folk medicine as a remedy for abdominal pain, bloody diarrhea, internal bleeding and gynecological disorders in Korea with the local name of "Aeyop". DA-9601 is a quality-controlled extract of dried aerial parts of Artemisiae Herba, which shows cytoprotection against various noxious agents (Oh et al., 1996a). The methods of extraction and manufacturing of DA-9601 were previously reported in detail (Yang, 1995).

The authors found that DA-9601 exerts mucoprotection through increased mucus and prostaglandin E_2 secretion from gastric mucosa and that nitric oxide mediates the cytoprotective action of DA-9601, at least in part (Oh et al. 1996b; Ryu et al., 1996). DA-9601 reduces ischemia/reperfusion injury of stomach and *Helicobacter pylori*-induced activation of human neutrophils (unpublished data). And eupatilin, an active ingredient of DA-9601, blocks completely *H. pylori*-induced leukotriene D_4 release from human neutrophils and Kato III cells at the concentration of 0.5 mM or above (Han et al., 1996).

These results indicate antioxidative properties of DA-9601 play an important role in its mode of action.

Though the etiology of inflammatory bowel disease (IBD) still remains obscure, it is known that radicals and other reactive oxygen species (ROS) are important components of the tissue damage pathway in IBD (Harris et al., 1992; Keshavarzian et al., 1992).

This study was conducted to evaluate the effect of DA-9601 in animal models of IBD.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250~300 g) and male

*To whom correspondence should be addressed.

ICR mice (28~33 g), aged 7 weeks, purchased from Charles River Japan (Kanagawa, Japan), were used in this study. The animals were kept under standard laboratory conditions and allowed access to rodent chow (Cheil, Korea), and drinking water.

Test materials

2,4,6-Trinitrobenzene sulfonic acid (TNBS, Sigma), dextran sodium sulfate (DSS, M.W. 40,000, ICN), acetic acid · glacial (Sigma), indomethacin (Sigma) were used to induce colonic damage, and DA-9601 (lot. L-07), 5-aminosalicylate (5-ASA, Acros), sulfasalazine (Sigma), prednisone (Aldrich) were used as therapeutics of experimental colitis. Enzyme immunoassay (EIA) kits were purchased from R&D (U.S.A.).

TNBS-induced colitis

Colitis was induced by the intracolonic administration of 30 mg of TNBS in 1 ml of 50% ethanol, as described previously (Morris et al., 1989). The rat was lightly anesthetized with ether inhalation and 8 cm long rubber cannula was inserted rectally for instillation of TNBS. 2 minutes after the instillation, the colon was rinsed with 2 ml of warm (36~37℃) saline in the same way. The rat was then allowed to recover from anesthesia and was returned to its cage. DA-9601 was administered orally at two dose levels (10 and 100 mg/kg), once a day for 7 days from 24 hours after the injury. 25 mg/kg of 5-ASA (mesalazine) or 1 mg/kg of prednisone was administered orally as a reference drug. Control rats were gavaged with 5% of hydroxypropylmethylcellulose (HPMC), a vehicle of DA-9601, during the same period.

Acetic acid-induced colitis

Rats were anesthetized with ether and 1 ml of 7% aqueous solution acetic acid (AA) was instilled as an enema via an 8 cm length of rubber cannula (Sharon and Stenson, 1993). Test materials including DA-9601, sulfasalazine and prednisone were treated orally for 4 days.

Indomethacin-induced colitis

Colitis was induced by subcutaneous injection of indomethacin (7.5 mg/kg) once a day for 2 days (Matsumoto et al., 1993). From 24 hours after the last injection of indomethacin, DA-9601 (5~125 mg/kg), sulfasalazine (50 mg/kg) or prednisone (1 mg/kg) was administered orally for 4 days.

Dextran sodium sulfate-induced colitis

Acute colitis was induced by feeding the mice for five days with 5% dextran sodium sulfate (DSS) dissolved in drinking water (Murthy et al., 1993). After 5 days, DSS was stopped, animals were returned to drinking plain tap water, and oral treatment with DA-9601 (5~125 mg/kg), sulfasalazine (50 mg/kg) or prednisone (1 mg/kg) was continued for extra 4 days.

Macroscopic examination

The rats were killed by cervical dislocation 24 hours after the last treatment, and a laparotomy was performed. The distal 10 cm of colon was removed and laid, mucosal side up, on a wax platform. In acetic acid- and DSS-induced colitis, macroscopic evaluation of damage was conducted by measuring the length of ulcerated colon. And in TNBS- and indomethacin-induced colitis, colonic damage was scored on a 0 (normal) to 10 (severe) scale, according to criteria described in Table I and elsewhere (Wallace et al., 1989), by an observer blinded to the treatment.

Histologic observation

Samples (4 mm × 1 cm) of grossly normal and grossly inflamed colonic tissue were fixed in neutral buffered formalin and processed by routine techniques prior to embedding in paraffin. Thick sections (4 μm) were mounted on glass slides and stained with hematoxylin and eosin. The sections were examined under a light microscope (BH-2, Olympus) by a veterinary pathologist unaware of the treatment and were assigned a damage score 0 (normal) to 5 (severe), as described by Wallace et al. (1989) and shown below:

0=no damage; 1=damage limited to the surface epithelium; 2=focal ulceration limited to the mucosa; 3=focal, transmural ulceration and inflammation; 4=extensive transmural ulceration and inflammation bordered by normal mucosa; 5=extensive transmural ulceration and inflamm-

Table I. Criteria for macroscopical scoring of colonic ulceration and inflammation in the rat

Score	Appearance
0	Normal
1	Localized hyperemia, no ulcers
2	Linear ulceration without hyperemia or bowel wall thickening
3	Linear ulceration with inflammation at one site
4	Two or more sites of ulceration and inflammation
5	Two or more sites of ulceration and inflammation, or one major site of damage extending more than 1 cm along the length of the colon
6~10	When an area of ulceration and inflammation extended more than 2 cm along the length of the colon, the score was increased by 1 for each additional cm of involvement

ation involving the entire section.

Inflammatory index

In TNBS-induced colitis experiment, samples (~100 mg) of grossly inflamed colonic mucosa were excised for determination of myeloperoxidase (MPO) activity and malondialdehyde (MDA) by the routine procedure with slight modification (Boughton-Smith et al., 1988; Wallace, 1988).

Cytokine Assay

In TNBS-induced colitis experiment, specimens of grossly inflamed colonic mucosa were excised and weighed, ×10 the weight of the tissue with sterile, ice-cold phosphate-buffered saline was then added to the samples. The tissues were homogenized on ice and the debris pelleted by centrifugation at 15,000×g for 15 min. The supernatant was diluted 1:10 and analysed for IL-1β, IL-6 and TNF-α contents in colonic mucosa with EIA kits.

Statistical Analyses

Results are given as mean±standard error of the mean. Kruskal-Wallis test was performed to determine the differences between the control group and experimental group. Dunnet's test (Dunnet, 1970) was used for multiple comparisons and StudentNewman-Keul's test (Newman, 1939) for pairwise differences. P values of ≤ 0.05 were considered statistically significant.

RESULTS

TNBS-induced colitis

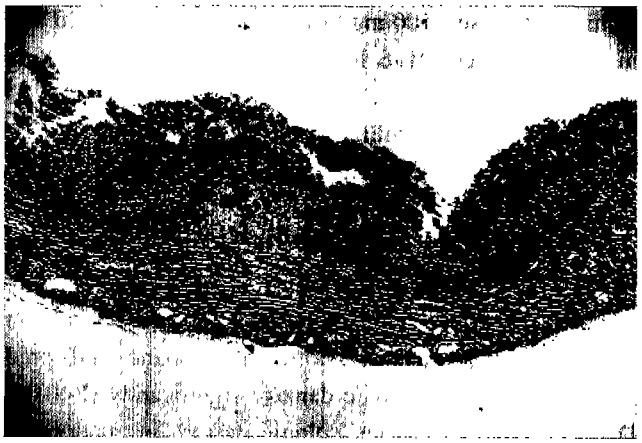

Fig. 1. Rat colonic tissue exposed to TNBS in 30% ethanol intrarectally. In affected regions of the bowel, total absence of epithelial cells, with the mucosal area instead occupied by pus cells and bacteria, was observed. Cellular infiltration to muscle layer was also seen. H-E stain, ×40.

Intracolonic administration of TNBS in a vehicle of 50% ethanol induced a severe colitis characterized by mucosal disruption, ulceration, hemorrhage and inflammatory cellular infiltration, which persisted throughout the experiment period. Areas of ulceration were frequently separated by macroscopically normal mucosa. Mucosal necrosis and transmural inflammation of the colon was most often noted underlying regions of macroscopic lesion (Fig. 1). Treatment with DA-9601 at both 10 mg/kg and 100 mg/kg markedly improved the colonic inflammation and mucosal regeneration (Fig. 2), whereas daily administration of mesalazine or prednisone did not significantly affect the incidence or severity of colonic damage, as assessed macroscopically (Fig. 3) or histologically (Fig. 4). In addition to reducing the extent of morphological damage, treatment with DA-9601 de-

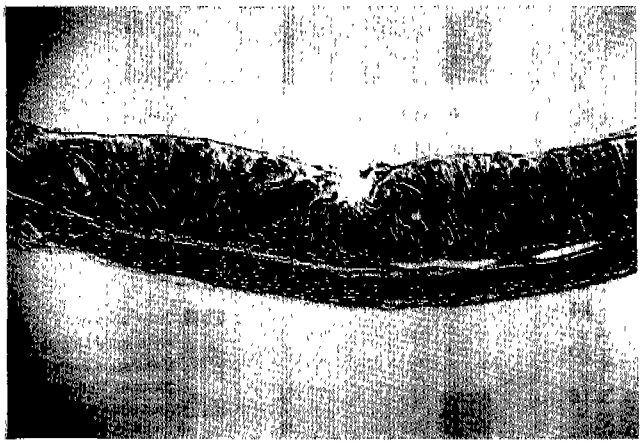

Fig. 2. Rat therapeutically treated with 10 mg/kg of DA-9601 daily for 7 days after enema of TNBS/ethanol showing mucosal regeneration. Note the ulcer surrounded by regenerated mucosal tissue. Mucus coating is observed on the surface of the mucosa. H-E stain, ×40.

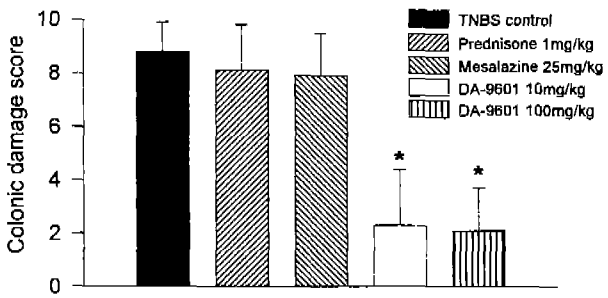

Fig. 3. Effects of oral treatment with DA-9601 (10 or 100 mg/kg) for 7 days on colonic damage score in TNBS-induced colitis. Each *bar* represents the mean±SEM. *P<0.05, Significantly difference from the TNBS control group, $n \geq 6$ rats per group.

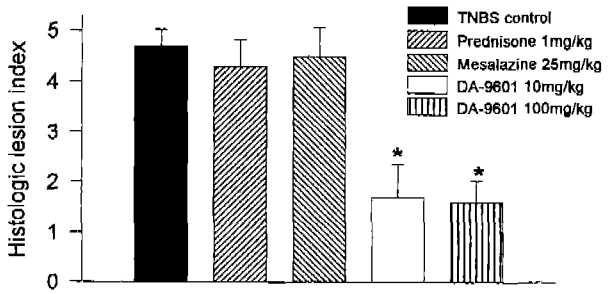

Fig. 4. Effects of DA-9601 (10 or 100 mg/kg) on histological lesion index in TNBS-induced colitis rats. Each *bar* represents the mean±SEM. *P<0.05 compared to TNBS control rats, n≥6 rats per group.

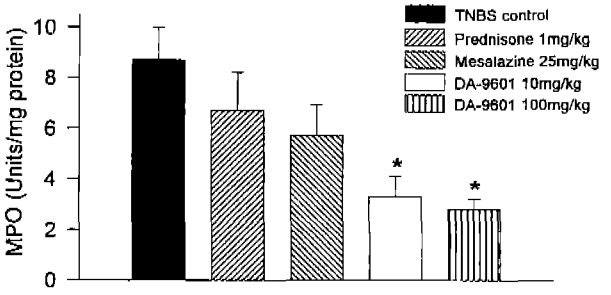

Fig. 5. Effects of DA-9601 on myeloperoxidase (MPO) activity of colonic mucosa. 8 days after induction of colitis. Each *bar* represents the mean±SEM. Asterisks denote significant difference between the group and TNBS control (P<0.05).

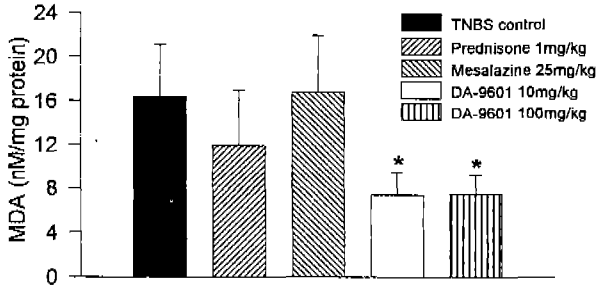

Fig. 6. Effects of DA-9601 on malondialdehyde (MDA) contents of colonic mucosa. 8 days after induction of colitis. Each *bar* represents the mean±SEM of at least six rats. *P <0.05; compared to control group.

creased mucosal MPO activity and MDA levels, in a dose-related manner, suggesting that DA-9601 reduces colonic damage through a mechanism related to reduction of neutrophil infiltration into the colon tissue and oxidative stress (Fig. 5, 6). However, neither mesalazine nor prednisone significantly affected the MPO activity and MDA levels of colon.

Reduced severity of inflammation by DA-9601 corresponded to the mucosal levels of proinflammatory cytokines (Fig. 7). DA-9601 decreased the colonic IL-1β and TNF-α slightly but significantly at both doses of 10 mg/kg and 100 mg/kg (P<0.05). In case of IL-6, the rats from high dose (100 mg/kg) of DA-9601 showed significantly reduced IL-6 level, whereas low dose (10 mg/kg) group did not.

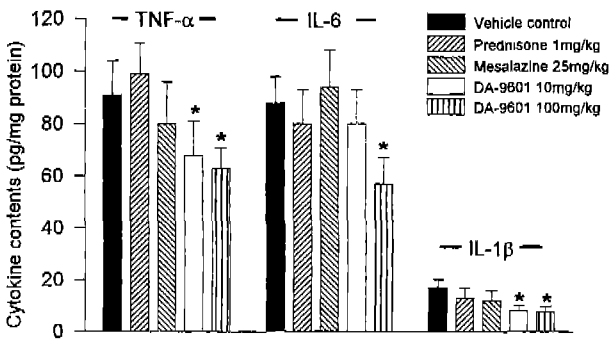

Fig. 7. Effects of DA-9601 on colonic mucosal contents of proinflammatory cytokines, including TNF-α, interleukin-6 (IL-6) and interleukin-1β (IL-1β). Each *bar* represents mean± SEM of at least six rats. *P0.05; compared to control group.

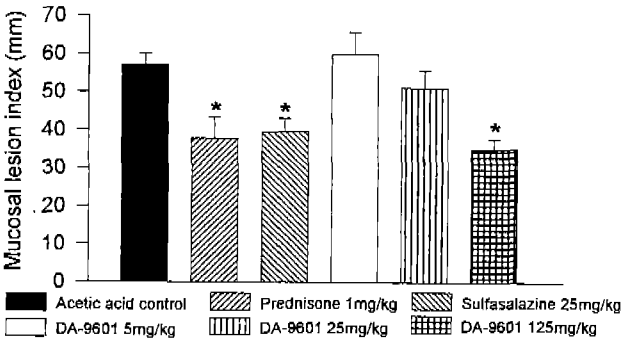

Fig. 8. Effect of DA-9601 on the mucosal lesion index during the acute phase of acetic acid-induced murine colitis. Each *bar* represents the mean±SEM of 7 to 8 rats. *P<0.05, Significantly differencc from thc acctic acid control.

Acetic acid-induced colitis

Colonic application of acetic acid (AA) produced diffuse necrotic change of mucous layer. At necropsy, thickening of bowel wall and hyperemic change of serosal surface were evident; however, in no instance was perforation identified. Fig. 8 illustrates the gross lesion index of colonic mucosa. Both sulfasalazine and prednisone ameliorated colonic damage significantly (P<0.05). DA-9601 improved the colonic lesion in a dose-dependent manner, and the efficacy of DA-9601 reached statistical significance at high dose (125 mg/kg). Histological assessment of colon samples confirmed the macroscopic scoring. Affected colon exhibited transmural in-

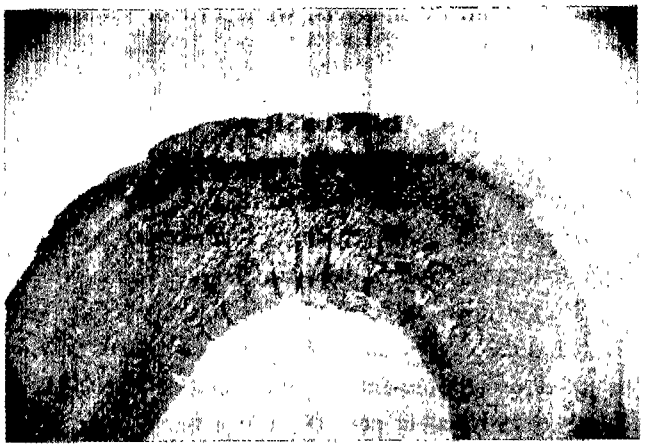

Fig. 9. Colonic injury 5 days after exposure to 7% acetic acid. Total absence of epithelial, transmural inflammation and thickened colonic wall were noted. Diffuse hemorrhage was also observed. H-E stain, ×40.

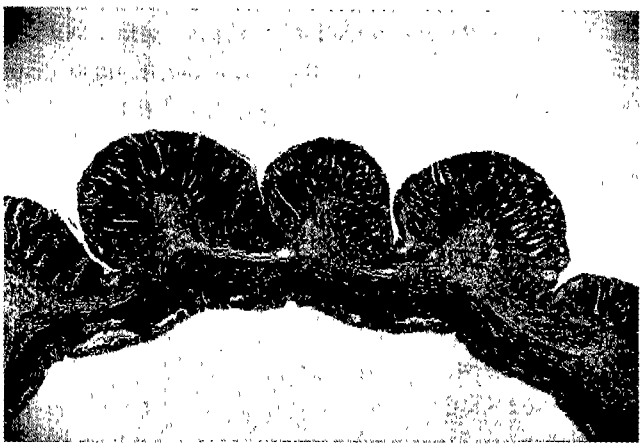

Fig. 10. Colon from a rat therapeutically treated with 125 mg/kg of DA-9601 for 4 days after insult of acetic acid enema. The mucosa appears healed except for focal erosions and submucosal infiltration of inflammatory cells. No inflammatory cells were observed in the muscle layers. H-E stain, ×40.

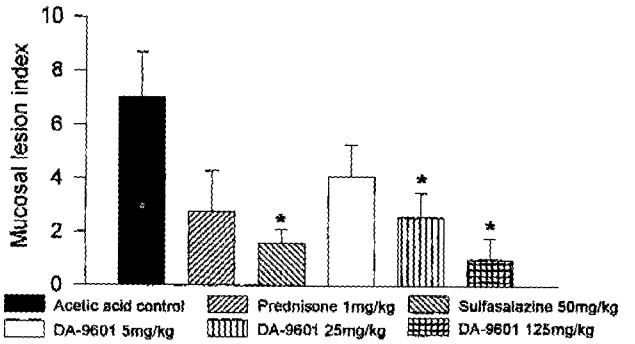

Fig. 11. Effect of DA-9601 on the mucosal lesion index during the acute phase of indomethacin-induced murine colitis. Each *bar* represents the mean±SEM of 7 rats. *represents P< 0.05 *vs.* indomethacin control.

Fig. 12. Indomethacin-injected rat showed thinning of mucosal layer and multifocal cryptic abscesses. Mucosal infiltration of mononuclear cells and fibroblasts was evident. Submucosal edema was also noted. H-E stain, ×100.

flammation with total necrosis of mucous membrane, submucosal edema and inflammatory cellular infiltration to submucosal and muscular layer in all animals treated with AA enema (Fig. 9). However, rats from DA-9601 treated group (125 mg/kg) showed only mild erosions of mucosa, though cellular infiltration of submucosa was similar to control group (Fig. 10).

Indomethacin-induced colitis

Indomethacin (INDO) produced moderate to severe mucosal damage in gastrointestinal tract including stomach, small and large intestine. Fig. 11 shows macroscopic lesion index of each group. Control rats showed multifocal linear ulcers of colonic mucosa with the lesion score of 7.0±1.7. All the test substances were proved to be effective against indomethacin-induced colitis. The lesion scores of sulfasalazine and high dose of DA-9601 were 1.6±0.5 and 1.0±0.8, respectively. DA-9601 also markedly attenuated the disease activity index, severity of intestinal adhesion and stricture formation (data not shown). Histologic examination revealed that diffuse cryptitis, well-demarcated ulceration and lymphoid hyperplasia are prominent features of colitis in control group. Both acute inflammatory cells (neutrophils and eosinophils) and chronic inflammatory cells (plasma cells and lymphocytes) were seen, but chronic inflammatory cells were more frequently observed in the affected mucosa (Fig. 12). DA-9601 treatment reduced the incidence of ulcers and cellular infiltration, as depicted in Fig. 13. Sulfasalazine and prednisone also attenuated the histologic

Fig. 13. Colon from a rat treated with 125 mg/kg of DA-9601 for 4 days after indomethacin exhibited reduced cellular infiltration of colonic mucosa. There was no evidence of thinning of mucosal layer. H-E stain, ×100.

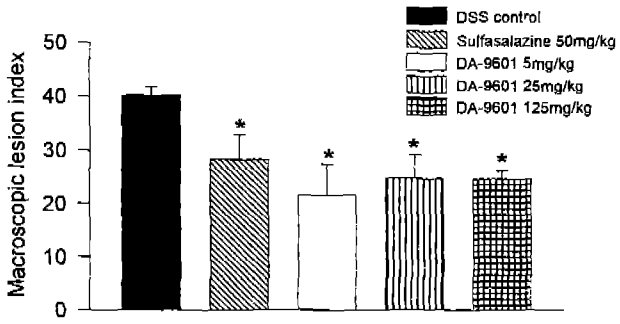

Fig. 14. Effects of DA-9601 on macroscopic lesion index of colonic mucosa of mice receiving 5% DSS for 4 days. Each *bar* represents the mean±SEM of at least seven mice. *$P<$ 0.05; compared to DSS control group.

lesion with no qualitative difference from DA-9601.

Dextran sodium sulfate-induced colitis

Bloody loose stools were observed in about half of the animals from 5 days after DSS drinking. At necropsy, animals received DSS for 5 days showed diffuse ulceration on colorectal mucosa. The lesion score of each test group is shown in Fig. 14. The length of ulcerated large intestine in vehicle control group was 40.2±1.6 mm. All three doses of DA-9601 decreased significantly the lesion score by 40~50% compared to treatment with vehicle ($P<0.05$). Sulfasalazine also reduced the lesion index to 28.3±4.6 mm, but failed to reach statistical significance. Microscopically, inflammation was observed throughout the entire length of the colon and rectum. Mucosal exfoliation and lymphoid hyperplasia were also evident in all mice receiving DSS for 5 days. The inflammation, however, in DA-9601 groups was limited to mucosa and spared deep muscular layers, while that of sulfasalazine group was not.

DISCUSSION

Inflammatory bowel disease (IBD) is an embracing term for the idiopathic chronic inflammatory conditions of the intestine, Crohn's disease (CD) and ulcerative colitis (UC), whose etiology remains unknown (Kim and Berstad, 1992; Thomson, 1996). Both can affect any age group but usually present in young adult patients. IBD affects 90~190 people per 100,000 in the western countries and 20~40 per 100,000 in the Asia, respectively, and its incidence is on the increase (Rao et al., 1987). Both CD and UC tend to be of a remitting and relapsing nature (Vernia et al., 1995). However, whilst UC is a mucosal disorder affecting only the colon or part of it; CD causes transmural inflammation which can affect any segment of the gastrointestinal tract with the terminal ileum being the region most commonly involved (Elson, 1995). The progress in IBD research has been hampered by the lack of suitable animal models of human disease, because many facets of IBD, including its etiology, are poorly understood (Podolsky, 1991).

Nonetheless the exact pathophysiology of IBD remains unclear, several animal models were developed (Kim and Berstad, 1992). The models of experimental colitis used in this study are most frequently used because they are inexpensive, reproducible, and readily available. Among them, TNBS model is more commonly used than others because it is relatively long-term (up to 6 weeks) and at least in part immune mediated.

There are considerable evidence suggesting that ROS play an important role in human IBD and experimental colitis (Conner et al., 1996; Grisham, 1994; Harris et al., 1992). In fact, many of the empirically effective IBD therapies target ROS (Babbs, 1992). For instance, sulfasalazine and mesalazine, an active component of sulfasalazine, which are contemporally useful drugs have potent antioxidant effect (Conner et al., 1996). In addition, an uncontrolled clinical study by Emerit et al. (1989) showed that administration of superoxide dismutase (SOD), a potent natural antioxidant, provided significant relief to patients with severe Crohn's disease. In TNBS model of our study, DA-9601 markedly decreased MDA levels compared to control with significant reduction of morphological lesion index, suggesting antioxid-

ative properties of DA-9601 play an important role, at least partly, in its mode of action. Measurement of MPO activity is a useful assay to quantify leukocyte infiltration and inflammation of the tissue (Sanchez et al., 1996; Yamada et al., 1992). In the present study, DA-9601 reduced colonic MPO activity, to 20~30% of control group, suggesting a decrease of neutrophil recruitment into the colon. The reduction of MDA and MPO by oral DA-9601 can explain, in part, the protective effect of DA-9601 against INDO-induced colitis, because INDO-induced mucosal damage is mediated by ROS (Parks, 1989; Zahavi et al., 1995), as well as depletion of endogenous prostaglandins (Whittle, 1981). The beneficial effects of treatment with DA-9601 were also evident from the data on cytokines. Initial tissue injury in inflamed gut renders inflammatory cytokine, including IL-1β, IL-6 and TNF-α, release by damaged epithelial cells and/or resident inflammatory cells (Mahida et al., 1991; McCafferty et al., 1992; Olson et al., 1993; Sategna-Guidetti et al., 1993). And these cytokines amplify the inflammatory response. In our study, daily administration of DA-9601 (100 mg/kg) significantly reduced tissue contents of three major inflammatory cytokines.

Enteric bacterial flora also play a significant role in colitis, disruption of the intestinal epithelial barrier may allow entry of luminal bacterial lipopolysaccharide into submucosal tissues (Azad-Khan et al, 1977; Muller et al., 1993). And it is regarded that control of luminal flora is helpful to the treatment of IBD (Hartley et al., 1996). Actually, metronidazole, an antibacterial drug, is currently used in the therapy of IBD, and a quinolone derivative, ciprofloxacin, is also reported to be effective against experimental colitis (Sutherland et al., 1991). We have not yet clarified the antimicrobial effect of DA-9601, which remains to be elucidated in the near future.

In this study, DA-9601 showed dose-related effect against animal colitis. However, in DSS model, we could not find dose-response correlation of DA-9601, although all doses (5, 25 and 125 mg/kg) were proved to be efficacious in reducing macroscopic lesion index. The reason(s) for this is not known, but it can be speculated that the differences in animal species and pathomechanism of animal models rendered low dose of the compound sufficient to improve gross lesion in this model.

In summary, DA-9601 was proved to be effective against all the animal models of IBD used in our study, and the healing observed with oral DA-9601 was comparable or superior to those observed with mesalazine, sulfasalazine and prednisone, which are commonly used drugs in the treatment of IBD (Courtney et al., 1992; Lauritsen et al., 1986; Miles and Grisham, 1994). In addition to the efficacy, DA-9601 is thought to be safe (Kim et al., 1996), as the original herb has been used as a traditional medicine in oriental countries, while the contemporary drugs have significant adverse effects (Cohen, 1996; Das et al., 1973; World et al., 1996). Based on these safety and efficacy data, it is thought that DA-9601 could be a useful drug in the therapy of IBD. In conclusion, the present results clearly demonstrate that DA-9601 attenuates experimental IBD either through reducing oxidative stress or by modulation of inflammatory cytokines.

Acknowledgement

This study was supported by a grant of the '96 Good Health R&D Project, Ministry of Health and Welfare, R. O.K.

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