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Pathophysiological Studies of Trinitrobenzene Sulfonic Acid-Induced Colitis in Syrian Hamsters (*Mesocricetus auratus*)

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We developed a colitis model in Syrian hamsters (*Mesocricetus auratus*) to investigate the relationship between colitis and neutrophil elastase (NE). Colitis was induced by a single intracolonic dose of trinitrobenzene sulfonic acid (TNBS; 90 mg/ml) dissolved in 15% (vol/vol) ethanol. The ulcer area, tissue myeloperoxidase (MPO) activity, and luminal NE activity all were increased on Days 1 and 5, corresponding with the acute inflammatory histopathological changes. These acute inflammatory parameters subsequently decreased by Day 14, and chronic inflammatory histopathological changes became evident. Recurrence of inflammation was not observed during the period up to Day 28. To evaluate our colitis model, the effects of prednisolone were examined. Prednisolone was administered orally once on the day before induction of colitis, and animals were treated twice daily thereafter. Although prednisolone had little effect on the tissue MPO activity, prednisolone inhibited the ulcer area and NE activity. In addition, the effects of an NE-specific inhibitor (ONO-6818) on our TNBS-induced colitis model were examined. In the subcutaneous treatment study, ONO-6818 was administered once before the induction of colitis. Although ONO-6818 had little effect on the tissue MPO activity, the ulcer area and NE activity were decreased in the ONO-6818-treated group. The inhibitory effects on the ulcer area and NE activity were confirmed after oral treatment with ONO-6818 after induction of colitis. We conclude that our colitis model is useful for investigating the relationship between colitis and NE, and inhibition of NE activity can prevent the progression of ulceration.

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is a group of intractable inflammatory conditions that are characterized by repeated episodes of exacerbation and remission (3). Active IBD is characterized by prominent neutrophil infiltration into the colonic mucosa and a high level of neutrophil elastase (NE) (2, 6, 23). Recently, NE has been suggested to play an important role in the exacerbation of IBD (20).

NE is a protease found in the azurophilic granules of neutrophils that shows broad-spectrum proteolytic activity against various proteins such as elastin and collagen types I through IV. Therefore, release of NE from neutrophils during inflammation leads to degradation of connective tissues and an increase in vascular permeability, thus causing tissue damage and organ failure (7, 10, 28). In patients with IBD, the fecal NE level is increased, and it shows a correlation with both disease activity and the fecal hemoglobin level (2, 23). As NE has a broad spectrum of proteolytic activity, it may well contribute to exacerbation of IBD. In addition, fecal NE in patients with IBD does not form a complex with α1-proteinase inhibitor (α1-PI) (21), which is a leading endogenous elastase inhibitor in serum, leading to NE–α1-PI imbalance (7, 10, 28). Consequently, NE activity may be increased in IBD patients, leading to the aggravation of inflammation (20).

Animal models can be used to investigate the role of NE in colitis in order to clarify the process underlying the exacerbation of human IBD. In addition, new therapeutic approaches, such as specific inhibitors of NE, may be developed using animal models. For this purpose, there is a need for a reliable animal model of colitis in which the level of NE activity in the colonic lumen or tissues resembles that in human IBD. However, the anti-NE activity of serum containing endogenous protease inhibitors varies widely between species. Takahara and colleagues (24) reported that the proteinase-inhibiting capacity of serum in humans and hamsters is 0.77 and 0.65 mg/ml, respectively, and the values for mice, rats, and guinea pigs are 3.40, 2.11, and 2.32 mg/ml, respectively (expressed as mg of trypsin bound per ml of serum). In addition, the proteinase-inhibiting activities of α1-PI and α-macroglobulin in human serum are 0.74 and 0.11 mg/ml, respectively, whereas the values for hamsters are 0.96 and 0.39 mg/ml. In contrast, the respective values for mice are 2.85 and 0.13 mg/ml, those for rats are 1.74 and 0.18 mg/ml, and those for guinea pigs are 1.97 and 0.05 mg/ml. Accordingly, because the total proteinase-inhibiting capacity of serum (which contains endogenous inhibitors) in hamsters is more similar to that in humans than mice, rats, and guinea pigs (24), a suitable animal colitis model might be developed using hamsters. On the other hand, because mice, rats, and guinea pigs have a higher proteinase-inhibiting capacity in sera than do humans or hamsters, the colitis models created using these animals are not suitable for investigating the role of NE in human IBD.

We previously demonstrated that the ulcer area and NE activity were increased in Syrian hamsters with acetic acid (AA)-induced colitis (8). However, this model has two drawbacks. First, the chronic inflammatory changes associated with healing (e.g., mononuclear cell infiltration and fibroblast proliferation) that

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occur in the chronic and remission phases of human IBD and TNBS-induced colitis in rats (3, 25, 29) are not observed in hamsters with AA-induced colitis. Second, the duration of inflammation is very short in this model. The ulcer area and NE activity peak at 24 h after the induction of colitis, and these parameters both return rapidly to near-normal by 48 h (8). Therefore, although the relationship between NE and the onset of ulceration in IBD can be investigated using the AA-induced colitis model, it is difficult to investigate the role of NE during the active phase of inflammation after the onset. In addition, when evaluating the effects of potential drugs for IBD using an AA-induced colitis model, the animals have to be pretreated because the duration of inflammation is so short (8, 9). However, from the viewpoint of assessing drugs for clinical application, the efficacy of treatment after the onset of inflammation is most important. Therefore, a colitis model that achieves a longer duration of inflammation than the AA-induced colitis model is needed.

In the present study, we attempted to develop a trinitrobenzene sulfonic acid (TNBS)-induced colitis model in Syrian hamsters. Colitis was induced by intracolonic administration of a solution of TNBS in ethanol. The changes in inflammatory parameters were determined at various times after the induction of colitis; histopathological changes also were evaluated. Furthermore, we examined the influence of prednisolone and a specific NE inhibitor (ONO-6818) in this colitis model. Prednisolone has the most powerful anti-inflammatory effect among the drugs generally used for human IBD (12). ONO-6818 is an orally active synthetic competitive inhibitor of human NE (Ki = 12 nM), which is at least 100-fold less active against various other proteases, including trypsin, proteinase 3, pancreatic elastase, plasmin, thrombin, collagenase, cathepsin G, and murine macrophage elastase (18, 19).

Materials and Methods

Animals. Male specific-pathogen-free Syrian hamsters (*Mesocricetus auratus*) (Japan SLC, Hamamatsu, Japan) weighing 94 to 132 g were used. These hamsters were certified by the vendor to be free of the following pathogens: Sendai virus, Hantavirus, murine pneumonia virus, lymphocytic choriomeningitis, *Pseudomonas aeruginosa*, *Salmonella* spp., *Pasteurella pneumotropica*, *Bordetella bronchiseptica*, *Streptococcus pneumoniae*, *Corynebacterium kutscheri*, *Clostridium piliforme*, *Mycoplasma* spp., *Giardia* spp., *Spironucleus* spp., *Syphacia* spp., and *Aspiculuris tetraptera*. Examination for *Helicobacter* spp. was not conducted. The animals were maintained in a room ventilated at 15 ± 5 100% air exchanges/h, which had a controlled temperature (23 ± 2°C), humidity (55% ± 10%), and lighting time (12:12-h light:dark cycle). The animals were housed at a maximum of five to a plastic cage (345 wide × 403 long × 177 mm high), and autoclaved, dried sawdust (Sankyo Labo Service, Tokyo, Japan) was used as the bedding. Standard pellet food for rodents, CRF-1 (Oriental Yeast Industry, Tokyo, Japan), and tap water were offered ad libitum. The animals were quarantined and acclimatized for 1 week after purchase. During this time, animals were observed once daily to assess their general condition and were weighed three times a week. Because no abnormalities in their general condition were observed in any animal, and their weight gain was satisfactory, their health was considered to be good, and these animals were grouped through the weight-stratified randomization method.

This study was conducted after receiving institutional animal care and use committee approval and was performed in compliance with the *Guidelines for Studies in Animals* of the Research Headquarters of Ono Pharmaceutical Co., Ltd., which is based on the "Guidelines for Animal Experimentation" of the Japanese Association for Laboratory Animal Science (11).

Reagents. ONO-6818 (*N*-[2-[5-(*tert*-Butyl)-1, 3, 4-oxadiazol-2-yl]-(1*RS*)-1-(methylethyl)-2-oxoethyl]-2-(5-amino-6-oxo-2-phenyl-6*H*-pyrimidin-1-yl) acetamide) was synthesized in our laboratory. TNBS was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Ethanol and aqueous hydrogen peroxide (H_2O_2) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Prednisolone 21-acetate, *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroaniline (Suc-Ala-Ala-Pro-Val *p*NA), and *o*-dianisidine were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Ethanol dose-determination study. In order to find the optimal dose of ethanol as a vehicle for TNBS, 0.1 ml each of 15% (vol/vol) and 50% (vol/vol) ethanol diluted with physiological saline was administered into the colonic lumen by using a flexible tube with the tip 1.5 cm proximal to the anus while the animal was under ether anesthesia, and then the anus was closed manually for 10 sec. Five days after administration, each ether-anesthetized (the ether can be replaced by other anesthetic agents) animal was exsanguinated by incising the thoracic aorta, and was autopsied to assess macroscopic changes of the colon. Intracolonic administration of 50% (vol/vol) ethanol caused early death of the hamsters, because of its toxicity. On the other hand, there were no obvious changes of in the general condition of the hamsters or colonic inflammation at autopsy after 15% (vol/vol) ethanol was administered.

TNBS dose-determination study. The day of colitis induction was designated as Day 0. In order to find the optimum dose of TNBS to induce colitis in Syrian hamsters, 0.1 ml of 90, 120, 150, or 200 mg/ml TNBS in 15% (vol/vol) ethanol in physiological saline (TNBS solution) was administered into the colonic lumen by the same method as used in the dose-determination study of ethanol. On Day 5, animals were autopsied, and macroscopic changes of the colon were evaluated. As a result, no changes of the animals' general condition were found in any TNBS dose group. However, intracolonic administration of TNBS at 120, 150, or 200 mg/ml caused severe adhesive peritonitis, and the inflamed colon could not be harvested from some animals because of the adhesions. In contrast, although slight adhesions were found in some animals given 90 mg/ml TNBS solution, the colon could always be harvested at autopsy.

Time-course study of colitis. From the results of the dose-determination study of TNBS, it was considered appropriate to use a 90 mg/ml TNBS solution in order to induce colitis in Syrian hamsters. To evaluate the time course of colitis in the hamsters, it was induced by using 90 mg/ml TNBS solution, and animals were autopsied on Days 1, 5, 14, and 28. Then the colon and rectum up to 3 cm from the anus were removed. After the resected colon was opened longitudinally and the lumen washed with 2 ml of physiological saline, the colon was photographed, and the developed image was used for determining the ulcer area. After centrifugation of the bowel washings (4°C, 1710 ×*g*, 20 min), the supernatant was stored at –80°C until measurement of the NE activity in the colonic lumen. After being washed and photographed, the isolated colon also was stored at –80°C for measurement of tissue myeloperoxidase (MPO) activity. The colonic histopathological examination was performed using a different

set of animals other than those used for the determination of ulcer area, MPO activity, and NE activity. On Days 1, 5, 14, and 28 after colitis induction, the colon and rectum were fixed in 10% buffered formalin (pH 7.2), dehydrated through a graded ethanol series, and infiltrated with xylene for embedding in paraffin. Sections (2 μm thick) were cut and stained with hematoxylin and eosin for light microscopy.

In the normal control group of these time-course studies, physiological saline was administered into the colon, and animals were autopsied on Day 5.

Effect of prednisolone on TNBS-induced colitis. Prednisolone 21-acetate was resuspended in a 0.5% (wt/vol) carboxymethyl cellulose sodium salt solution (0.5% CMC), and the dose for each animal was set at 10 mg/kg. The dosing volume was set at 10 ml/kg. Syrian hamsters were pretreated orally with prednisolone by using a flexible gavage tube once on the day before induction of colitis and twice daily from the day of induction to the day before autopsy. Normal and colitis control animals received 0.5% CMC. Colitis was induced using 90 mg/ml TNBS solution; normal control animals received intracolonic administration of physiological saline. Sampling of colonic tissues and washings were performed on Day 5. Then the ulcer area, tissue MPO activity, and luminal NE activity were determined.

Effect of ONO-6818 on TNBS-induced colitis. In the subcutaneous treatment study, ONO-6818 at 1000 mg/kg was administered subcutaneously once 2 h before the induction of colitis. In the oral treatment study, Syrian hamsters were treated orally with ONO-6818 at 25 and 100 mg/kg twice daily from the day after colitis induction to the day before autopsy. In these studies, the dosing volume was set at 10 ml/kg. Colitis was induced using 90 mg/ml TNBS solution, and normal control animals received intracolonic administration of physiological saline; sampling of colonic tissues and washings were performed on Day 5. ONO-6818 was resuspended in olive oil in the subcutaneous treatment study and in 0.5% CMC in the oral treatment study. Normal and colitis control animals were subcutaneously or orally dosed with the appropriate vehicle. In order to evaluate the efficacy of ONO-6818, the ulcer area, tissue MPO activity, and luminal NE activity were determined.

Determination of the ulcer area. Developed photographic slides of the colon were processed using image processing software (Adobe PhotoShop 4.0J, Adobe Systems, San Jose, Calif.) after being scanned into a computer by using a Minolta QuickScan 35 (Minolta, Osaka, Japan). The ulcerated region was copied with PhotoShop, and the ulcer area (cm^2) was measured using image analysis software (NIH Image 1.61/fat; National Institutes of Health, Bethesda, Md.).

Assay of tissue MPO activity. As an index of the neutrophil count, tissue MPO activity was determined in the colon (13). Briefly, each isolated colon was placed in 3 ml of 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5% (wt/vol) cetyltrimethylammonium bromide and was homogenized. After centrifugation of the homogenate (4°C, 1710 ×*g*, 20 min), the supernatant was diluted fivefold with potassium phosphate buffer. To 0.05 ml of the diluted sample was added 1.4 ml of 0.00107% H_2O_2 diluted with potassium phosphate buffer and mixed. To this mixture was added 0.05 ml of 0.03 M *o*-dianisidine solution, and changes of the absorbance at 450 nm were determined for 60 sec (DU7400 SpectroPhotometer; Beckman, Toyonaka, Japan). Then the tissue MPO activity was determined from the increment of absorbance.

Assay of NE activity. NE activity was measured using the synthetic substrate Suc-Ala-Ala-Pro-Val *p*NA, which is highly specific for NE, by the method described previously (30). Briefly, a sample was incubated for 24 h at 37°C in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and 0.001 M of the substrate dissolved in 1-methyl-2-pyrrolidone. After incubation, *p*NA release was measured spectrophotometrically (DU7400 SpectroPhotometer) at 405 nm as an indicator of NE activity.

Pharmacokinetics study. To evaluate the systemic exposure to ONO-6818, the plasma drug concentration was determined in Syrian hamster. ONO-6818 was administered subcutaneously or orally through the same time course as in the subcutaneous (1000 mg/kg) or oral (25 mg/kg) dosing studies for efficacy. A blood sample was drawn from the jugular vein, and plasma was separated by centrifugation (4°C, 1,710 ×*g*, 20 min). The plasma sample was deproteinated with acetonitrile and assayed by high performance liquid chromatography (column, CAPCELL PAK C18 UG120 4.6 × 250 mm; mobile phase, 20 mM Na_2HPO_4 (pH 8):CH_3CN = 65:35; flow rate, 1 ml/min; detection, 302 nm). The area under the concentration–time curve (AUC) for 24 h was calculated by the trapezoidal method.

Statistical analysis. Values are expressed as the mean ± the standard error of the mean. The significance of differences in each parameter was assessed using a two-tailed Student's *t* test with significance assigned at $P < 0.05$. A two-tailed Dunnett's *t* test with significance assigned at $P < 0.05$ was used when ONO-6818 groups treated by oral administration were compared with a colitis control group.

Results

Time course of colitis. After colitis was induced using 90 mg/ml TNBS solution, macroscopic observation of the colon on Days 1 and 5 revealed ulceration, bowel wall thickening, and hemorrhage (Fig. 1), whereas these changes showed progressive resolution on Days 14 and 28. The sites of inflammation and ulceration ranged from the perirectal region to 3 cm proximal to the anus. There were some animals that had two or more separate sites of ulceration (so-called "skip-segment ulceration") and a cobblestone-like appearance of the mucosa in the affected region. Measurement of inflammatory parameters showed that the ulcer area, tissue MPO activity, and luminal NE activity were increased significantly ($P < 0.001$) on Day 1 compared with the normal control group (Fig. 2), and the marked elevation of these parameters continued up to Day 5. On Day 14, all of these parameters were decreased to near-normal, although values slightly higher than normal persisted on Day 28. However, recurrence of inflammation was not observed during the period up to Day 28.

Histopathological findings. On histopathological examination, various changes indicating severe acute inflammation were seen on Days 1 and 5, including crypt abscesses, neutrophil infiltration, hemorrhage, ulceration, erosion, and edema (Table 1 and Fig. 3). Colonic ulceration, erosion, edema, and hemorrhage were observed in the regions where neutrophil infiltration was prominent. Chronic inflammatory changes, such as mononuclear cell infiltration and fibroblast proliferation, were detected initially on Day 5. On Day 14, ulceration and neutrophil infiltration were still found, but these changes were less marked than on Day 5.

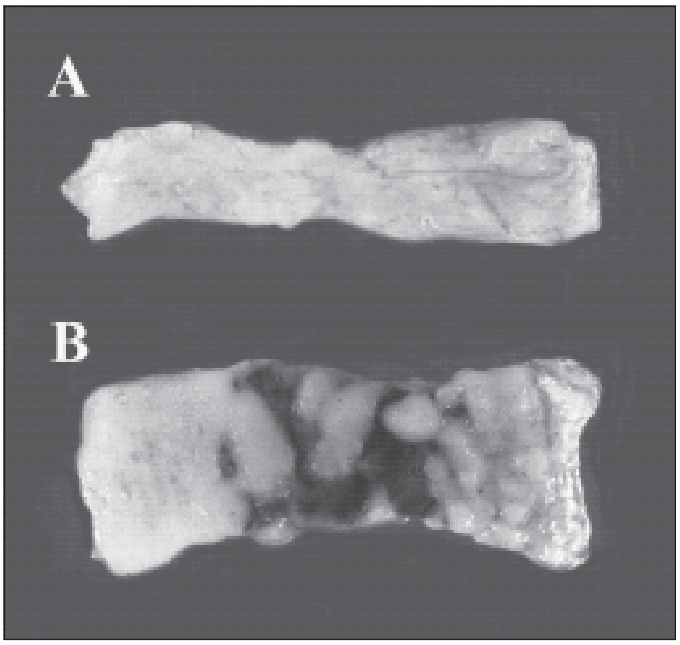

Figure 1. Colonic ulceration induced by intracolonic administration of 90 mg/ml TNBS in 15% (vol/vol) ethanol in saline. No inflammatory changes are observed in a normal control colon (A). Bowel wall thickening, ulceration, and cobblestone-like mucosa are observed in a hamster with TNBS/ethanol-induced colitis on Day 5 (B).

Instead, evidence of chronic inflammation, such as mononuclear cell infiltration and fibrosis, were more striking on Day 14. Epithelial regeneration, which was considered to represent spontaneous tissue repair, also was observed on Day 14. On Day 28, the spontaneous healing was more evident than on Day 14, and completely healed normal-looking mucosa also was found on Day 28.

Effect of prednisolone. Because the colitis time course study showed that the increase of ulcer area, tissue MPO activity, and NE activity continued until Day 5, we evaluated the effects of prednisolone on Day 5. Ulcer area, tissue MPO activity, and luminal NE activity were significantly increased on Day 5 in the colitis control group compared with the normal control group (Fig. 4). In the prednisolone-treated group, the ulcer area was decreased by 53% compared with that in the colitis control group, and this difference was significant ($P < 0.05$). In contrast, prednisolone had little effect on the tissue MPO activity, and the difference between the groups was not statistically significant. However, luminal NE activity was decreased by 62% in the prednisolone-treated group compared with the colitis control group, and the difference between the groups was significant ($P < 0.05$).

Effect of subcutaneous treatment with ONO-6818. The ulcer area, NE activity, and tissue MPO activity of the colitis control group were all significantly increased ($P < 0.001$) on Day 5 compared with those of the normal control group (Fig. 5). Subcutaneous administration of ONO-6818 caused significant decreases of the ulcer area and NE activity ($P < 0.01$ and 0.001, respectively) but had little effect on tissue MPO activity. The percent inhibition of the ulcer area and NE activity was 61 and 84%, respectively.

To investigate the correlation between luminal NE activity and the ulcer area, the ulcer area was plotted against luminal NE activity for the colitis-induced animals and those treated with subcutaneous ONO-6818. A positive correlation was found

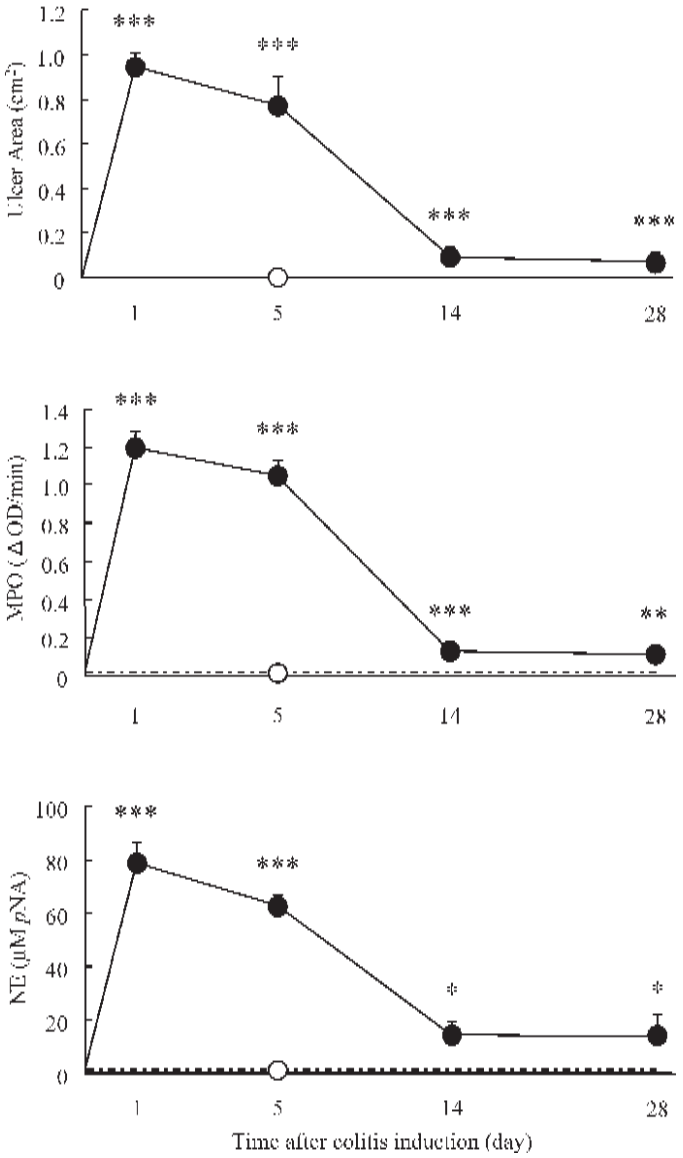

Figure 2. Time course of colonic inflammatory parameters in our TNBS-induced colitis model using Syrian hamsters. Animals that received either 90 mg/ml TNBS in 15% (vol/vol) ethanol (filled circle) or saline (normal control, open circle) were euthanized at the indicated time points, and ulcer area, tissue MPO activity, and luminal NE activity were measured. Each point represents the mean ± standard error of seven animals. Values with asterisks were significantly higher than those of normal controls (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

between these parameters in the colitis control group and the ONO-6818-treated group (Fig. 6).

Effect of oral treatment with ONO-6818. The ulcer area, NE activity, and tissue MPO activity of the colitis control group were all significantly increased ($P < 0.001$) on Day 5 compared with those of the normal control group (Fig. 7). At doses of 25 and 100 mg/kg, oral ONO-6818 had a significant ($P < 0.05$ and 0.001, respectively) dose-dependent inhibitory effect on the ulcer area. The percent inhibition of the ulcer area by doses of 25 and 100 mg/kg was 42 and 70%, respectively. ONO-6818 also significantly ($P < 0.001$) decreased the NE activity by 95 and 96% at doses of 25 and 100 mg/kg, respectively. On the other hand, oral ONO-6818 did not have any inhibitory effect on tissue MPO activity.

Table 1. Time course of colonic histopathological changes in Syrian hamsters with TNBS-induced colitis (i.e., animals with histopathological changes)

Intracolonic administration		Saline	TNBS*			
Days after induction		Day 5	Day 1	Day 5	Day 14	Day 28
No. of animals examined		5	5	5	4	4
Mucosa	Crypt abscesses	0	2	0	0	0
	Abscesses	0	1	5	0	0
	Erosions	0	4	0	0	0
	Ulcers	0	3	5	4	2
	Epithelial regeneration	0	0	0	4	4
Lamina propria	Neutrophil infiltration	0	3	5	0	0
	Hemorrhage	0	3	1	0	0
Sub-mucosal layer	Neutrophil infiltration	0	5	5	4	3
	Hemorrhage	0	0	3	0	0
	MNC infiltration†	0	0	3	4	3
	Edema	0	5	4	4	2
	Fibroblast proliferation	0	0	5	0	0
	Fibrosis	0	0	0	4	3
Muscle layer	Neutrophil infiltration	0	0	2	0	0
	Fibroblast proliferation	0	0	2	0	0
Serosa	Neutrophil infiltration	0	0	2	0	0

*TNBS = 90 mg/ml TNBS in 15% (vol/vol) ethanol/saline.
†Mononuclear cell infiltration.

Pharmacokinetics. The pharmacokinetic profile of ONO-6818 is shown in Fig. 8. In the group given a subcutaneous dose of 1000 mg/kg, the AUC was 12.1 μg·h/ml. In the group given 25 mg/kg orally, the AUC was 6.3 μg·h/ml.

Discussion

As a working hypothesis for the etiology and pathogenesis of IBD, it has been presumed that an increase of mucosal permeability allows a luminal antigen to enter the lamina propria that is not adequately cleared by the mucosal immune system of IBD patients (22, 27). Applying this hypothesis, TNBS–ethanol-induced colitis models have been developed in several laboratory animals, such as rats and mice (16, 17). TNBS is a hapten, and when coupled to high molecular weight substances like colonic tissue proteins, it has been shown to elicit an immune response (5, 15). Ethanol causes breakdown of the mucosal barrier when instilled into the colon, so combined administration of TNBS plus ethanol to rats results in colonic inflammation and ulceration that persists for a relatively long period (14, 16). In the present study, we demonstrated that intracolonic administration of TNBS mixed with ethanol to Syrian hamsters caused acute inflammation and a subsequent healing phase associated with chronic pathological changes. The duration of acute inflammation was at least five times longer than in hamsters with AA-induced colitis (8). This prolonged duration of inflammation allowed us to perform a post-treatment study of a specific NE inhibitor, which could not be done in the AA-induced colitis model (9).

Although 120 mg/ml TNBS in 50% (vol/vol) ethanol generally has been used to induce colitis in rats (16), we used lower concentrations of TNBS and ethanol for Syrian hamsters. Because intracolonic administration of 50% (vol/vol) ethanol alone was fatal for these hamsters, it was considered inappropriate to use such a high concentration of ethanol as the vehicle of TNBS. In addition, intracolonic administration of TNBS at 120 mg/ml or more in 15% (vol/vol) ethanol caused very severe colonic inflammation, and sometimes the colon could not be harvested because of adhesive peritonitis. Therefore, 90 mg/ml TNBS in 15% (vol/vol) ethanol was found to be a suitable combination to induce reproducible inflammation and ulceration of the colon without causing death or excessively severe inflammation in the present model. In addition, when the 90 mg/ml TNBS solution was used to induce colitis, skip ulceration and cobblestone-like mucosa were observed during the active phase of inflammation. Because these lesions are similar to those seen in active Crohn's disease and because the same changes were observed in rats with TNBS–ethanol-induce colitis (3, 16), we suggest that our colitis model mimics the active phase of Crohn's disease.

Although numerous studies of TNBS-induced colitis in rats, mice, and guinea pigs have been reported, there has been no investigation of colonic NE activity in these animal models. In a preliminary study, we attempted to measure the luminal NE activity of TNBS-induced colitis in rats. However, there was no increase of luminal NE activity on the day after induction of colitis despite the marked ulceration, hemorrhage, and increased tissue MPO activity (data not shown). Accordingly, it seems that TNBS-treated rats develop colitis independent of NE, so a rat model is not useful for investigating the relationship between colitis and NE activity. The reason for the lack of increased NE activity in the rat model is unclear, but it may be related to the fact that the anti-NE activity of serum (which contains endogenous proteinase inhibitors) is higher in rats than humans or hamsters (24). In a rat TNBS model, leakage of serum from the inflammatory lesions may inhibit luminal NE activity.

On the other hand, in our Syrian hamster colitis model, colonic luminal NE activity was increased during both the acute and subacute phases. This finding suggests that there was abundant NE in the inflamed regions that was not endogenously inhibited by the serum. This situation may be related to the fact that the anti-NE activity of serum is relatively lower in hamsters than rats (24). In addition, the increase of the ulcer area on Days 1 and 5 occurred in parallel with a significant increase of luminal NE activity (Fig. 2), resembling the correlation between fecal NE levels and disease activity in human IBD (2, 23). Therefore, we suggest that an increase of "free NE" activity contributed to the progression of inflammation by a direct proteolytic effect on colonic tissues and that inhibition of "free NE" is important to pre-

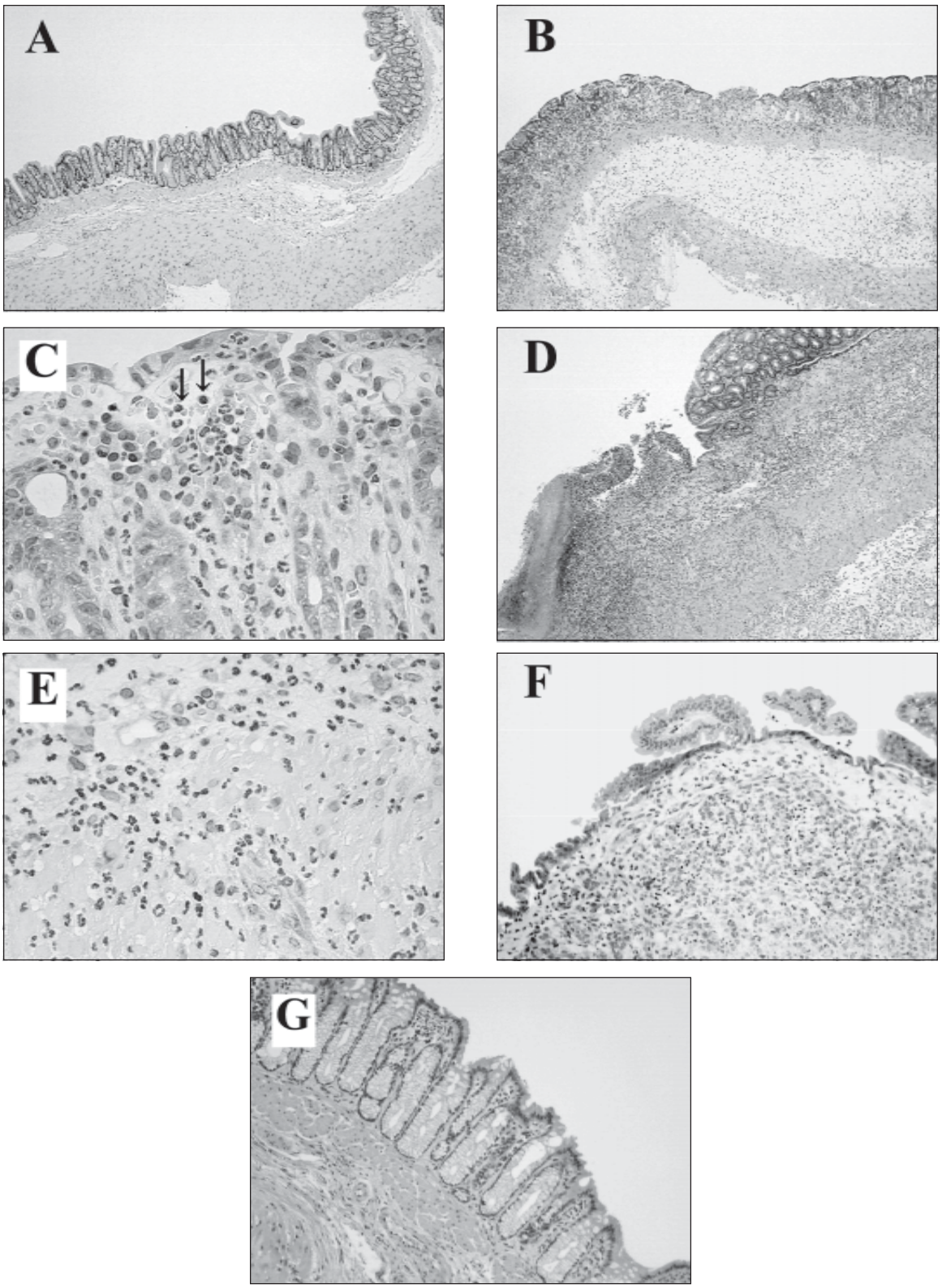

Figure 3. Histopathological features of hematoxyilin- and eosin-stained sections of the colon. No inflammatory changes are seen in normal control mucosa (magnification, ×40) (A). On Day 1 after induction of colitis, inflammatory changes (erosion, neutrophil infiltration, and edema) are observed, and neutrophils mainly infiltrate in submucosal layer (magnification, ×40) (B). In addition, neutrophils (arrows) infiltrate the lamina propria on Day 1 (magnification, ×400) (C). On Day 5 after induction of colitis, inflammatory changes (ulceration, abscesses, hemorrhage, transmural neutrophil infiltration, and mononuclear cell infiltration, and edema) are observed (magnification, ×40) (D). Numerous neutrophils infiltrate in submucosal layer and muscle layer on Day 5 (magnification, ×400) (E). Epithelial regeneration and fibrosis was observed on Day 14 (magnification, ×100) (F). Completely healed normal-looking mucosa was found on Day 28 (magnification, ×100) (G).

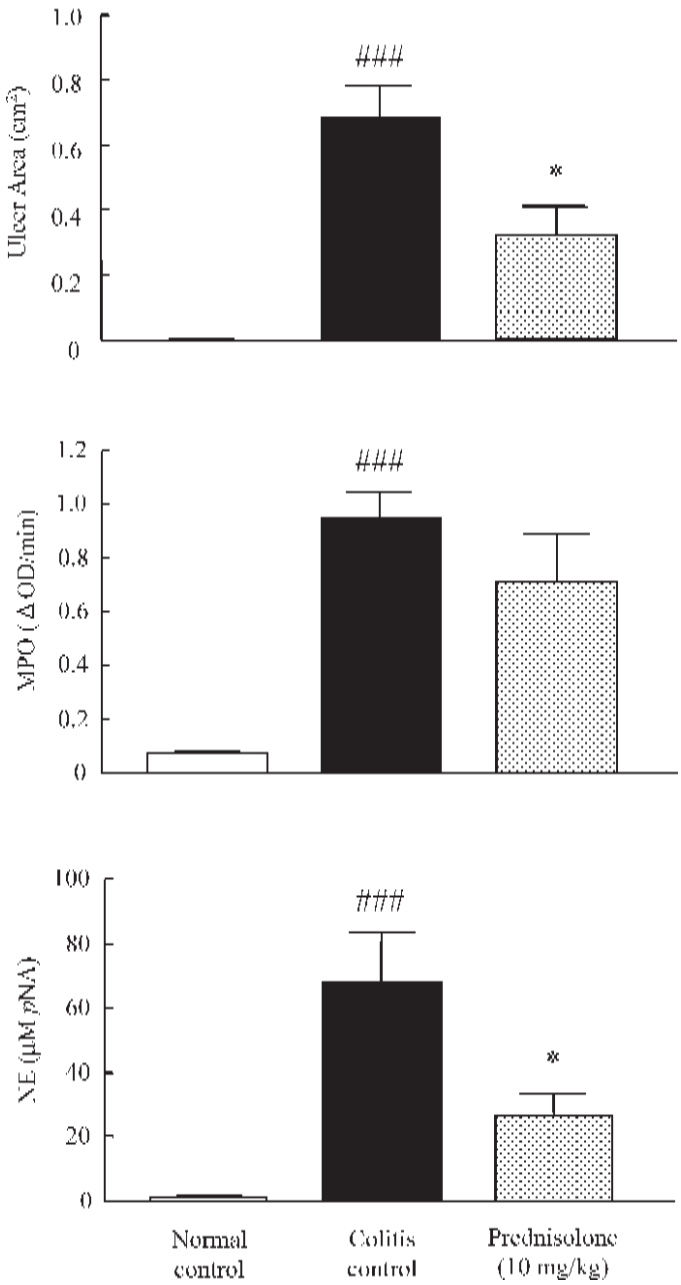

Figure 4. Effects of prednisolone on inflammatory parameters in our TNBS-induced colitis model. Syrian hamsters were pretreated orally with prednisolone once on the day before induction of colitis and twice daily from the day of induction to the day before autopsy, which was performed on Day 5. Normal and colitis control animals received vehicle only. Colitis was induced using 90 mg/ml TNBS solution; normal control animals received saline. Each bar represents the mean ± standard error of nine animals. Values with "#" are significantly higher than those for the normal control ($^{###}$, $P < 0.001$). Values with asterisks are significantly lower than those for the colitis control (*, $P < 0.05$).

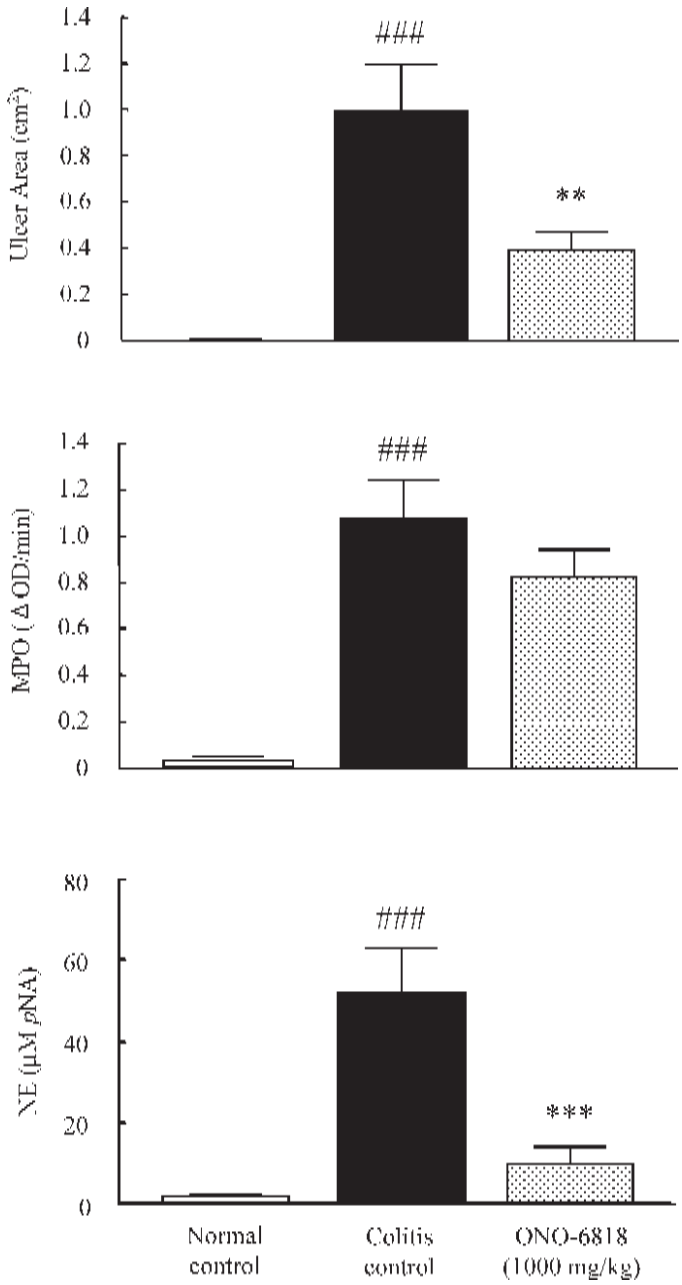

Figure 5. Effects of subcutaneous treatment with ONO-6818 on inflammatory parameters in our TNBS-induced colitis model. ONO-6818 was subcutaneously administered once 2 h before the induction of colitis, and autopsy was performed on Day 5. Normal and colitis control animals were subcutaneously administered the vehicle of ONO-6818. Colitis was induced using 90 mg/ml TNBS solution, and while normal control animals received saline. Each bar represents the mean ± standard error of 16 animals. Values with "#" are significantly higher than those for the normal control ($^{###}$, $P < 0.001$). Values with asterisks are significantly lower than those for the colitis control (**, $P < 0.01$; ***, $P < 0.001$).

vent the progression of colitis. This mechanism is supported by the finding that a specific NE inhibitor (ONO-6818) was able to inhibit ulceration in our colitis model (Fig. 5).

Although the mechanisms provoking neutrophil infiltration and subsequent NE release at sites of colonic inflammation are still unclear, the role of factors such as pro-inflammatory cytokine may be clarified using the present TNBS-induced colitis model. In AA-induced colitis models, the nonspecific nature of the initial mucosal injury that precedes the inflammatory response is generally considered to be a drawback (4). Unlike AA-induced colitis model, the inflammation that occurs in TNBS-induced colitis has an immunologic basis (4, 5, 15). Because murine colitis induced by TNBS treatment has been shown to be related to a Th1 cytokine response, neutrophil infil-

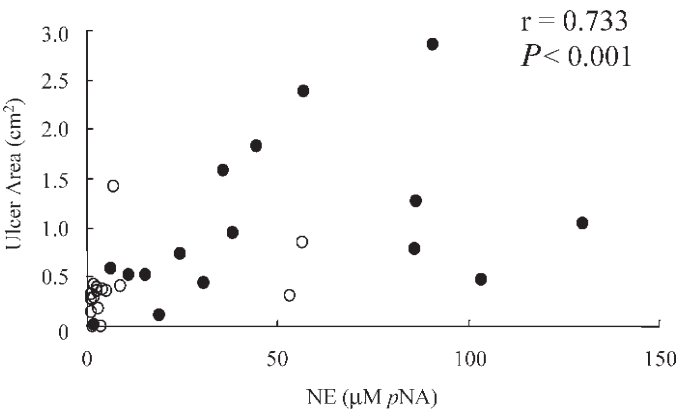

Figure 6. Correlation between luminal NE activity and ulcer area in colitis-induced animals. Correlation was calculated with Spearman's rank-correlation coefficient. Symbols express luminal NE activity and ulcer area in each animal in colitis control group (filled circle) and in ONO-6818 group treated by subcutaneous route (open circle).

tration might be due to the production of Th1 cytokines, such as interferon-γ (17), but further studies are needed to clarify the exact mechanisms involved.

In the acute phase of TNBS-induced colitis in rats and in human IBD, the main changes are neutrophil accumulation in the colonic mucosa, formation of crypt abscesses, degeneration of colonic epithelial cells, and development of erosions (3, 6, 25, 29). In the chronic and remission phases, however, mononuclear cell infiltration, fibroblast proliferation, and fibrosis are mainly observed (3, 25, 29). In our Syrian hamster TNBS-induced colitis model, the ulcer area, tissue MPO activity, and luminal NE activity were all significantly increased on both Days 1 and 5, and histopathological changes such as neutrophil infiltration, crypt abscesses, edema, erosions, and ulceration were seen concurrently. On Day 5, chronic inflammatory changes were also observed, including mononuclear cell infiltration and fibroblast proliferation. These findings suggest that the changes seen on Days 1 and 5 in our colitis model mimic the acute and subacute phases of human colitis, respectively. On Day 14, in parallel with the decrease of tissue MPO and luminal NE activity and the disappearance of neutrophil infiltration, mononuclear cell infiltration was more evident than on Day 5. The ulcer area showed a decline toward normal range on Day 14, so it is unlikely that mononuclear cells (macrophages and lymphocytes) contribute to tissue destruction and ulceration in this model. In order to determine whether the chronic inflammatory changes associated with healing on Day 14 could relapse into active inflammation, histopathological examination also was performed on Day 28. It was found that spontaneous healing continued on Day 28, and completely healed normal-looking mucosa was also seen. The relapse of colitis observed in human IBD probably does not occur in our hamster model; rats with TNBS-induced colitis also lack this relapse phase (4).

We examined the effect of prednisolone as a way of assessing the adequacy of our colitis model. Prednisolone has the most powerful anti-inflammatory effect among the agents used to treat human IBD, and is the first-line drug for acute exacerbations of IBD. As shown in Fig. 4, prednisolone therapy had little effect on tissue MPO activity, but ulcer area and luminal NE activity were significantly decreased in the prednisolone-treated

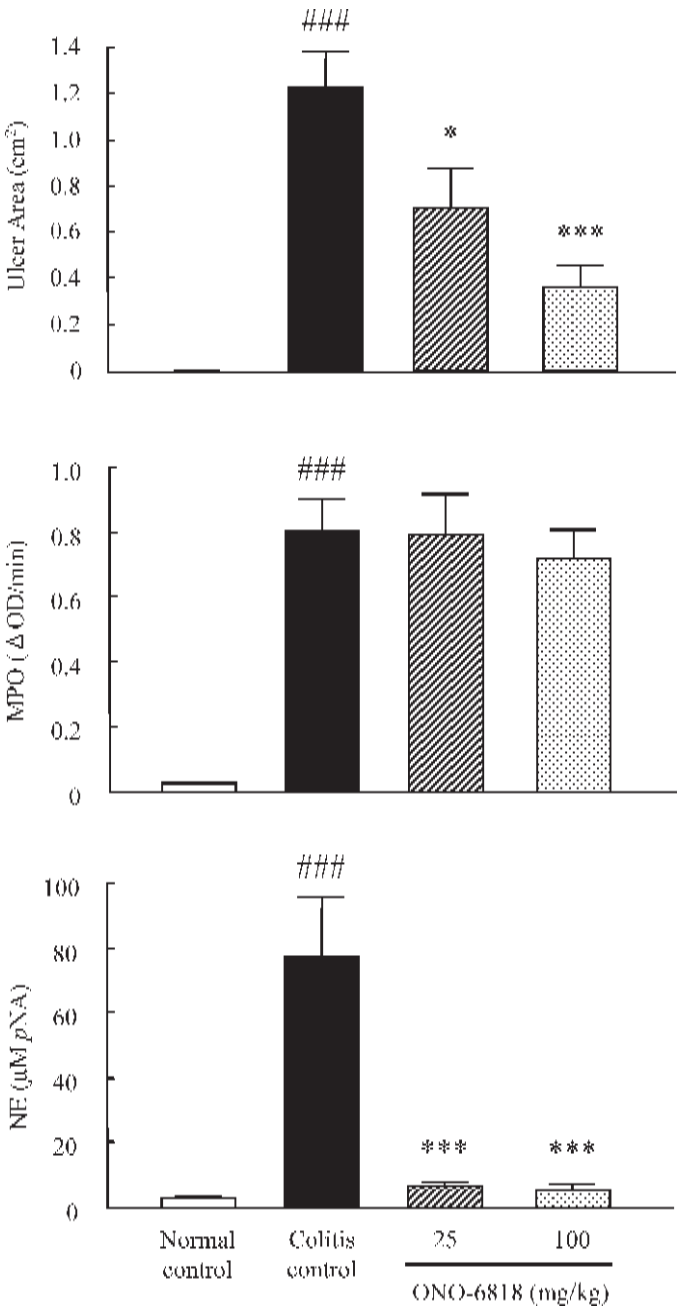

Figure 7. Effects of oral post-treatment with ONO-6818 on inflammatory parameters in TNBS-induced colitis model. Syrian hamsters were post-treated orally with ONO-6818 twice daily from the next day of colitis induction to the day before autopsy, which was performed on Day 5. Normal and colitis control animals were orally administered the vehicle of ONO-6818. Colitis was induced using 90 mg/ml TNBS solution, and while normal control animals received saline. Each bar represents the mean ± standard error of 16 animals. Values with "#" are significantly higher than those for the normal control (###, $P < 0.001$). Values with asterisks are significantly lower than those for the colitis control (*, $P < 0.05$; ***, $P < 0.001$).

group. Because it has been reported that release of NE is inhibited by prednisolone in vitro (1), this decrease of luminal NE activity with prednisolone therapy in our colitis model may have been due to the blocking of NE release from infiltrating neutrophils. The ulcer area was also reduced by prednisolone treat-

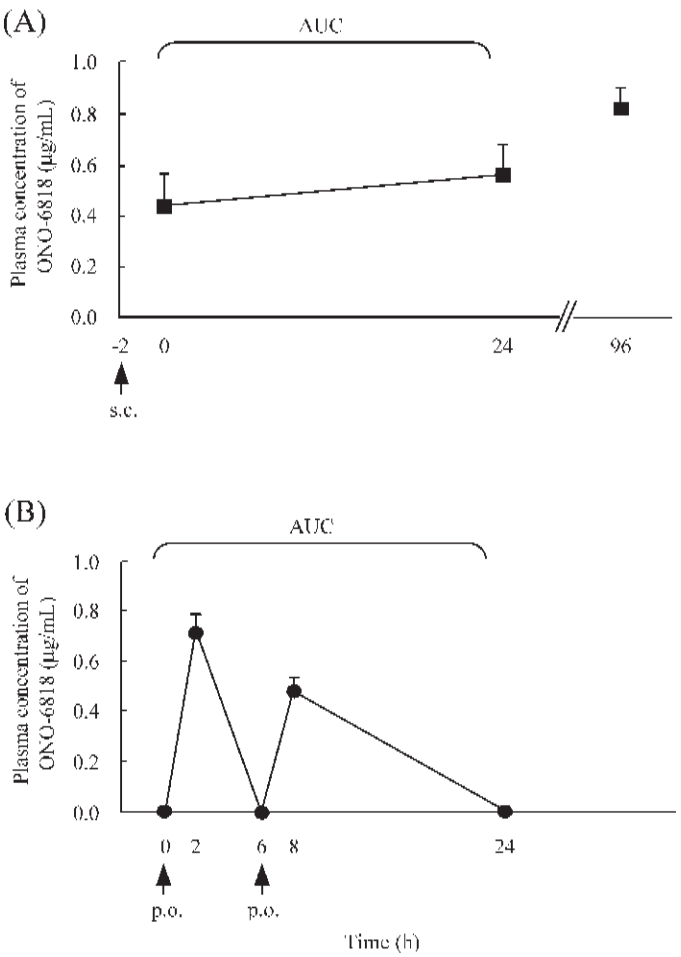

Figure 8. Pharmacokinetic profiles of Syrian hamster treated with ONO-6818 through the same time course as the studies for efficacy (mean ± standard error of three animals). Arrows indicate the time points of administration. The area under the concentration–time curve (AUC) from 0 to 24 h was calculated by the trapezoidal method. (A) ONO-6818 was administered subcutaneously at 1000 mg/kg (filled square). (B) ONO-6818 was administered orally at 25 mg/kg (filled circle).

ment. As mentioned above, the proteolytic activity of NE contributes to aggravation of inflammation, so the decrease of the ulcer area after prednisolone administration probably was attributable to inhibition of NE activity.

We also showed that treatment with a specific NE inhibitor (ONO-6818) could prevent the progression of colonic inflammation in our model. Like prednisolone, ONO-6818 had little effect on tissue MPO activity, whereas the ulcer area and luminal NE activity both decreased markedly in the ONO-6818-treated group. As shown in Fig. 6, there was a significant correlation between luminal NE activity and ulcer area in the colitis-induced animals in the subcutaneous treatment study. These findings suggest that colonic tissue damage was decreased because ONO-6818 reached the colon via the blood stream and inhibited tissue NE activity. Our data also suggest that NE has no role in the processes of neutrophil adhesion to the vessel wall/rolling and tissue infiltration because ONO-6818 administration did not inhibit tissue MPO activity.

In order to clarify the potential for clinical application of NE inhibitors to treat IBD, we investigated the effect on the ulcer area of oral ONO-6818 administration after the induction of colitis, which was impossible to evaluate in hamsters with AA-induced colitis (9). Although ONO-6818 had no effect on tissue MPO activity, the ulcer area and luminal NE activity were decreased in the treated group. Two important conclusions about the relationship between NE and colitis can be drawn from these results. First, NE may contribute to the persistence and progression of colonic tissue injury during the active phase of inflammation after the onset. Second, the clinical application of NE inhibitors seems to have potential. The inhibitory effect on the ulcer area of post-treatment with ONO-6818 is important with respect to clinical application, because drugs for IBD are administered after the onset of inflammation. In addition, an orally active agent offers several advantages for the treatment of human IBD, the most important of which is the ability to inhibit NE activity in parts of the gastrointestinal tract that are not easily accessible by enema. For example, a topically administered compound (enema) would probably be of little value for treatment of inflammation in the jejunum or ileum (26).

We also attempted to determine the effective plasma concentration of ONO-6818, because the drug was transported via the blood to inhibit colonic tissue NE activity and consequently reduce the ulcer area. The effective plasma concentration (AUC) of ONO-6818 was found to be at least 6.3 μg·h/ml (at a dose of 25 mg/kg orally; Fig. 8B). It is also worth noting that the AUC at 1000 mg/kg subcutaneously (12.1 μg·h/ml; Fig. 8A) was twofold higher than that at 25 mg/kg orally, because this difference accounts for the greater inhibitory effect on the ulcer area at 1000 mg/kg subcutaneously than at 25 mg/kg orally. The reason why ONO-6818 at 25 mg/kg orally achieved complete inhibition of NE activity in colonic washings but had only a moderate effect on the ulcer area presumably was because the unabsorbed drug inhibited NE activity in the washings. This hypothesis is supported by the fact that the bioavailability of ONO-6818 is less than 51% in various animals, and not all of a dose is absorbed (18).

We conclude that the present model of TNBS-induced colitis in Syrian hamsters is useful for investigating the relationship between IBD and NE, because the increase of luminal NE activity was well correlated with the colonic ulcer area. We demonstrated that a novel NE inhibitor, ONO-6818, could inhibit colonic ulceration in this model, suggesting that inhibition of NE activity is important to prevent the progression of ulceration in colitis.

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