Effect of DA-6034, a Derivative of Flavonoid, on Experimental Animal Models of Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) is a multifactorial disorder with unknown etiology and pathogenesis. DA-6034, 7-carboxymethyloxy-3', 4', 5-trimethoxy flavone, is a synthetic flavonoid known to possess anti-inflammatory activity. This study was performed to evaluate the oral therapeutic effect of DA-6034 in three experimental animal models of IBD: two chemical-induced IBD models of rats and the human leukocyte antigen (HLA)-B27 transgenic rat model known to develop spontaneous colitis without the use of exogenous agents. Acute chemical colitis was induced by intracolonic instillation of 1.2 ml of 4% acetic acid solution. Prednisolone (1 mg/kg), sulfasalazine (100 mg/kg) and DA-6034 (0.3~3 mg/kg) were orally administered twice daily for 6 days in these rats. In addition, chronic chemical colitis was induced by intracolonic administration of trinitrobenzene sulfonic acid (TNBS) 30 mg in 50% ethanol and agents were orally administered for 6 or 20 days. In chemical-induced IBD models, all of these agents reduced the severity of colitis and specially, DA-6034 (3 mg/kg) showed more potent effect than other drugs in macroscopic lesion score. In HLA-B27 transgenic rats, DA-6034 (3 mg/kg) and prednisolone (0.5 mg/kg) were treated orally twice daily for 6 weeks. The HLA-B27 transgenic rats showed only mild colitis, compared with the chemical-induced colitis models. DA-6034 ameliorated the loose stool and decreased microscopic damage, which is the important indicator of this model. In conclusion, oral therapy of DA-6034 attenuated the macroscopic and histologic damages of the colon in all three experimental models of IBD, which suggest that DA-6034 could be a promising drug in the treatment of IBD.

Key words: Inflammatory bowel disease, DA-6034, TNBS, Acetic acid, HLA-B27 transgenic rat

INTRODUCTION

Flavonoids have been established as potentially useful anti-inflammatory agents, in view of their inhibitory effects on a number of cells of the immune system such as macrophages, neutrophil, mast cell and the enzymes involved in triggering or amplifying the inflammatory reaction (Havsteen, 1983; Middleton and Kandaswami, 1993; You *km*, 1999). Eupatilin, a flavonoid derivative, is a main component of the extract of *Artemisiae* species, a Korean folk medicine used in the treatment of chronic diarrhea (Wu, 1985; Ahn *et al.*, 1997). DA-6034, 7-carboxymethyloxy-3', 4', 5-trimethoxy flavone, is a synthetic derivative of eupatilin. Although the anti-inflammatory mechanism of eupatilin or DA-6034 is poorly understood, rectal administration of DA-6034 has shown to be effective in trinitrobenzene sulfonic acid (TNBS)-induced colitis (Chang *et al.*, 1998). This study was conducted to evaluate the oral therapeutic effect of DA-6034 in three experimental Inflammatory bowel disease (IBD) models: acetic acid-induced acute colitis of rats, TNBS-induced chronic colitis of rats, and human leukocyte antigen (HLA)-B27 transgenic rats.

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MATERIALS AND METHODS

Animals

HLA-B27 transgenic rats expressing HLA-B27 and β2-microglobulin genes of human and Fisher 344 rats were purchased from Taconic (Germantown, NY, USA) at 10~11 weeks of age. Male Sprague-Dawley rats (250~300 g, 7~8 weeks of age) were purchased from

Charles River Japan (Kanagawa, Japan). The animals were housed in individual cages, maintained with a 12-hour light/dark cycle and allowed free access to rodent chow and drinking water.

Materials and reagents

DA-6034 (7-carboxymethyloxy-3', 4', 5-trimethoxy flavone) was synthesized at Research Laboratories, Dong-A Pharmaceutical Co. (Kyunggi-do, Korea). Acetic acid-glacial, 2, 4, 6-trinitrobenzene sulfonic acid, sulfasalazine and prednisolone were purchased from Sigma (St. Louis, USA).

Acetic acid-induced colitis

Acute colitis was induced in rats by a modification of the previously published method (MacPherson and Pfeiffer, 1976, 1978). Rats were anesthetized with ether and 1.2 ml of 4% acetic acid solution (pH 2.3) was instilled intraluminally via 8 cm length of rubber cannula. After a 1-day recovery period, DA-6034 was administered orally at three dose levels (0.3, 1 and 3 mg/kg) twice a day for 6 days (n=10). Sulfasalazine (100 mg/kg) and prednisolone (1 mg/kg) were administered orally as re-ference drugs in each group of rats (n=10). An acetic acid control group and a saline control group were also included for reference.

TNBS-induced colitis

Chronic colitis was induced by intracolonic administration of 30 mg of TNBS in 0.5 ml of 50% ethanol as described previously (Morris *et al.*, 1989). DA-6034 was administered orally at two dose levels (1 and 3 mg/kg), once a day for 6 days (acute phase) or 20 days (chronic phase) from day 1 after injury (n=10). 100 mg/kg dose of sulfasalazine or 1 mg/kg of prednisolone was administered orally as reference drugs in each group of rats (n=10). A TNBS control group and a saline control group were also included for reference.

HLA-B27 and β2 microglobulin transgenic rats

Transgenic rats expressing HLA-B27 and β2-microglobulin genes of human have been produced from Fisher 344 rats using a recombinant DNA technology. They spontaneously develop multi-organ diseases manifested by colitis, arthritis, orchitis, and psoriasiform changes of skin and nails that are similar to the human spondyloarthropathies associated with the HLA-B27 and β2 microglobulin genes (Hammer *et al.*, 1990). The onset of the colitis is between 6 to 20 weeks of age, and the disease is chronic and progressive. We obtained 18 transgenic rats at 10~11 weeks of age and divided into 3 groups. DA-6034 (3 mg/kg) and prednisolone (0.5 mg/kg) as a reference drug were administered orally twice a day for 6 weeks. A transgenic control group and a normal Fisher 344 group were included for reference and rats from each group were treated with saline during the same period.

Gross observation

Loss of body weight and diarrhea (loose stool and/or diarrhea) are among the important clinical signs of the colitis. In rats with acetic acid- and TNBS-induced colitis, the body weight and the frequency of diarrhea were measured daily. In HLA-B27 transgenic Fisher rats, the stool consistency was assessed according to the scale shown in Table I.

Macroscopic examination and colon weight measurement

At 24 hours after the last drug treatment, the rats were sacrificed under ether anesthesia and a laparotomy was performed. A portion of the colon (10 cm) showing a greater damage was isolated, weighed and then opened by a longitudinal incision. In acetic acid-induced colitis, a macroscopic evaluation of the damage was conducted by measuring the area of ulceration of the colon. The colonic damage in rats with TNBS-induced colitis and HLA-B27 transgenic rats, was scored according to the criteria (Table II) by two observers who were blinded to the treatment.

Histologic evaluation

Table I. Stool consistency evaluation

Score	Consistency of stool
0	Normal stool: well formed pellets, rigid as normal
1	Loose stool: formed pellet with moisture, soft stool keeping in stool shape
2	Loose stool: stools that have abnormal form with much moisture, more soft stool loosing in shape
3	Watery diarrhea

Table II. Criteria of macroscopic scoring of the colonic lesion

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

Table III. Histological lesion score of the colonic lesion

Criteria	Severity	Score
Ulceration	No ulcer, epithelization	0
	Small ulcers < 3 mm	1
	Large ulcers > 3 mm	2
Inflammation	None	0
	Mild	1
	Moderate	2
	Severe	3
Depth of Lesion	None	0
	Submucosa	1
	Muscularis propria	2
	Serosa	3
Fibrogenesis	None	0
	Mild	1
	Severe	2

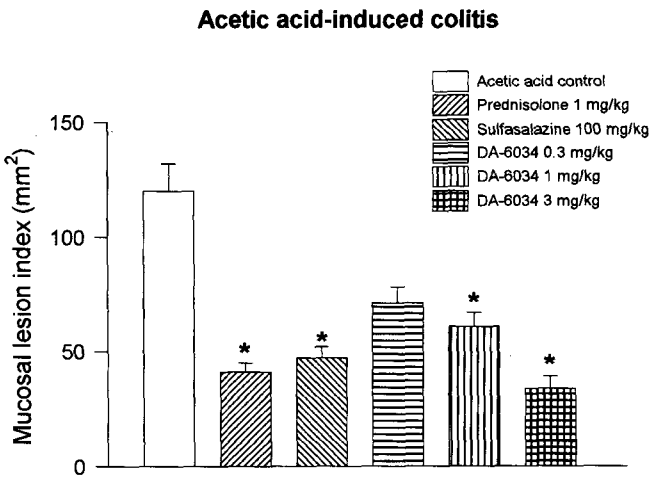

Fig. 1. The therapeutic effect of DA-6034, sulfasalazine and prednisolone on the mucosal lesion index in acetic acid-induced colitic rats. Each bar represents the mean±S.E. of 10 rats.
* represents $p<0.05$ vs. control.

Samples of the inflammed colonic tissues(4 mm×1 cm) were obtained from HLA-B27 tansgenic rats, 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. Sections were examined under a light microscope (BH-2, Olympus) by a veterinary pathologist who unaware of the treatment. The damage score was assigned according to the criteria shown in Table III.

Statistical analysis

All data were expressed as the mean±S.E. Comparisons of the nonparametric data, e.g., damage scores, were made by the Mann-Whitney rank-sum test. Parametric data statistical significance was set at $p<0.05$.

RESULTS

Acetic acid-induced colitis

Rats with acetic acid-induced colitis exhibited a loss of body weight and diarrhea for the first three days, then recovered slowly in the remainder periods of the experiment. The colonic damage by acetic acid showed diffuse necrotic changes of the mucosal layer. At necropsy, a diffuse thickening of the bowel wall and hyperemic changes of the serosal surface were evident; however, no perforation was identified. Both prednisolone and sulfasalazine ameliorated colonic damage significantly ($p<0.05$). DA-6034 improved the colonic lesion in a dose-dependent manner, and the efficacy of DA-6034 reached a statistical significance at doses>1 mg/kg (Fig. 1). Further, DA-6034 at 3 mg/kg dose significantly increased the degree of the weight gain (Table IV) and reduced the frequency of diarrhea (data not shown). DA-6034, as with other reference drugs, reduced the colonic weight (Table IV).

TNBS-induced colitis

Intracolonic administration of TNBS (30 mg) dissolved in 50% ethanol induced a severe colitis in rats. The rats with TNBS-induced colitis exhibited a weight loss and diarrhea for the first three days, and then slowly recovered in the remainder periods of the experiment. The colonic lesion of TNBS-induced colitis showed a diffuse hemorrhagic necrosis, edema and deep ulceration. The gross damage score in control rats was 9.0 at 1 week (acute

Table IV. Effects of DA-6034 on body weight gain and colon weight in acetic acid-induced colitic rats

Group	Dose (mg/kg)	No. of animal	Acetic acid-induced IBD (1 week)	
			Body weight gain(g/day)	Colon weight (g/10 cm)
Control	-	9	2.1±0.6	1.10±0.05
Prednisolone	1	10	0.7±0.4	1.05±0.05
Sulfasalazine	100	9	3.4±0.6	0.94±0.06*
DA-6034	0.3	10	2.7±0.6	0.92±0.09*
DA-6034	1	10	3.1±0.5	0.99±0.05
DA-6034	3	10	4.5±0.5*	0.93±0.04*

Data are expressed as mean±S.E. *represents $p<0.05$ *vs.* control.

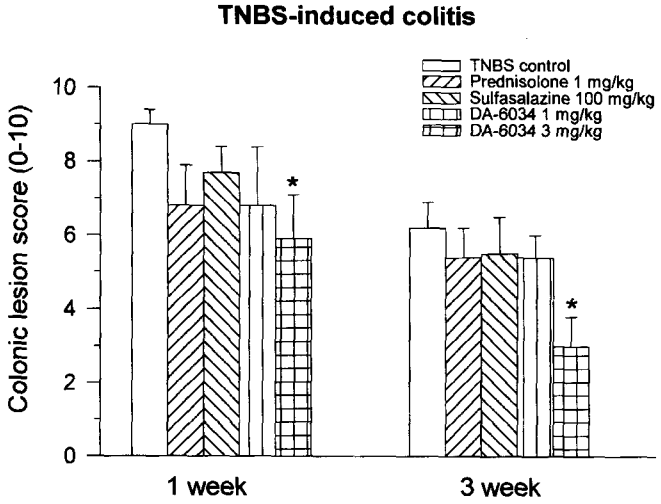

Fig. 2. The therapeutic effect of DA-6034, sulfasalazine and prednisolone on the damage score index in TNBS-induced colitic rats. Each bar represents the mean±S.E. of 10 rats. * represents $p<0.05$ vs. control.

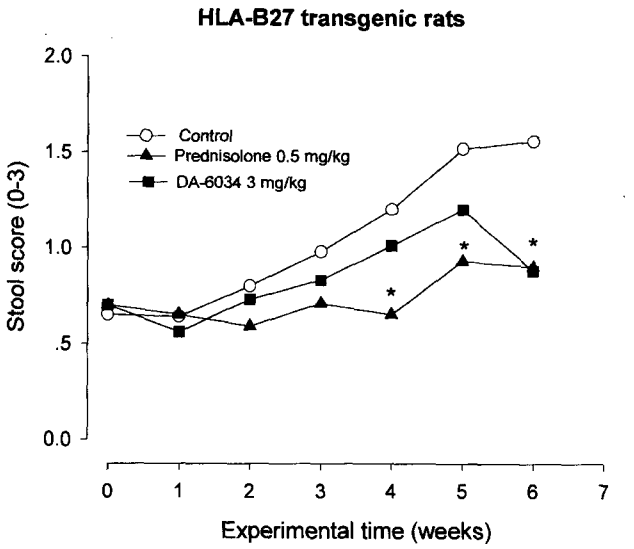

Fig. 3. Effects of DA-6034 on the stool consistency in HLA-B27 transgenic rats. Each data point represents the mean±S.E. of 6 rats. * represents $p<0.05$ vs. control.

stage) and 6.2 at 3 weeks (chronic stage). The oral administration of DA-6034 at 3 mg/kg of dose attenuated the weight loss and markedly improved the colonic lesion compared with that of control. The effects were more favorable than those of prednisolone and sulfasalazine (Fig. 2). Further, treatment with DA-6034 at 3 mg/kg dose reduced the colonic weight (Table V), and reduced the frequency of diarrhea (data not shown).

HLA-B27 transgenic rat

Transgenic control rats treated with saline developed loose stools and/or diarrhea (loose stool more than 1.0 stool score) spontaneously at 12~13 weeks of age, and the stool score was continuously increased in the remainder periods of the experiment. However, no clinical signs were observed in non-transgenic Fisher 344 rats. Treatments with DA-6034 (3 mg/kg) showed tendency of improved stool consistency during the experimental periods, and significantly decreased the stool score at 6 weeks compared with the control group. The reference drug, prednisolone, also significantly ameliorated stool score after 4 weeks of treatment (Fig. 3). Macroscopicfindings of the colon of the transgenic rats showed a colonic dilatation and bowel wall thickening with edema Lymph node swelling was also observed. However, mucosal lesions such as erosions or ulcerations were not observed in any transgenic or non-transgenic Fisher 344 rats. The macroscopic lesion score of the colon showed no difference among groups of transgenic rats. In histological examination of the colon, transgenic control rats showed diffuse chronic inflammatory cell infiltration (mainly lymphocytes) which extended into the submucosal layer. Multifocal erosions with fibrosis were also observed in some severe cases (Fig. 4B), whereas non-transgenic Fisher 344 rats showed a normal mucosa appearance (Fig. 4A). Trans-genic rats treated with prednisolone or DA-6034 showed lesions of inflammatory cell infiltration or ero-sions, but the degree of lesions was significantly less than that of transgenic control rats (Fig. 4C and 4D). The total histological lesion score of HLA-B27 transgenic control rats was significantly elevated. In contrast, treat-ment with DA-6034 and prednisolone significantly reduced the total

Table V. Effects of DA-6034 on body weight gain and colon weight in TNBS-induced colitic rats

Group	Dose (mg/kg)	No. of animal	1 week		3 week	
			Body weight gain (g/day)	Colon weight (g/10cm)	Body weight gain (g/day)	Colon weight (g/10cm)
Control	-	10	1.6±1.5	2.14±0.26	5.9±0.5	1.46±0.20
Prednisolone	1	10	2.2±1.4	1.93±0.38	5.4±0.4	1.40±0.20
Sulfasalazine	100	10	2.4±2.0	1.82±0.27	6.6±0.8	1.52±0.40
DA-6034	1	10	4.0±1.4	1.92±0.47	6.3±0.6	1.48±0.24
DA-6034	3	10	6.0±0.8*	1.72±0.45	6.4±0.7	1.11±0.14

Data are expressed as mean±S.E. * represents $p<0.05$ vs. control.

Table VI. The therapeutic effects of DA-6034 and prednisolone on histological findings of colonic lesion in HLA-B27 transgenic rats

Group	Ulceration (0-2)	Inflammation (0-3)	Depth of lesion (0-3)	Fibrosis (0-2)	Total (0-10)
Normal[a]	0.0±0.0	0.4±0.2	0.3±0.2	0.0±0.0	0.6±0.3
Control[b]	1.3±0.2#	2.5±0.2#	2.3±0.5#	1.7±0.2#	7.8±0.9#
Prednisolone	0.5±0.2	1.5±0.4	1.0±0.0	0.8±0.3	3.7±0.7*
DA-6034	0.3±0.2*	1.2±0.2*	1.0±0.0	0.2±0.2*	2.7±0.5*

Data are expressed as mean±S.E.
#represents $P<0.05$ vs. normal. *represents $p<0.05$ vs. transgenic control.
[a]represents normal Fisher 344 group
[b]represents transgenic Fisher 344 group

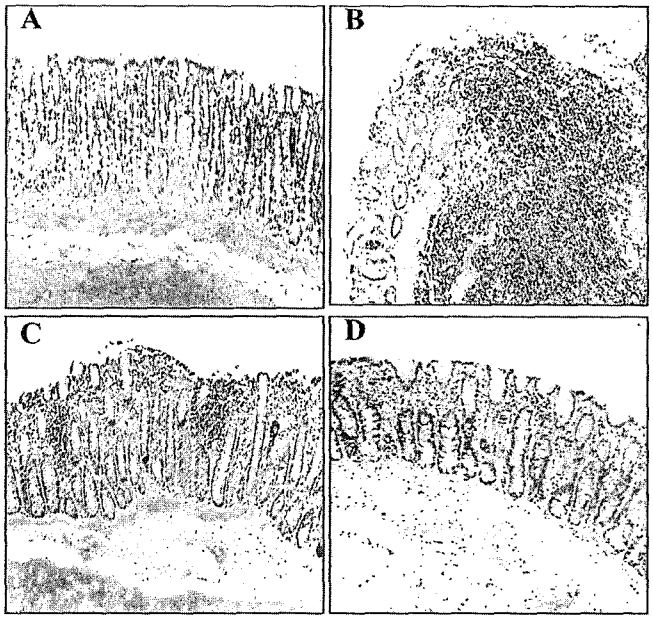

Fig. 4. Histology of the colon of Fisher 344 normal rats and HLA-B27 transgenic rats. (A) Fisher 344 normal rats showed normal mucosa (×100). (B) HLA-B27 transgenic control rats showed severe inflammatory cell infiltration from the mucosa layer to the submucosa and ulceration with bowel wall thickening (×100). (C) HLA-B27 transgenic rats treated with prednisolone (0.5 mg/kg) showed multifocal inflammatory cell infiltration in mucosa and lamina propria (×100). (D) HLA-B27 transgenic rats treated with DA-6034 (3 mg/kg) showed no remarkable abnormal lesion (×100).

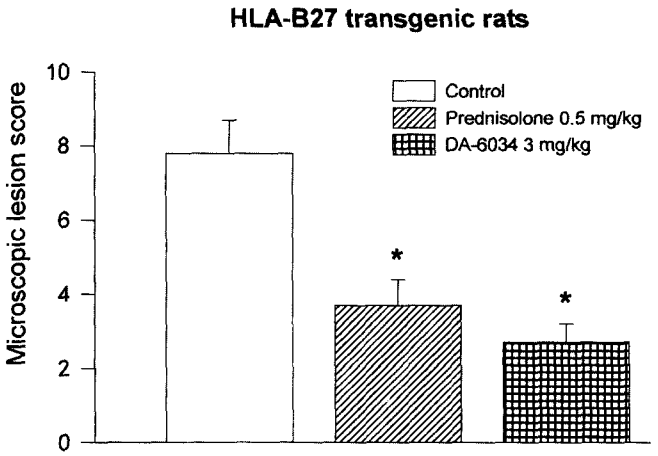

Fig. 5. The therapeutic effects of DA-6034 and prednisolone on the microscopic damage score in HLA-B27 transgenic rat. Each bar represents the mean±S.E. of 6 rats. *represents $p<0.05$ vs. transgenic control.

histological lesion score. The efficacy of DA-6034 was greater than that of prednisolone in the chronic progressive colitis of HLA-B27 transgenic rats (Fig. 5 and Table VI).

DISCUSSION

The incidence of IBD, especially the Crohns disease has been increasing over the last few decades (Calkins and Mendeloff, 1995). Yet, no significant progress has been made in the development of new therapeutic agents for the treatment of IBD. Traditional therapy including sulfasalazine, prednisolone and immunomodulatory agents such as azathioprine and methotrexate generally help improve the symptom and maintain disease remission. These drugs, however, have side effects such as drug toxicity and immunosuppression, etc. An adequate animal model is necessary in evaluating of the therapeutic effect of new drugs as well as understanding of the etiology and pathogenesis of the disease. However, no ideal experimental model for IBD exists at present (Elson *et al.*, 1995). There are multiple components contributing to IBD; genes, environment, specific immunity, innate immunity, nonspecific inflammation and wound healing process (Elson *et al.*, 1995). The models of chemical-induced colitis are most frequently used because they are inexpensive, reproducible, and readily available. Acetic acid-induced colitis model is useful for study of nonspecific inflammation and wound healing (Elson *et al.*, 1995). It shares many histologic features of ulcerative colitis including mucosal edema, neutrophil infiltration of the mucosa, and mucosal ulceration. However, there are major differences between the acetic acid-induced colitis model and ulcerative colitis. Acetic acid-induced colitis resolves rapidly and has only the histologic features of acute inflammation, whereas ulcerative colitis develops and resolves more slowly with the histologic features of mixture of

acute and chronic inflammation (Fedorak *et al.*, 1990; Sharon *et al.*, 1985). A loss of body weight and an increase in colon weight are important signs at acute stage of IBD in experimental animals (Son *et al.*, 1998; Wallace and Keenan, 1990). In our experiment, DA-6034 attenuated these symptoms significantly. Moreover, DA-6034, like prednisolone and sulfasalazine, showed significantly improved colonic lesions probably via modulation of nonspecific inflammation.

TNBS/ethanol-induced colitis model is a more suitable IBD experimental model than acetic acid-induced colitis. The acute mucosal injury produced by the barrier breaker, ethanol, resolves quickly (Wallace *et al.*, 1985), but is followed by a more chronic phase of inflammation through the delayed-type hypersensitivity reaction by TNBS, acting as a hapten (Hoshino *et al.*, 1992; Morris *et al.*, 1989). This model shares many histologic features of Crohn's disease; transmural necrosis, focal ulcer, focal basal cryptitis and fibrosis. Hence, this model is more suitable for Crohn's disease than ulcerative colitis. Because the TNBS-induced colitis model has persisted relatively long periods (Morris *et al.*, 1989), we evaluated the therapeutic effects at two stages, acute (1 week) and chronic (3 weeks) stages. Treatment with DA-6034 (3 mg/kg) markedly improved the colonic lesions significantly ($p<0.05$) at both acute stage and chronic stage. In these two chemical-induced colitis models, DA-6034 at a dose of 3 mg/kg was found to exhibit potent therapeutic efficacy.

Effects of DA-6034 found in the HLA-B27 transgenic rats were interesting, since this model is based on the expression of human genes known to be associated with several human inflammatory disorders (Elson *et al.*, 1995; Hammer *et al.*, 1990). This IBD model possesses three major advantages over the chemical-induced colitis models using TNBS or acetic acid. First, no exogenous agents are required for the induction of colitis. Second, the time-consuming and tedious nature of survival is not required. Third, the inflammation is chronic and progressive without spontaneous resolution of the inflammation, thereby allowing for long period observations. But, this model has main disadvantages such as the lack of either relapses or remissions and the absence of the acute neutrophilic component of human IBD. There appeared no difference in the gross findings of colitis among the groups of transgenic rats. It may be due to the colitis being mild. However, microscopic findings of the colitis in these rats revealed significant differences among groups. Treatment with DA-6034 (3 mg/kg) for 6 weeks markedly improved the colitis of this model and the efficacy of DA-6034 is somewhat greater than predniso-lone. A long term use of prednisolone in the treatment of IBD is rather limited due to its severe side effects. In this regard, our findings that the long term treatment of DA-6034 showed good therapeutic efficacy without side effects are very encouraging.

Some flavonoids such as quercetin and rutin have been studied in various IBD experimental models (Galvez, 1996; Sanchez *et al.*, 1996). Flavonoids have been reported to possess a number of biochemical and pharmacological activities affecting the function of enzyme systems critically involved in the immune response (Middleton *et al*, 1993) and the generation of inflammatory processes, namely tyrosine and serine-threonine protein kinases, phospholipase A, phospholipase C, lipoxygenase, cyclooxygenase, etc (Feriola *et al*, 1989; Laughton *et al*, 1991; Lee *et al*, 1982; Welton *et al*, 1986). Flavonoids have been recognized as the anti-oxidatives and free radical-scavenging agents (Bors *et al*, 1990; Nakayama *et al*, 1993). But some flavonoids are known to possess mutagenicity and cytotoxicity as well (Das *et al.*, 1994). In Korean folk medicine, *Artemisia* species has been used for the treatment of chronic diarrhea and further eupatilin, a flavonoid derivative, has been recently suggested to be a probable effective component of extract of the *Artemisia* species (Wu, 1985; Ahn *et al.*, 1997). DA-6034 is a synthetic eupatilin derivative. In this study, DA-6034 was found to be effective in the treatment of colitis in three experimental animal models of IBD. Therapeutic efficacy of DA-6034 was comparable or superior to prednisolone and sulfasalazine. In addition, unlike other conventional drugs with significant side effects, DA-6034 has been shown to be a safe flavonoid derivative (Cohen, 1996; Das *et al.*, 1973; World *et al.*, 1996). Based on these findings, DA-6034 appears to be an excellent drug candidate in the treatment of IBD. Although DA-6034 has anti-inflammatory effect, the detailed mechanism of its action remains unknown. Further studies are needed to examine whether DA-6034 exerts its beneficial effects in humans.

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