

## Suppression of Dextran Sulfate Sodium-Induced Colitis in Kininogen-Deficient Rats and Non-peptide B<sub>2</sub> Receptor Antagonist-Treated Rats

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**ABSTRACT**—Various proinflammatory mediators are believed to be involved in the processes and symptoms of ulcerative colitis (UC). To determine whether endogenous kinin enhances the severity of UC, we induced experimental colitis (EC) in kininogen-deficient mutant rats and tested the effect of a non-peptide B<sub>2</sub> receptor antagonist. EC was induced in male kininogen-deficient Brown Norway-Katholiek rats (BN-Ka) and normal Brown Norway-Kitasato rats (BN-Ki) with 5% dextran sulfate sodium (DSS). Sprague-Dawley rats (SD) were also used. Colon length, body weight and hematocrit were determined for 7 days. Effects of FR173657, an orally active B<sub>2</sub> antagonist, were tested. The colon length was shortened in BN-Ki with DSS treatment, but not in BN-Ka, and the difference between their lengths was significant. The hematocrit value was also reduced in BN-Ki, and the difference in hematocrit between BN-Ki and BN-Ka was significant. In SD, shortening of the colon and reduction in hematocrit were also observable, and both were blunted by FR173657. The survival rate in SD given DSS for 7 days was 68%, but FR173657 treatment restored it significantly to 100%. These results suggest that the endogenous kinins generated from the kallikrein-kinin system have a significant role in the development of EC.

**Keywords:** Bradykinin, B<sub>2</sub>-receptor antagonist, Kininogen-deficient rat, Dextran sulfate sodium, Experimental colitis

The inflammatory bowel diseases (IBD), which include ulcerative colitis (UC) and Crohn's disease, are multifactorial diseases of unknown etiology (1). Various proinflammatory mediators, such as nitric oxide, prostaglandins, leukotrienes, platelet activating factor, neuropeptides, and inflammatory cytokines have been considered to play a role in the inflammatory processes and symptoms of UC (2, 3), but the precise mechanisms and mediators remain to be elucidated (4). Recent results (5–7) using knockout mice that are defective in interleukin (IL)-2, IL10 and T-cell receptors suggested the significance of the immune system in the development of IBD-like symptoms. However, the plasma proteolytic cascades are also reported to be important factors in causing IBD-like pathological conditions (8, 9).

Endogenous kinins, such as bradykinin (BK) and kallidin

(lysyl-BK), which are potent proinflammatory and vasoactive peptides, are produced by a proteolytic cascade, the kallikrein-kinin system. BK and kallidin are physiological ligands for the bradykinin B<sub>2</sub> receptor; and they are released from their precursors, the kininogens, by the proteolytic activity of plasma kallikrein after contact activation via the Hageman factor (factor XII of the coagulation pathway) and by tissue kallikrein, respectively (10). Various physiological or pathological conditions such as tissue damage, ischemia, heat, cold, low pH, and negatively charged surfaces can activate i) the plasma kallikrein-kinin system via the Hageman factor to release bradykinin and ii) the tissue kallikrein-kinin system via the release or secretion of tissue kallikrein (10). BK is a very potent mediator of vasodilatation and vascular permeability (10), the most potent chemical mediator of pain sensation (10, 11), and a major spasmogen in the smooth muscle of many organs including the gut (10, 12, 13). These direct actions of BK may at least partly explain the pathogenesis of UC. Further-

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more, BK is also known to induce or increase other proinflammatory factors such as nitric oxide (10), prostaglandins (10, 14), leukotrienes (10, 15), platelet-activating factor (10), neuropeptides (16), and inflammatory cytokines (10, 17–21). These interactions between BK and other substances may facilitate the development of UC, and BK may have a crucial role in it.

To our knowledge, in spite of the potent proinflammatory activity of kinins, the involvement of the kallikrein-kinin system in the development of experimental enterocolitis (8, 9, 22) and colitis (4) has been demonstrated, but the number of reports is limited. In a model of granulomatous enterocolitis induced in a genetically susceptible strain of Lewis rats by peptidoglycan-polysaccharide polymers isolated from group A streptococci, the activation of the plasma kallikrein kinin system was presumed to have occurred, because of the consumption of the precursor proteins plasma prekallikrein and high-molecular-weight kininogen (9, 23–25). However, they did not use the specific antagonists for kinin receptors, and no actual contribution of kinin to that enterocolitis was demonstrated in their report. Utilizing another widely used model of UC induced by dextran sulfate sodium (DSS), only one paper described the effect of a selective antagonist for bradykinin B<sub>2</sub> receptor (4). The antagonist used in their study was a second-generation antagonist for B<sub>2</sub> receptor, Hoe140, which is a peptide analogue of BK and has a limited application because of its chemical properties (26, 27).

In the present study, to demonstrate the contribution of endogenous kinins generated from the kallikrein-kinin system to DSS-induced experimental colitis (EC), we used i) Brown Norway-Katholiek rats (BN-Ka), which do not generate kinin because of the lack of kininogens due to a one-point mutation in their genes (28), and ii) an orally active non-peptide B<sub>2</sub> antagonist, FR173657 ((*E*)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyloxy)methyl]phenyl]-*N*-methylaminocarbonylmethyl]acrylamide), which was recently developed as a novel pharmacological tool for in vivo analysis (29). The present results will provide strong evidence for the contribution of endogenous kinin in the process of DSS-induced EC in rats.

## MATERIALS AND METHODS

### *Rats*

Male BN-Ka, which do not have kinin precursor proteins, kininogens, and normal Brown Norway-Kitasato rats (BN-Ki) from the same strain (weighing approximately 300 g) were used for induction of the EC in the present experiment. Male Sprague-Dawley rats (SD) (Japan SLC, Shizuoka), weighing 80–100 g, were also used. The rats were kept under standard laboratory conditions and were

maintained on a controlled 12-h dark/light cycle (light on from 8 AM to 8 PM). They were housed in groups of three animals per cage, and allowed free access to animal chow (CE-2; Nippon Clea, Tokyo). Before induction of EC, all animals were given sterile distilled water as drinking water. All experiments were performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

### *Induction of colitis*

EC was induced by providing rats with sterile distilled water containing 5% DSS (mol wt 5000; Sigma Chemical Co., St. Louis, MO, USA) as drinking water for consumption ad libitum throughout the experiment.

### *Administration of FR173657 to rats*

FR173657 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka). The non-peptide bradykinin B<sub>2</sub> receptor antagonist was dissolved in 5% gum arabic and was orally administered (30 mg/kg, twice a day) to SD rats from the first day of the experiment (day 0). To control SD rats given vehicle only, 5% gum arabic were orally administered. The rats were killed on days 3, 5 and 7 for measurement of the colon length (30), body weight and hematocrit.

### *Measurement of the colon length*

After the body weight was measured, a blood sample was promptly taken from the carotid artery of the rats under light ether anesthesia. After the rats were killed by exsanguination, the entire colon from the ascending colon to the rectum was isolated, cut longitudinally, wiped free of feces, and placed on a plastic board. Then the colon length was measured, as reported previously (30).

### *Activation of plasma kallikrein-kinin system by DSS in rat plasma*

Rat citrated plasma (200  $\mu$ l) (31) was acidified to pH 2.0 followed by incubation for 15 min at 37°C. It was then neutralized to pH 7.8. The plasma was added to a polypropylene tube containing 180  $\mu$ l of 50 mM Tris-HCl (pH 7.8), EDTA-2Na (2 mg), *o*-phenanthroline (400  $\mu$ g) and DSS (100  $\mu$ g). The mixture was incubated for 0, 15 and 30 min at 37°C. The reaction was stopped by the addition of trichloroacetic acid (4.0% of the final concentration). Proteins in the mixture were removed by a subsequent centrifugation (4°C, 1500  $\times$ g, for 30 min). The bradykinin released in the supernatant was measured by ELISA (MARKIT-M Bradykinin; Dainippon Pharmaceutical Co., Ltd., Osaka), as reported previously (32).

### *Statistical analyses*

Values are expressed as the mean  $\pm$  S.E.M. For comparison of data from multiple groups, one-way ANOVA fol-

lowed by post-hoc Scheffe's test was used. For analysis of survival rates, Fisher's test was used for comparison. In Figs. 2 and 3, comparisons were made between BN-Ka and BN-Ki and between day 0 and day 3 to 7 after administration of DSS.

## RESULTS

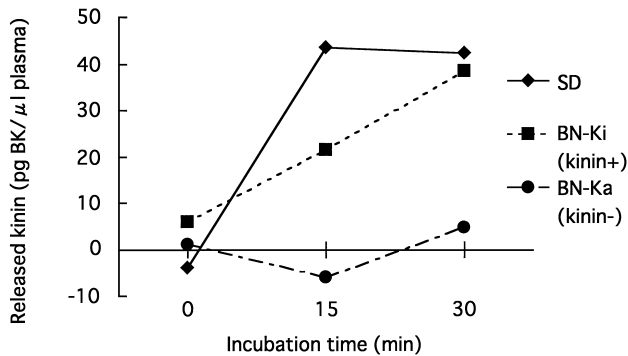
### *In vitro experiment: generation of kinins from rat plasma after the addition of DSS*

To test the ability to generate kinins from rat plasma used in the present study, we incubated plasma with DSS in vitro. BK was immediately released after the addition of DSS from the plasmas prepared from SD and BN-Ki, in which the plasma levels of kininogen were normal. However, we did not detect substantial amounts of kinins in kininogen-deficient BN-Ka even after the addition of DSS (Fig. 1).

### *In vivo experiment 1: comparison of development of DSS-induced EC between BN-Ka and BN-Ki*

The colon length in BN-Ki and BN-Ka without DSS treatment were  $23.9 \pm 0.1$  cm ( $n = 6$ ), and  $24.4 \pm 0.2$  cm ( $n = 9$ ), respectively (Fig. 2, day 0). After DSS treatment, that from BN-Ki was shortened significantly to  $21.6 \pm 0.1$  cm ( $n = 5$ ) at day 5 and to  $20.2 \pm 0.1$  cm ( $n = 6$ ) at day 7. By contrast, the colon length from BN-Ka was not significantly shortened by DSS treatment. The difference of colon length receiving 5% DSS between BN-Ki and BN-Ka was significant at day 5 and at day 7 (Fig. 2, ANOVA,  $P < 0.01$  and  $P < 0.01$ , respectively).

During the treatment of DSS, marked macroscopic bleeding was observed in the lumen of the colon in BN-Ki, whereas that in BN-Ka was negligible. To estimate the

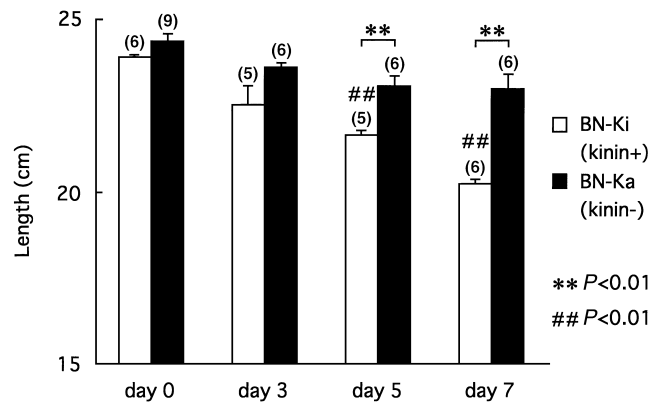


**Fig. 1.** Kinin release from rat plasma by DSS in vitro. Rat plasma was incubated with DSS. Bradykinin released in the incubation mixture was measured by ELISA for BK. DSS, dextran sulfate sodium; BK, Bradykinin; SD, Sprague-Dawley; BN-Ka, Brown Norway-Katholiek rats; BN-Ki, Brown Norway-Kitasato rats. These results are representative data from three experiments.

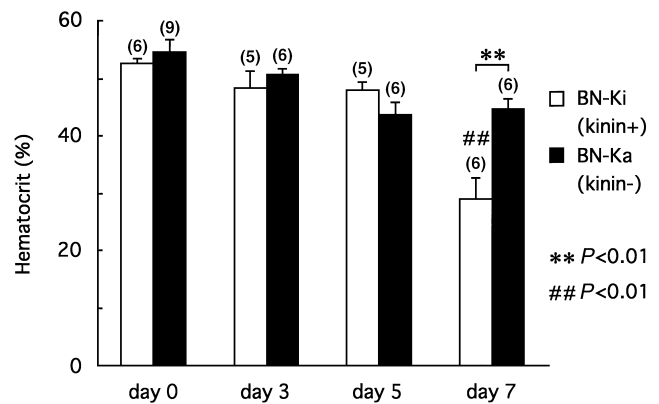
severity of the bleeding from the colon, the hematocrit value of these rats was determined. The hematocrit value in BN-Ki at day 7 was  $28.9 \pm 3.6\%$  ( $n = 6$ ), which was significantly less than that in BN-Ki at day 0 ( $53.6 \pm 1.2\%$ ,  $n = 6$ ). By contrast, we did not see any reduction in the hematocrit value in BN-Ka ( $52.3 \pm 1.9\%$  ( $n = 9$ , day 0) vs  $44.6 \pm 1.9\%$  ( $n = 6$ , day 7)). The difference in hematocrit between BN-Ki and BN-Ka at day 7 was statistically significant (Fig. 3, ANOVA,  $P < 0.01$ ).

### *In vivo experiment 2: effects of FR173657 on DSS-induced EC in SD rats*

To evaluate the contribution of  $B_2$  signaling in the



**Fig. 2.** Colon length after DSS administration as drinking water in BN-Ka and BN-Ki. The colon length (mean  $\pm$  S.E.M.) were determined at day 0, day 3, day 5 and day 7. ##, comparison with day 0; \*\*, comparison between BN-Ka and BN-Ki (ANOVA,  $P < 0.01$ ). DSS, dextran sulfate sodium; BN-Ka, Brown Norway-Katholiek rats; BN-Ki, Brown Norway-Kitasato rats. Numbers of observations are indicated in parentheses on columns.



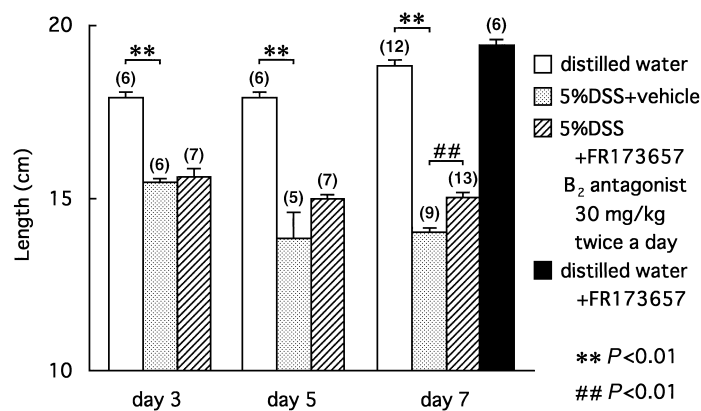
**Fig. 3.** Hematocrit values after DSS administration as drinking water in BN-Ka and BN-Ki. The hematocrit values (mean  $\pm$  S.E.M.) were determined at day 0, day 3, day 5 and day 7. ##, comparison with day 0; \*\*, comparison between BN-Ka and BN-Ki (ANOVA,  $P < 0.01$ ). DSS, dextran sulfate sodium; BN-Ka, Brown Norway-Katholiek rats; BN-Ki, Brown Norway-Kitasato rats. Numbers of observations are indicated in parentheses on columns.

development of EC induced by DSS, we orally administered FR173657 to SD. DSS administration caused the EC as determined by the shortening of the colon. The mean lengths of the colon in control SD that received distilled water without DSS were  $17.9 \pm 0.2$  cm ( $n=6$ ) at day 3,  $17.9 \pm 0.2$  cm ( $n=6$ ) at day 5, and  $18.8 \pm 0.2$  cm ( $n=12$ ) at day 7. DSS treatment significantly shortened the colon to  $15.5 \pm 0.1$  cm ( $n=6$ ) at day 3,  $13.8 \pm 0.8$  cm ( $n=5$ ) at day 5, and  $14.0 \pm 0.1$  cm ( $n=9$ ) at day 7, in comparison with the control SD that received distilled water without DSS. Daily oral administration of FR173657 from the first day of DSS treatment significantly blocked the shortening of the colon to  $15.0 \pm 0.2$  cm ( $n=13$ ) at day 7, compared with that in DSS-treated SD receiving vehicle solution (5% gum arabic) for FR173657 (Fig. 4, ANOVA,  $P<0.01$ ).

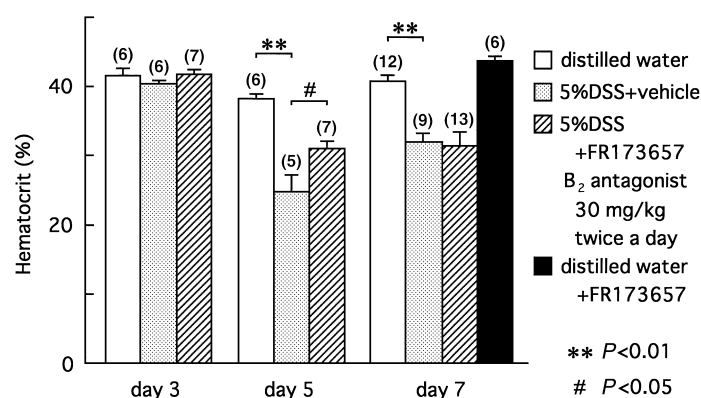
Hematocrit values at day 3 were not changed in SD receiving either distilled water or 5% DSS as drinking water. However, 5 days and 7 days after DSS treatment,

they fell significantly, to  $24.8 \pm 2.6\%$  ( $n=5$ ) and  $32.1 \pm 1.2\%$  ( $n=9$ ), respectively, compared with the values in SD receiving distilled water without DSS (day 5,  $38.3 \pm 0.7\%$  ( $n=6$ ); day 7,  $40.9 \pm 1.0\%$ , ( $n=12$ )) (Fig. 5, ANOVA,  $P<0.01$ ). The reduction in hematocrit at day 5 was significantly blocked by daily oral administration of FR173657 to  $31.1 \pm 1.1\%$  ( $n=7$ ) (Fig. 5, ANOVA,  $P<0.05$ ).

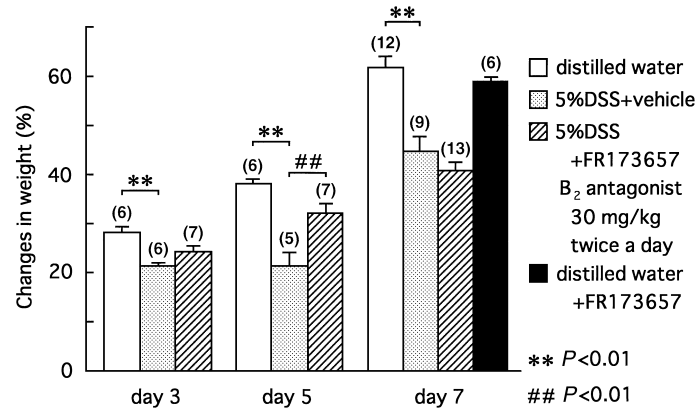
During the experimental period, the mean body weight of SD receiving distilled water without DSS increased because of the spontaneous growth (at day 3,  $28.1 \pm 1.2\%$  ( $n=6$ ); at day 5,  $38.1 \pm 1.2\%$  ( $n=6$ ); at day 7,  $62.0 \pm 2.3\%$  ( $n=12$ )). With 5% DSS administration in drinking water, they were reduced significantly, to  $21.3 \pm 0.7\%$  ( $n=6$ ) at day 3,  $21.3 \pm 2.9\%$  ( $n=5$ ) at day 5, and  $44.8 \pm 3.1\%$  ( $n=9$ ) at day 7, respectively (Fig. 6, ANOVA,  $P<0.01$ ). FR173657 administration restored body weight increase significantly, to  $32.2 \pm 2.0\%$  ( $n=7$ ) at day 5 (Fig. 6,



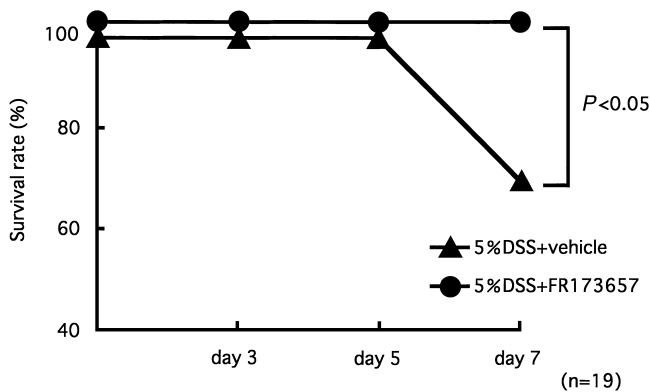
**Fig. 4.** Colon length in SD rats treated with DSS. The colon length (mean  $\pm$  S.E.M.) were determined at day 3, day 5 and day 7. \*\*, comparison with control rats receiving distilled water; ##, comparison between FR173657 and vehicle (ANOVA,  $P<0.01$ ). SD, Sprague-Dawley rats; DSS, dextran sulfate sodium. Numbers of observations are indicated in parentheses on columns.



**Fig. 5.** Hematocrit values in SD rats treated with DSS. The hematocrit values (mean  $\pm$  S.E.M.) were determined at day 3, day 5 and day 7. \*\*, comparison with control rats receiving distilled water; #, comparison between FR173657 and vehicle (ANOVA,  $P<0.01$ ,  $P<0.05$ ). SD, Sprague-Dawley rats; DSS, dextran sulfate sodium. Numbers of observations are indicated in parentheses on columns.



**Fig. 6.** Body weight in SD rats treated with DSS. Changes in body weight (mean %  $\pm$  S.E.M.) were determined at day 3, day 5, and day 7. \*\*, comparison with control rats receiving distilled water; ##, comparison between FR173657 and vehicle (ANOVA,  $P < 0.01$ ). SD, Sprague-Dawley rats; DSS, dextran sulfate sodium. Numbers of observations are indicated in parentheses on columns.



**Fig. 7.** Survival rate (%) in SD rats treated with DSS. The survival rates (%) were determined at day 0, day 3, day 5 and day 7. Comparison between FR173657-treated rats and vehicle-treated rats. (Fisher test,  $P < 0.05$ ). SD, Sprague Dawley rats; DSS, dextran sulfate sodium. Nineteen rats were used for each experimental group.

ANOVA,  $P < 0.01$ ).

The survival rate at day 7 in SD receiving 5% DSS was 68.4%, and FR173657 restored the rate significantly, to 100% (Fig. 7, Fisher,  $P < 0.05$ ).

## DISCUSSION

In the present study, we were the first to report that DSS-induced EC was suppressed in rats genetically defective in kininogens (which are components of the kallikrein-kinin system) and consequently lacking the capacity for kinin generation (Figs. 2 and 3). The very low plasma levels of high-molecular-weight kininogen and low-molecular-weight kininogen in this strain were attributed to these rats' inability to secrete these kininogens from the liver, due to one-point mutation of alanine163 to threonine

in the kininogen moiety, although the hepatic cells of the mutant BN-Ka produced kininogens with one-point mutation (28). Comparison of BN-Ka with normal BN-Ki allowed us to study the pathophysiological roles of endogenous kinins in many situations, including inflammation (33, 34).

Zeitlin and Smith reported relatively high levels of active tissue kallikrein in acutely inflamed tissue from UC patients, suggesting that kinins can be generated from low-molecular-weight kininogen during active human disease (12). Furthermore, the involvement of the kallikrein-kinin system in the development of experimental IBD models that mimic human Crohn's disease was also reported (8, 9, 35). Although it was reported that the activation of the plasma kallikrein-kinin system was evaluated from the consumption of plasma prekallikrein and high-molecular-weight kininogen (8, 35), the actual contribution of kinins to enterocolitis should be tested using adequate biological and pharmacological approaches (36). It is well known that kinins are very readily degraded to the inactive peptides (37) and that the above precursor proteins are degraded to the small molecules without generating kinins in some inflammatory situations (38, 39). The use of reliable antagonists for kinin receptors and genetically biased experimental animals which lack both the ability of kinin generation (28) and that of kinin receptor signaling (40) is believed to be a sound approach to obtain evidence of the contribution of endogenous kinin to EC, as shown in the present study.

The bradykinin  $B_2$ -receptor antagonist used in the present study was FR173657, which was developed as the first non-peptide antagonist that shows good bio-availability when administered orally (29). The previously developed non-peptide  $B_2$  antagonist WIN64338 showed antagonizing activity in different species but with much

lower bioavailability than FR173657. Therefore, there was a limitation to its use *in vivo* (41). FR173657, when used at the dosage in the present study, effectively suppressed the plasma exudation in acute exudative inflammation and bradykinin-induced hypotension in rats (42). With the use of this antagonist, we could prevent the development of DSS-induced EC (Figs. 4–6). Furthermore, this compound blocked the loss of body weight and completely restored the survival rate. These results, together with those obtained from the mutant BN-Ka, clarified the contribution of kinins in the development of DSS-induced EC.

The mechanism of kinin generation in the DSS-induced UC model was not tested precisely in this study. However, once kinin generation was activated as a result of the activation of the kallikrein-kinin system, plasma exudation was enhanced by kinins. Increased plasma exudation resulted in the increase in the supply of kininogens to the inflammatory sites. A kinin-related positive feedback loop in plasma exudation may be present where inflammation was introduced. In the lumen of the colon in DSS-treated rats, the activation of Hageman factor may be induced by the contact with DSS, which has a negative charge and can activate Hageman factor to generate kinin from high-molecular-weight kininogen. In fact, in an *in vitro* experiment (Fig. 1), in the presence of DSS, kinin generation occurred in plasma taken from SD and BN-Ki, but not in that from BN-Ka. In contrast, the epithelial cells of the intestine were reported to contain the tissue kallikrein (12, 22, 43, 44). Once the damage to the intestinal mucosa is induced, the tissue kallikrein may be released from the epithelial cells (43). Alternatively, the stimuli to increase the secretion of kallikrein during DSS-treatment may also facilitate the release of tissue kallikrein. It cannot be ruled out that the released tissue kallikrein may also generate kinins from low molecular weight kininogen, since the intestine is rich in tissue kallikrein (12, 22, 43, 44).

The action of kinins is certainly mediated by B<sub>2</sub> receptors, judging from the preventive effect of B<sub>2</sub> antagonist on EC (Figs. 4–6). However, it was reported that the expression of B<sub>1</sub> receptor, another subtype of BK receptor, in the vascular tissues was up-regulated after the tissue injury (45). Furthermore, the altered frequency of a promotor polymorphic allele of B<sub>1</sub> receptor gene in patients with IBD, unlike that in healthy volunteers, was reported, suggesting the involvement of B<sub>1</sub> receptor in the pathogenesis of IBD (46). Endogenous ligands for B<sub>1</sub> receptor are des-Arg<sup>9</sup>-BK and des-Arg<sup>10</sup>-kallidin, which are degradation products of BK and kallidin, respectively (10). Since kinin-degrading enzymes are present in many tissues including intestinal tissue (47), the possible involvement of B<sub>1</sub> receptors in the pathogenesis of DSS-induced EC cannot be ruled out when kinins are generated. In fact, the effect of FR173657 was significant but partial, even though as much

as 30 mg/kg was administered (Figs. 4–6). Since several rats died at day 7, parameters from those severe animals could not be added to the data. Therefore, significant effects of FR173657 on hematocrit and body weight were observed at day 5, but not at day 7. FR173657 seems to specifically attenuate DSS-induced EC, since administration of the antagonist to normal rats without drinking of DSS did not affect the colon length, hematocrit, and body weight (Figs. 4, 5 and 6, respectively). Compared with the effect of FR173657, the difference of the severity of DSS-induced EC between BN-Ka and BN-Ki was more clear. EC was not induced in BN-Ka (Figs. 2 and 3), suggesting that the lack of generation of kinins in BN-Ka resulted in the lack of signaling on both B<sub>2</sub> and B<sub>1</sub> receptors. These results indicate a significant role of the kinin receptors in the development of DSS-induced EC.

The results in the present study suggest strongly that the presence of kinins reflect a pathophysiological response to intestinal tissue injury. The mechanism of enhancement of EC by kinins was not clear in the present study. However, previous reports have described the possible involvement of kinin in production of the inflammatory cytokine IL-1 in DSS-induced EC, since the production of IL-1 from macrophages (19), mononuclear cells (20), and fibroblasts (19, 21) was up-regulated by BK *in vitro*. It has frequently been reported that the significant role of IL-1 in the development of experimental enterocolitis was identified using IL-1 antibody (48) and soluble IL-1 receptors (49). Neutrophil accumulation may also involve the pathogenesis of enterocolitis. Kallikrein inhibitor was reported to inhibit the extravasation of neutrophils in another IBD model induced by indomethacin. Kallikrein itself is known to have a chemotactic activity, and to stimulate the release of neutrophil elastase (50, 51). As kininogen deficiency was responsible for the lack of activation of the contact activation system that generates active plasma kallikrein, the neutrophil-dependent tissue injury may be reduced in BN-Ka. This may explain the marked reduction in the severity of EC in BN-Ka.

The contribution of endogenous kinins to DSS-induced EC, judging from the difference between BN-Ki and BN-Ka and the effects of FR173657 (Figs. 2–7), was seen in the late phase of this experimental period (day 5 or day 7). Thus, the endogenous kinins may have a role in the maintenance of EC rather than in its initiation in this model.

In conclusion, the present results suggest that the endogenous kinins generated from the kallikrein-kinin system facilitate the development of EC and that the agents controlling the kallikrein-kinin system will be useful in therapeutic interventions for treating UC.

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