

## TJN-419 Improves Dextran Sulfate Sodium-Induced Colitis *via* Inhibition of Interleukin-12 Release

Chiharu SADAKANE,<sup>a,b</sup> Junichi KOSEKI,<sup>a</sup> Yayoi INAGAKI,<sup>a,b</sup> Yoshihiro HASEGAWA,<sup>a</sup> Shoichiro SHINDO,<sup>a</sup> Hirofumi MARUYAMA,<sup>c</sup> Shuichi TAKEDA,<sup>a</sup> Hiroshi TAKEDA,<sup>b</sup> and Tomohisa HATTORI<sup>\*,a</sup>

<sup>a</sup> Tsumura & Co., Tsumura Research Laboratories; 3586 Yoshiwara, Ami-machi, Inashiki-gun, Ibaraki 300–1192, Japan;

<sup>b</sup> Department of Pathophysiology and Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University; N12 W6, Kita-ku, Sapporo 060–0812, Japan; and <sup>c</sup> Iwaki Meisei University; 5–5–1 Chuodai Iino, Iwaki, Fukushima 970–8551, Japan. Received September 15, 2009; accepted October 9, 2009

We investigated the association of interleukin-12 (IL-12) with development of dextran sulfate sodium (DSS)-induced colitis in mice, and examined the effects of TJN-419, a synthetic compound derived from acteoside, on this process. Enhanced IL-12 production in lipopolysaccharide (LPS)-stimulated macrophages was dose-dependently inhibited by addition of TJN-419 to culture medium, and this effect was abolished by pretreatment with PD98059, an inhibitor of extracellular-regulated kinase. We then assessed the effect of TJN-419 or a neutralizing antibody against murine IL-12 in a DSS-induced colitis model in C57 BL/6 mice. Colitis was induced by 5% DSS solution given as drinking water. Treatment with the anti-IL-12 antibody was performed intravenously and TJN-419 was administered orally. We also investigated the effect of TJN-419 on erosion in the rectum in a DSS-induced colitis model in rat. The IL-12 level in the rectum was significantly enhanced and the IL-10 level was significantly decreased in animals with DSS-induced colitis compared with untreated controls. Intravenous injection of the anti-IL-12 antibody and oral administration of TJN-419 inhibited clinical symptoms in DSS-induced colitis. TJN-419 also inhibited the increase in IL-12 and suppressed the area of erosion in the rectum in DSS-induced colitis in rats. These results indicate that IL-12 has a possible role in development of DSS-induced colitis and that TJN-419 is effective for treatment of this disease model *via* inhibition of IL-12 production.

**Key words** TJN-419; dextran sulfate sodium-induced colitis; interleukin-12; clinical index

Ulcerative colitis is a representative inflammatory intestinal disease that shows early-stage clinical manifestations of diarrhea and mucous/bloody stool, with repeated reactivation, recurrence and progression to the chronic state. An immunological onset mechanism has been suggested and progression involves infiltration of macrophages and neutrophils in the large intestine. A colitis model induced by dextran sulfate sodium (DSS) is widely used for pharmacological evaluation of drug efficacy or examination of disease mechanism in ulcerative colitis. The characteristics of this model include an acute lesion occurring up to 1 week after the start of ingestion of a DSS solution accompanied by symptoms such as loss of body weight, bloody stool and diarrhea, followed by infiltration of inflammatory cells into the colonic mucosa or epithelial cell layer.<sup>1)</sup>

A role of cytokines in the rectum has also been shown in inflammatory colitis models.<sup>2–4)</sup> Among these cytokines, interleukin-12 (IL-12) is a regulator of Th1 cytokines, which enhance production of interferon gamma (IFN- $\gamma$ ) and activate cellular immunity. In humans, data on IL-12 expression in ulcerative colitis is still somewhat controversial, but increased gene expression and secretion of IL-12 have been reported in rodent ulcerative colitis. A recent study of IL-12 p35 knockout mice provided some evidence for the role of IL-12 in ulcerative colitis. However, more studies are needed because there is no direct evidence associating IL-12 with the development of ulcerative colitis. Therefore, the first objective of this study was to demonstrate the involvement of the Th1/Th2 cytokine balance in the DSS-induced ulcerative colitis model in mouse. We then determined the rectum IL-12 and IL-10 concentrations in this model to evaluate the effect of an anti-IL-12 antibody.

Herbal medicines are known to contain compounds that

have immunological effects. Acteoside is a component of crude drugs such as Rehmannia Root, Chinese artichoke and *Leucosceptum japonicum*, and is reported to be effective against DSS-induced colitis through suppression of cytokine production from mesenteric lymph node cells.<sup>5)</sup> Acteoside has a complex structure in which caffeic acid and phenethyl alcohol are bound together by two sugars, and it is unlikely that the unchanged compound exerts an effect *in vitro*. TJN-419 is a synthetic compound derived from acteoside that suppresses IL-12 production *in vitro*.<sup>6)</sup> In the second part of this study, the effects of TJN-419 on symptoms and the histological effect of TJN-419 on colonic mucosal erosion were evaluated in rodent DSS-induced colitis.

### MATERIALS AND METHODS

**Experimental Animals** C57BL/6N male mice aged 6 weeks and Sprague-Dawley (SD) male rats aged 7 weeks (Charles River Laboratories Japan, Inc.) were used in the study. The animals were moved into individual cages lined with filter paper and floor litter one week before the start of the experiment and given food and water *ad libitum* in an air-conditioned animal room (lighting: 7 a.m.—7 p.m.). All experiments were performed between 9 a.m. and 6 p.m. within the guidelines of the Experimental Animal Ethics Committee of Tsumura & Co.

**Chemicals and Antibodies** The cinnamide derivative TJN-419 (4-((E)-2-(N-(3-pyridylmethyl)carbamoyl)ethenyl)-phenoxy)acetate, Fig. 1) and its analogs (Table 1) are synthetic compounds derived from acteoside. These compounds were synthesized in the Research Laboratories of Tsumura & Co. We have previously reported the structure–activity relationship of TJN-419.<sup>6)</sup> In the current study, TJN-419

\* To whom correspondence should be addressed. e-mail: hattori\_tomohisa@mail.tsumura.co.jp

(Tsumura & Co., Ibaraki, Japan), FK506 (Astellas Pharma Inc., Tokyo, Japan) or salazosulfapyridine (SASP) (Sigma-Aldrich, St. Louis, MO, U.S.A.) was suspended in a 0.5% solution of carboxymethylcellulose sodium (CMCNa) (Maruishi Pharmaceutical, Japan). Mouse immunoglobulin G (IgG) and anti-IL-12 neutralizing antibody (R&D Systems, Minneapolis, MN, U.S.A.) were dissolved in sterile physiological saline for administration at a dose of 20  $\mu$ g/mouse. PD98059, an inhibitor of extracellular regulated kinase (MAP kinase kinase I or MEKI), was obtained from Promega (Madison, WI, U.S.A.).

**Preparation of the DSS-Induced Colitis Model in Mouse** Animals in the colitis group were given a 5% solution of DSS *ad libitum* in drinking water until sacrifice. Control animals were given distilled water *ad libitum*. All animals were weighed prior to the start of the experiment. TJN-419 at 5, 25 or 125 mg/kg/d or FK506 at 5 mg/kg/d was administered orally twice a day for the first 5 d. Anti-IL-12 antibody was administered intravenously once a day at 20  $\mu$ g/mouse. After administration on day 5, the floor litter and filter paper were exchanged. The stools left on the filter paper on day 6 were observed for bleeding and diarrhea in a blinded manner and the stool consistency score was determined. For investigation of the effect of TJN-419 on cytokine levels in the rectum of mice with DSS-induced colitis, TJN-

419 at 25 mg/kg or SASP at 25 mg/kg was administered twice a day for 5 d and the rectum was isolated and a homogenate was prepared using PBS on day 1, 3 or 5.

**Preparation of the DSS-Induced Colitis Model in Rat** Since histological evaluation of rectum tissues is difficult in the mouse DSS-induced colitis model, a similar model was prepared in rat.<sup>7)</sup> Ten rats selected randomly were assigned to the control group and given distilled water *ad libitum* until sacrifice. All other rats were given a 3% aqueous solution of DSS *ad libitum* from a water supply bottle. Bloody stool was observed in more than 90% of the rats on day 9 after starting ingestion of DSS. On this day, the rats were randomly assigned to groups (10 rats per group) with a similar mean body weight in each group. Thereafter, the rats were given 1% DSS solution *ad libitum* and TJN-419 (0.5, 1.5 or 5 mg/kg) or SASP (50 mg/kg) was administered orally twice a day (at 9:30–11:00 and 16:00–17:30, except on the first day, when only one dose was given) for 14 d. The dose of each test compound was determined in preliminary experiments and doses that gave reproducible results were used. Effects were seen at lower dose levels compared with the mouse model, particularly for TJN-419.

Representative symptoms in the colitis model (body weight decrease, bleeding and stool consistency) were evaluated using the method of Melgar *et al.*<sup>1)</sup> with slight modifications. Body weight was measured on the day of induction (day 1) and on days 4, 5 and 6. The weight decrease score was determined as the change from the weight on day 1, with decreases of 0–5%, 5–10%, and 10–20% scored as 1, 2 and 3 points, respectively. The bleeding score was determined from observation of stools left on the filter paper on day 6, with the extent of hemorrhage scored as follows: no

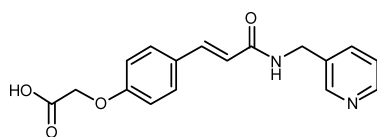


Fig. 1. Chemical Structure of TJN-419

Table 1. *In Vitro* Profile of TJN-419 Derivatives

Name	Chemical structures	IL-12 (inhibition %)	IL-10 (inhibition %)
TJN-419		64.2	-23.9
ACL-P432		48.6	-7
ACL-P435		61.4	23.1
ACL-U559		14.5	23.8
ACL-U575		-20.5	-3.8
ACL-U579		-27.6	3.2

hemorrhage, 0 points; trace of hemorrhage, 1 point; traces of hemorrhage at one to two sites, 2 points; traces of hemorrhage at several sites, 3 points; and definite hemorrhage at several sites, 4 points. The stool consistency score was also obtained by observation of the stools left on the filter paper on day 6, with the extent of diarrhea scored as follows: no diarrhea, 0 points; slight change of shape, 1 point; no shape, 2 points; and watery stool, 3 points. In evaluation of bleeding and stool consistency, the evaluator was blinded to the identity of each mouse by randomization, and the identity was only revealed after the evaluation. The clinical index was defined as follows to reflect the overall disease severity: clinical index = (weight decrease score + bleeding score + stool consistency score) / 3.

**Determination of IL-12 and IL-10 Levels in Mouse Rectum** The isolated rectum was washed with physiological saline and homogenated using phosphate buffered saline (PBS). The homogenate was centrifuged at 3000 rpm for 15 min and cytokine levels were determined in the supernatant using mouse IL-12 (p70 or p40) and IL-10 ELISA kits (Life Technologies Inc.).

**Immunostaining of IL-12 in the Rectum and Appendix** Paraffin sections were prepared from mouse tissues obtained on day 5 after induction of colitis and subjected to immunostaining using an ABC-Elite Peroxidase Stain kit (Vector Lab., Burlingame, CA, U.S.A.) or the anti-IL-12 monoclonal antibody kits mentioned above.

**In Vitro Effect of TJN-419 and Analogs on Mouse Intraperitoneal Macrophages** Two milliliters of 3% Brewer thioglycollate culture medium (DIFCO) was administered intraperitoneally to C57BL/6 mice and macrophages were collected by intraperitoneal injection of 5 ml Hank's balanced salt solution 3 d after administration. The macrophages were washed with Hank's solution and then incubated (100000 cells/ml) with or without TJN-419 at various concentrations or analogs at a dosage of 10  $\mu\text{mol/l}$  ( $n=3$ ) for 24 h in a  $\text{CO}_2$  incubator with stimulation with 10 ng/ml lipopolysaccharide (LPS) (from *Escherichia coli* 055:B5; Sigma). IL-12 (p70 or p40) and IL-10 levels were then determined in the supernatant using the methods described above. In experiments in-

volving ERK1/2 inhibition, macrophages were preincubated with PD98059 (10  $\mu\text{mol/l}$ ), an inhibitor of MEK1 (ERK), for 30 min in the presence or absence of TJN-419 (50  $\mu\text{mol/l}$ ) and then stimulated with LPS for 24 h. Optical density was measured using Tetracolor One (Seikagaku-kogyo, Tokyo, Japan) to evaluate the number of surviving cells in each well.

**Fixation of Large Intestinal Sections** After blood sampling, the large intestine (colon and rectum) was isolated and immediately fixed by injection of a 10% neutral formalin solution into the lumen for expansion, followed by standing in physiological saline for at least 1 h. Thereafter, the intestinal tube was cut open along the mesenterium attachment and fixed in 10% neutral formalin in a stretched state for one week.

**Determination of the Erosion Area in Colonic Mucosa** The large intestine specimen fixed in 10% neutral formalin was washed with streaming water for 5 min and further washed with distilled water 3 times. Then, the specimen was pretreated in a 3% acetic acid solution for 5 min, stained in a 1% Alcian Blue solution for 20 min, and washed 4–5 times with 3% acetic acid until no further Alcian Blue dissolved. The stained specimens were given random serial numbers and photographed. An image analysis system (WinRoof v. 3.1, Mitani Co., Japan) was used to measure areas of deep blue staining, which reflect areas of erosion, on the photographs in a blinded manner.

**Statistical Analysis** Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett test. For evaluation of symptom scores, the statistical analysis for vehicle vs. test compound-treated animals was conducted using a Steel analysis. Data are presented as means  $\pm$  standard error for each group. Differences between groups with  $p < 0.05$  were judged to be significant.

## RESULTS

**Expression of IL-12 in the Rectum and Appendix** In normal mice, only a trace of positive IL-12 staining was seen in the stroma of the rectum and appendix (Fig. 2). In mice with DSS-induced colitis, the number of IL-12-positive cells

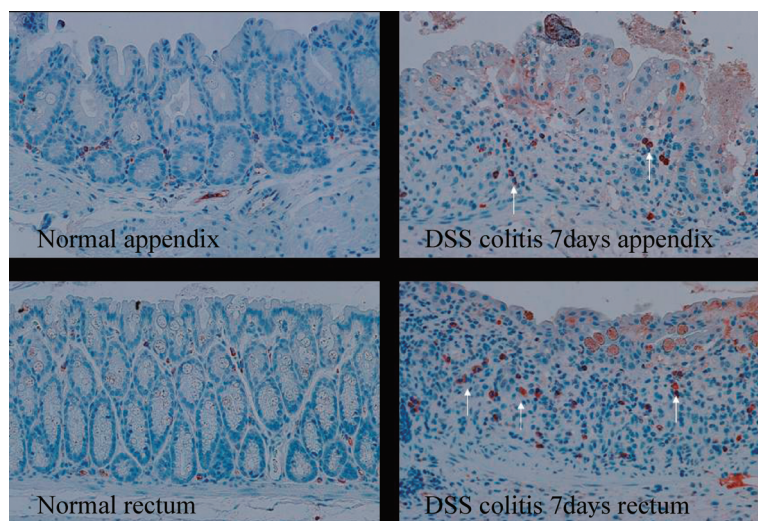


Fig. 2. Immunoperoxidase Staining for IL-12 in the Appendix and Rectum of Normal Mice and Mice with DSS-Induced Colitis  
Arrows indicate areas positive for IL-12 (p70).

(shown by arrows in Fig. 2) in the rectum and appendix clearly increased on day 7 after induction of colitis. These cells were thought to be infiltrating inflammatory cells.

**Time-Course of IL-12 and IL-10 Levels in the Rectum in the Colitis Mouse Model** The IL-12 level clearly increased from day 5 after induction and further increased until day 7 in mice with DSS-induced colitis. In contrast, the IL-10 level decreased significantly on day 2 and day 5 after induction and recovered to the normal level on day 7 (Fig. 3).

**Effect of Anti-IL-12 Neutralizing Antibody** Mice with DSS-induced colitis that were administered anti-IL-12 antibody showed a tendency for suppression of increases in weight loss and bleeding scores, and had significant suppres-

sion of increases in stool consistency score and clinical index (Fig. 4).

**Effects of TJN-419 or Analogs on Production of IL-12 and IL-10 by Peritoneal Macrophages** Inhibition of IL-12 production by TJN-419 at 10  $\mu\text{mol/l}$ , ACL-P432, and ACL-P435 was approximately same intensity, compared with control (Table 1). Inhibition of IL-12 production was attenuated or disappeared for molecules with a shorter chain attached to the benzene ring, based on the results for TJN-419, ACL-U559, and ACL-U575. This suggests that the distance between the carboxyl group and pyridine ring influences the activity. Introduction of a methyl group at the 6-position of the pyridine ring (ACL-U579) eliminated inhibition of IL-12 production. The methyl group may inhibit an interaction of the N atom in the pyridine ring, which suggests that this N atom has an important role in activity.

Production of IL-12 (p70) by macrophages with addition of 10% fetal calf serum (FCS) plus stimulation with LPS was suppressed by TJN-419 in a dose-dependent manner (Fig. 5A). The IL-12 p40 protein concentration from macrophages was also enhanced by LPS compared to the non-stimulated group ( $47.3 \pm 4.1$  pmol/l), and addition of TJN-419 to the culture medium markedly inhibited the increase in IL-12 p40 (Fig. 5B). On the contrary, IL-10 production was significantly increased by stimulation with LPS and was not influenced by TJN-419 (Fig. 5C). To examine the role of ERK1/2 MAP kinase in the mechanism of action of TJN-419, we analyzed the effect of TJN-419 on IL-12 p40 production in LPS-stimulated macrophages pre-treated with PD98059, a specific inhibitor of MEK1 (ERK). Inhibition of ERK1/2 MAP kinase by PD98059 significantly enhanced production of IL-12 p40 by LPS-stimulated macrophages in the absence or presence of TJN-419 (Fig. 5D). TJN-419 at 50  $\mu\text{mol/l}$  showed

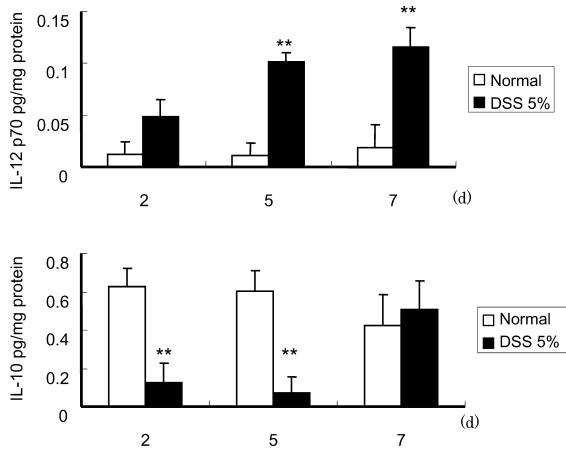


Fig. 3. Changes in IL-12 (p70) or IL-10 Levels in Rectum Homogenates from Mice with DSS-Induced Colitis

Each column shows the mean  $\pm$  S.E. of 5 mice. \*\* Significant difference for normal (saline-treated) vs. DSS-induced colitis mice ( $p < 0.01$ , Student *t*-test).

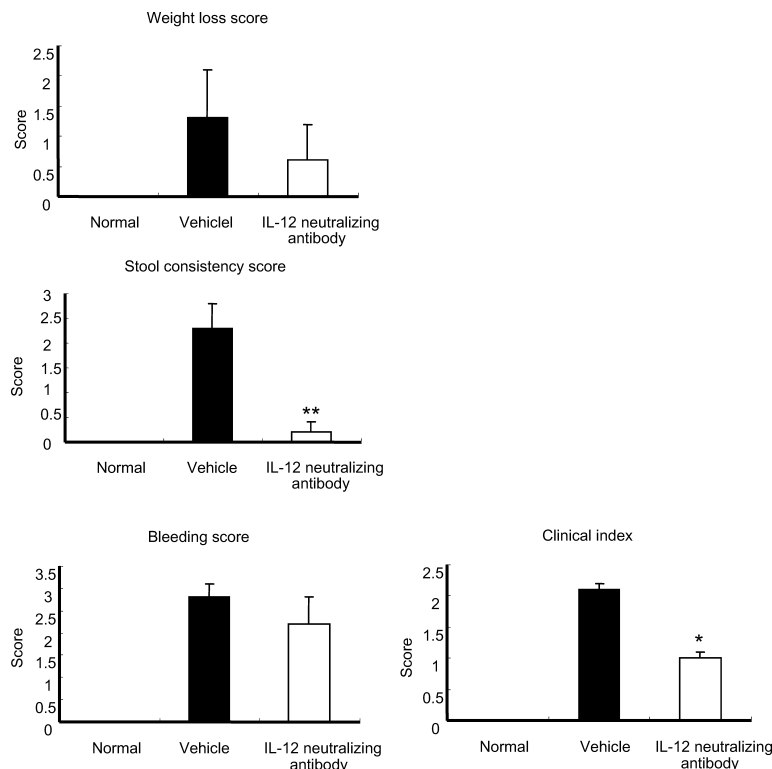


Fig. 4. Effect of a Neutralizing Antibody for Murine IL-12 (p70) on Clinical Parameters in DSS-Induced Colitis in Mice

Each column shows the mean  $\pm$  S.E. of 5 mice. \*  $p < 0.05$ , \*\*  $p < 0.01$  for control (mouse IgG-treated) vs. anti-IL-12-treated mice (Steel test).



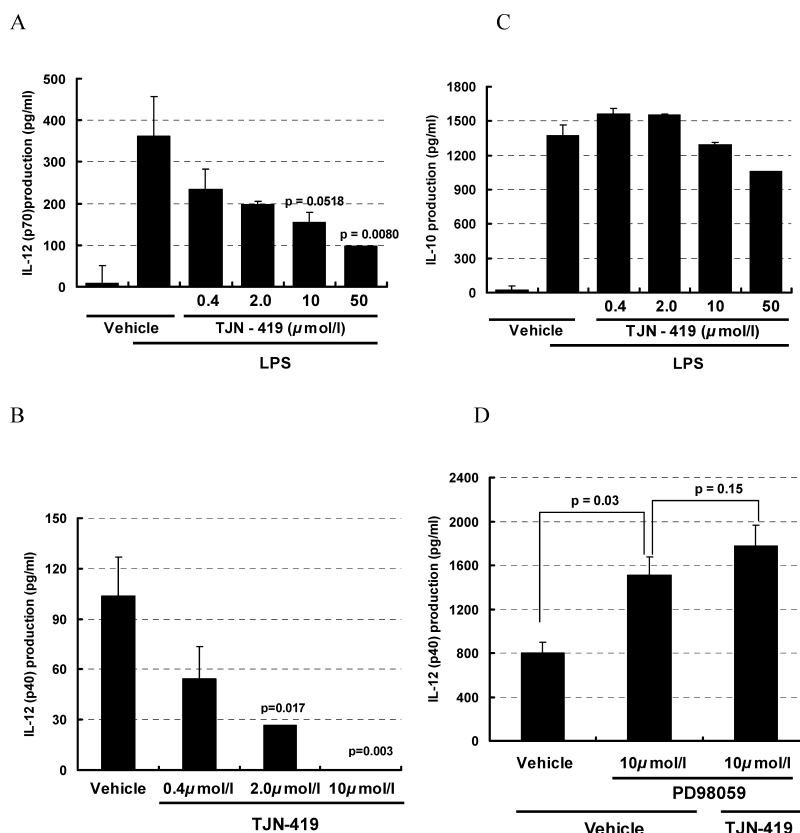


Fig. 5. *In Vitro* Assays of IL-12 and IL-10 Release from Brewer Thioglycollate-Stimulated Peritoneal Exudate Cells

Effects on IL-12 p70 (A), p40 (B) and IL-10 (C) production from LPS-stimulated macrophages. (D) Effect of TJN-419 on the increase in IL-12 p40 production caused by pre-treatment with PD98059. There were significant differences between controls (LPS-stimulated) and test compound-treated animals (Dunnett test).

no cytotoxicity (cell death: controls, 27.0% vs. TJN-419, 19.5%).

**Effect of TJN-419 on Symptom Scores in the Mouse DSS-Induced Colitis Model** The weight decrease scores were  $0.17 \pm 0.24$  in control mice,  $1.25 \pm 0.25$  in untreated colitis mice, and  $0.38 \pm 0.26$  in colitis mice treated with 5 mg/kg TJN-419 ( $p < 0.05$  vs. untreated colitis). These scores were  $0.50 \pm 0.27$  in colitis mice treated with 25 mg/kg TJN-419 and  $0.50 \pm 0.27$  in colitis mice treated with 125 mg/kg TJN-419, with a tendency for a decrease in score compared to untreated colitis mice. The weight loss score with FK506 treatment was  $0.75 \pm 0.25$ . The stool consistency score was  $0 \pm 0$  in control mice,  $1.75 \pm 0.31$  in untreated colitis mice, and  $1.75 \pm 0.37$ ,  $1.38 \pm 0.38$  and  $0.88 \pm 0.35$  with TJN-419 treatment at 5, 25 and 125 mg/kg, respectively. The scores for the higher TJN-419 doses showed a tendency for a decrease compared to the untreated colitis mice. With FK506, the stool consistency score was  $0.38 \pm 0.26$  ( $p < 0.01$  vs. untreated colitis mice). The bleeding score was  $0 \pm 0$  in control mice,  $2.13 \pm 0.4$  in untreated colitis mice, and  $2.00 \pm 0.47$ ,  $1.58 \pm 0.35$  and  $1.75 \pm 0.37$  with TJN-419 treatment at 5, 25 and 125 mg/kg, respectively. The scores for the higher TJN-419 doses showed a tendency to decrease compared with untreated colitis mice. With FK506, the bleeding score was  $0.75 \pm 0.31$  ( $p < 0.05$  vs. untreated colitis mice).

**Effect of TJN-419 on the Clinical Index in the Mouse DSS-Induced Colitis Model** The clinical index increased in DSS-induced colitis mice compared with controls (Fig. 6) and was reduced by treatment with TJN-419 (25 or 125 mg/kg). The effect on the clinical index was stronger

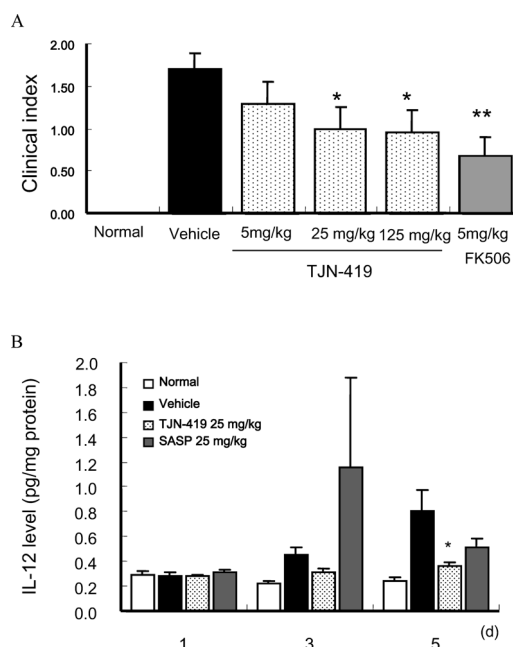


Fig. 6. Effects of TJN-419 on Clinical Index (A) and IL-12 Level (B) in the Rectum of Mice with DSS-Induced Colitis

Each column shows the mean  $\pm$  S.E. of 5–8 mice. \*  $p < 0.05$ , \*\*  $p < 0.01$  for control (distilled water) vs. treated mice (Dunnett test or Steel test).

with FK506 (Fig. 6A). The IL-12 level increased in the rectum homogenate of colitis mice from days 1 to 5 and was significantly suppressed by TJN-419 on day 5 (Fig. 6B).

**Effect of TJN-419 on the Rectum Erosion Area in the**

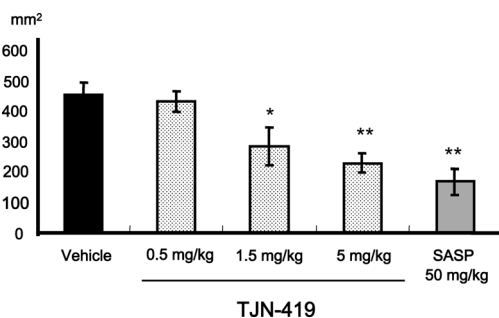


Fig. 7. Effects of TJN-419 and Salazosulapyridine (SASP) on DSS-Induced Colitis in Rats

The compounds were administered orally (5 ml/kg) twice a day for 14 d, except for the first day of administration. Each column shows the mean  $\pm$  S.E. for 8 rats. \* $p < 0.05$ , \*\* $p < 0.01$  vs. vehicle (Dunnett test).

**Rat DSS-Induced Colitis Model** As shown in Fig. 7, TJN-419 suppressed the increase of the rectum erosion area in a dose-dependent manner. A similar suppressive effect was seen for salazosulapyridine at 50 mg/kg. TJN-419 also showed a tendency for suppression of the decrease in large intestine length in DSS-induced colitis in rat (data not shown).

## DISCUSSION

In this study in rodent DSS-induced colitis models, production of IL-12 was enhanced in infiltrating cells and aggravation of clinical parameters was suppressed by blocking the function of IL-12 with an anti-IL-12 neutralizing antibody. TJN-419, a synthetic compound derived from acteoside, also showed suppression of IL-12 and improvement of DSS-induced colitis in mice and rats. Melgar *et al.*<sup>1)</sup> have shown that DSS-induced colitis causes more severe damage in C57BL/6 mice (regarded as Th1-dominant) than in BALB/c mice, and also found that IL-12 level in the large intestine is high in this colitis model. We also found an elevated level of IL-12 p70 in the DSS-induced colitis model, and it has also been shown that DSS-induced colitis is mild in IL-12p35(-/-) mice compared with wild type mice.<sup>8)</sup> These findings suggest that IL-12 is involved in development of DSS-induced colitis in Th1 predominant mice. However, the effects of direct inhibition of IL-12(p70) in DSS-induced colitis have not been evaluated.

In this study, an immunohistological investigation of DSS-induced colitis showed that IL-12-positive cells accumulated in the appendix and large intestine and that IL-12 in the tissue homogenate increased from day 5, when clinical symptoms such as bloody stool and diarrhea started to become significant. Furthermore, we found that IL-10 clearly decreased in the early stage after onset (days 2 to 5). Cytokines such as IL-10 can negatively regulate IL-12 production. These results suggest that enhanced IL-12 production at topical inflammation sites in the large intestine and the early decrease of IL-10 may lead to Th1 predominance in the mouse colitis model. In addition, since the severity of DSS-induced colitis was alleviated by administration of an anti-IL-12 antibody, IL-12 appears to be involved in the disease, at least in the acute stage.

Acteoside has been reported to be effective against immunological inflammatory diseases such as nephritis,<sup>9)</sup> and

suppression of cytokine production by acteoside has also been found in DSS-induced colitis.<sup>5)</sup> We discovered TJN-419 as an acteoside derivative based on its suppressive effect on IL-12 production from macrophages.<sup>6)</sup> In the current study, addition of TJN-419 to macrophage culture medium inhibited the increase in release of IL-12, but not IL-10, without causing cytotoxicity. Based on the structure-activity relationships of 6 compounds, the distance between the N atom of the pyridine ring and the terminal carboxyl group is important for inhibitory activity against IL-12 production.

TJN-419 also suppressed the increase of the clinical index in the mouse DSS-induced colitis model. These results suggest that TJN-419 influences colitis symptoms in this model by suppressing IL-12 production without influencing IL-10. The detailed mechanisms of the inhibitory action of TJN-419 on IL-12 release are unclear. The p35 and p40 subunits of IL-12 are encoded by two genes that are each regulated by a different stimulator. It is believed that the p40 protein synthesized in macrophages through activation of CD40 signaling can be released into the extracellular space, but that the p35 protein cannot be released independently. TJN-419 inhibited increased release of bioactive IL-12 p70 and p40. Previous reports show that ERK1/2 is a negative regulator of production of IL-12 p40, but not of IL-10, by LPS-stimulated macrophages,<sup>10)</sup> and we also found a significant increase in IL-12 production from macrophages pretreated by PD98059, an ERK1/2 inhibitor. Interestingly, TJN-419 failed to suppress IL-12 production from macrophages pretreated with PD98059. These results suggested that TJN-419 may suppress transcription of the IL-12 p40 subunit *via* activation of ERK1/2 kinase, thus limiting the amount of biologically active p70 heterodimer.

Histological evaluation of large intestine tissues is difficult in the mouse DSS-induced colitis model because of the small amount of tissue and the high death rate. Therefore, a rat DSS-induced colitis model was used for histological evaluation. In this model, TJN-419 significantly decreased the erosion area in the colonic mucosa. These results also suggest a role of IL-12 in rodent DSS-induced colitis. Efficacy of TJN-419 for erosion was found at a lower dose in rat DSS-induced colitis (erosion area with TJN-419 at 10 mg/kg,  $182.6 \pm 46.5$  mm<sup>2</sup> vs. untreated colitis,  $388.7 \pm 32.6$  mm<sup>2</sup>;  $p < 0.01$ ) compared to that in the mouse model. However, TJN-419 at 50 mg/kg failed to improve colitis (erosion area  $376.0 \pm 33.1$  mm<sup>2</sup>). We do not have sufficient data to explain why TJN-419 at a lower dose was more effective in improving histopathological damage. One possible reason is differences in susceptibility to TJN-419 due to genetic background influencing absorption or distribution.

IL-12 is well known to activate cellular immune responses through differentiation of Th1 cells and IFN- $\gamma$  production, but the relationship between DSS-induced colitis and cellular immunity has not been widely investigated. It is conceivable that IFN- $\gamma$  production enhanced by IL-12 is involved in this disease, since IFN- $\gamma$  deficient mice do not develop DSS-induced intestinal inflammation.<sup>11)</sup> In addition, heterozygous mice without suppressor of cytokine signaling 1 (SOCS1), a critical inhibitor of IFN- $\gamma$  signaling, develop more severe DSS-induced colitis than DSS-treated wild type mice.<sup>12)</sup> We also found a high INF- $\gamma$  level in the large intestine in the DSS-induced colitis model at 7 d after induction

( $0.057 \pm 0.02$  vs.  $0 \pm 0$  pg/ml in controls). We suggest that DSS deposited in the large intestine may be recognized as a foreign substance, and that induction of NK cells may induce tissue impairment. Further studies are needed to clarify the mechanism of stimulation of Th1 cell differentiation by DSS.

## REFERENCES

- 1) Melgar S., Karlsson A., Michaelsson E., *Am. J. Physiol. Gastrointest. Liver Physiol.*, **288**, G1328—G1338 (2005).
- 2) Boismenu R., Chen Y., *J. Leukoc. Biol.*, **67**, 267—278 (2000).
- 3) Hans W., Scholmerich J., Gross V., Falk W., *Eur. Cytokine Netw.*, **11**, 67—74 (2000).
- 4) Duchmann R., Schmitt E., Knolle P., Meyer zum Buschenfelde K. H., Neurath M., *Eur. J. Immunol.*, **26**, 934—938 (1996).
- 5) Hausmann M., Obermeier F., Paper D. H., Balan K., Dunger N., Menzel K., Falk W., Schoelmerich J., Herfarth H., Rogler G., *Clin. Exp. Immunol.*, **148**, 373—381 (2007).
- 6) Hasegawa Y., Shindou S., Hattori T., U.S. Patent 6413995 (2002).
- 7) Gaudio E., Taddei G., Vetusch A., Sferra R., Frieri G., Ricciardi G., Caprilli R., *Dig. Dis. Sci.*, **44**, 1458—1475 (1999).
- 8) Takagi H., Kanai T., Okazawa A., Kishi Y., Sato T., Takaishi H., Inoue N., Ogata H., Iwao Y., Hoshino K., Takeda K., Akira S., Watanabe M., Ishii H., Hibi T., *Scand. J. Gastroenterol.*, **38**, 837—844 (2003).
- 9) Hattori T., Fujitsuka N., Shindo S., *Nippon Jinzo Gakkai Shi*, **38**, 202—212 (1996).
- 10) Fernando C., Leyre M., Docagne F., Guaza C., *Br. J. Pharmacol.*, **145**, 441—448 (2005).
- 11) Ito R., Shin-Ya M., Kishida T., Urano A., Takada R., Sakagami J., Imanishi J., Kita M., Ueda Y., Iwakura Y., Kataoka K., Okanoue T., Mazda O., *Clin. Exp. Immunol.*, **146**, 330—338 (2006).
- 12) Horino J., Fujimoto M., Terabe F., Serada S., Takahashi T., Soma Y., Tanaka K., Chinen T., Yoshimura A., Nomura S., Kawase I., Hayashi N., Kishimoto T., Naka T., *Int. Immunol.*, **20**, 753—762 (2008).