Effects of the Neutrophil Elastase Inhibitor (ONO-6818) on Acetic Acid Induced Colitis in Syrian Hamsters

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ABSTRACT. Neutrophil elastase (NE) released from neutrophils during inflammation is related to tissue disturbance and organ failure. We investigated the effects of an orally active NE inhibitor, ONO-6818, on acetic acid induced colitis in Syrian hamsters. The ulcer area, hemoglobin level in the colonic lumen, NE activity, and tissue myeloperoxidase (MPO) activity in the colitis control animals were significantly increased compared to the normal control ones. Either oral or subcutaneous treatment with ONO-6818 had significant inhibitory effects on the ulcer area, hemoglobin level and NE activity in the colonic lumen, but ONO-6818 did not have a significant inhibitory effect on tissue MPO activity. We conclude that NE is closely related to the development of inflammation in acetic acid-induced colitis in Syrian hamsters and that the condition is improved by the inhibition of NE.

KEY WORDS: colitis, neutrophil elastase, orally active inhibitor, Syrian hamster.

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease, is an intractable inflammatory disease that is characterized by repeated exacerbations and remissions [2]. The inflammatory reaction in IBD is characterized by prominent colonic mucosal neutrophil infiltration and the presence of a high neutrophil elastase (NE) level [1, 3, 15].

NE is a protease that is known to have a broad-spectrum proteolytic action on various proteins, such as elastin and types I-IV collagen. Release of NE during inflammation is related to degradation of connective tissues and an increase in vascular permeability, thus causing tissue damage and organ failure [4, 6, 16]. It has been reported that α1-protease inhibitor (α1-PI), an endogenous inhibitor of NE, is inactivated by reactive oxygen species (ROS) released from neutrophils, resulting in attenuation of the NE inhibitory activity of α1-PI and a consequent increase in NE activity (NE/α1-PI imbalance) that leads to the aggravation of inflammation [9, 18]. These processes have been suggested to play an important role in the progression of various inflammatory diseases, such as chronic obstructive pulmonary disease (COPD) and IBD [8, 12].

Various findings have been obtained concerning the relationship between IBD and NE. Briefly, the fecal NE level is increased and shows a correlation with disease activity and with the fecal hemoglobin level in IBD patients [1, 15]. Furthermore, NE in the stool does not form a complex with α1-PI, causing an imbalance between NE and α1-PI activity [13]. Therefore, orally bio-available NE inhibitor may be a potential new therapeutic agent for IBD.

ONO-6818 is an orally active synthetic competitive inhibitor of human NE (Ki=12 nM) and is at least 100-fold less-active against other proteases such as trypsin, proteinase 3, pancreatic elastase, plasmin, thrombin, collagenase, cathepsin G, and murine macrophage elastase [11]. In the laboratory animal models of COPD with hamsters, ONO-6818 inhibited NE-induced lung hemorrhage, and also inhibited endotoxin-induced increase in leukocyte counts, protein levels and NE activity in bronchoalveolar lavage fluid [10].

In the present study, we investigated the effects of ONO-6818 on acetic acid induced acute colitis in Syrian hamsters. This experimental colitis model shows similar histopathological features to human IBD, and the increase in luminal NE activity in the colon is closely correlated with the colonic ulcer area [5].

MATERIALS AND METHODS

Animals: Male Syrian hamsters weighing 89–126 g (SLC, Hamamatsu, Japan) were used. The animals were maintained in an air conditioned animal room, and were housed individually in plastic cages. Standard food pellet for rodents and tap water were offered ad libitum. All studies were conducted after institutional animal care and use committee approval and were in compliance with “Guidelines for Studies in Animals” in our facilities.

Reagents: ONO-6818 (N-[2-[5-(tert-Butyl)-1, 3, 4-oxadiazol-2-yl]-1, 3, 4-oxadiazol-2-yl]-1-(methylethyl)-2-oxoethyl]-2-(5-amino-6-oxo-2-phenyl-6H-pyrimidin-1-yl) acetamide) was synthesized in our laboratory. Acetic acid (AA) and aqueous hydrogen peroxide (H2O2) were purchased from Wako Pure Chemical Industries (Osaka, Japan). N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroaniline (Suc-Ala-Ala-Pro-Val pNA), and o-dianisidine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3,3’,5,5’-tetramethylbenzidine (TMBZ) was from Dojindo Laboratories (Kumamoto, Japan).

Induction of colitis: After pentobarbital anesthesia, 1 volume % AA diluted with distilled water was administered into the colonic lumen at 10 mL/kg with a flexible tube, the tip of which was set 4 cm from the anus, and then the anus...
was clipped for 30 min. The normal control animals received an intracolonic administration of distilled water.

**Administration of ONO-6818:** In an oral dosing study, ONO-6818 was administered with a stomach tube at 18 and 1 hr before and 6 hr after the induction of colitis. ONO-6818 at 10, 30 and 100 mg/10 mL/kg was suspended in 0.5 w/v % carboxymethyl cellulose sodium salt solution (0.5% CMC). In a subcutaneous dosing study, ONO-6818 was administered once 2 hr before the induction of colitis. ONO-6818 at 1,000 mg/10 mL/kg was suspended in olive oil. The control animals were orally or subcutaneously administered 0.5% CMC or olive oil, respectively.

**Autopsy and sampling:** At 24 hr after the induction of colitis, under ether anesthesia, each animal was exsanguinated to death by incising the thoracic aorta, and the colon and rectum up to 7 cm from the anus were removed. After opening the isolated colon longitudinally and washing the colonic lumen with 5 mL of physiological saline, the opened colon was photographed. The developed photo slide was used for determining the ulcer area. After centrifugation of the bowel washing (4°C, 1,710 × g, 20 min), the supernatant was stored at −80°C until use. The isolated colon was also stored at −80°C.

**Determination of the ulcer area:** After each developed photo slide of the colon was assigned a randomized number, the measurement of the ulcer area was done in a blind manner. These slides were processed with image processing software (Adobe PhotoShop (R) 4.0J; Adobe Systems, San Jose, CA, U.S.A.), and the ulcer area (cm²) was measured with image analysis software (NIH Image 1.61/fat; NIH, Bethesda, MD, U.S.A.).

**Determination of the hemoglobin level in the colonic lumen:** The hemoglobin level in the bowel washing was determined by the method previously described previously [19]. Briefly, 20 µL of supernatant of bowel washing was incubated in 2 mL of 45% acetic acid containing TMBZ (0.5 mg/mL) and H₂O₂ (0.05%) for 25 min at room temperature, and then 5 mL of 10% acetic acid was added. After the mixture was incubated for 20 min, the absorbance at 660 nm was measured spectrophotometrically. Whole blood hemolyzed with distilled water was used as the standard. The hemoglobin level is expressed as an equivalent volume of whole blood with distilled water was used as the standard. The hemoglobin level was determined spectrophotometrically. The area under the concentration-time curve (AUC) was calculated by the trapezoidal method.

**Statistical analysis:** Values are expressed as the means ± SE. The significance of differences in each parameter was assessed by two-tailed Student’s t-test with significance assigned at p<0.05. But two-tailed Dunnett’s t-test was used with significance assigned at p<0.05. when ONO-6818 groups treated by oral administration were compared with a colitis control group.

**RESULTS**

**Effects of oral treatment with ONO-6818:** Ulcer area, hemoglobin level, NE activity, and tissue MPO activity in the colitis control group were significantly increased at 24 hr after induction of colitis compared to the normal control group (Table 1). ONO-6818 had significant inhibitory effects on the ulcer area (Fig. 1) and hemoglobin level at doses of 30 mg/kg or more. The percent inhibition of the ulcer area for 30 and 100 mg/kg doses was 65 and 63%, respectively. The inhibitory effect on the hemoglobin level was comparable to the inhibitory effect on the ulcer area, and the percent inhibition of hemoglobin level for the 30 and 100 mg/kg doses was 61 and 66%, respectively. ONO-6818 also significantly decreased the NE activity by 76, 80 and 93% at 10, 30 and 100 mg/kg, respectively. On the other hand, ONO-6818 did not have a significant inhibitory effect on tissue MPO activity.

**Effects of subcutaneous treatment with ONO-6818:** The ulcer area, hemoglobin level, NE activity, and tissue MPO activity in the colitis control group were significantly increased at 24 hr after induction of colitis compared to the normal control group (Table 2). ONO-6818 caused a significant decrease in the ulcer area, hemoglobin level and NE activity, but did not affect the tissue MPO activity. The inhibitory effects of the ulcer area or hemoglobin level were
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In parallel with the inhibitory effect of NE activity. The percent inhibition of the ulcer area, hemoglobin level and NE activity were 70, 76 and 75%, respectively.

To know the correlation between the luminal NE activity and ulcer area, the ulcer area was plotted against the luminal NE activity in the colitis-induced animals and in those pretreated with ONO-6818 subcutaneously. There was a positive correlation between them in a colitis control group and ONO-6818 treatment group (Fig. 2).

Pharmacokinetic study: Pharmacokinetic profiles of ONO-6818 are shown in Fig. 3. In the oral dosing groups at 10 and 30 mg/kg, AUC was 2.8 and 9.5 µg*h/mL, respectively. In the subcutaneous dosing group at 1,000 mg/kg, AUC was 11.2 µg*h/mL.

Table 1. Effects of oral treatment with ONO-6818 on acetic acid induced colitis in Syrian hamsters at 24 hr after induction of colitis (mean ± SE of 15 to 16 animals)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose level</th>
<th>Ulcer area (cm²)</th>
<th>Hemoglobin (µL/mL)</th>
<th>NE activity (µM pNA)</th>
<th>MPO activity (AOD/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>–</td>
<td>0.00 ± 0.00</td>
<td>0.08 ± 0.03</td>
<td>1.5 ± 0.3</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Colitis control</td>
<td>–</td>
<td>0.24 ± 0.08***</td>
<td>0.29 ± 0.05***</td>
<td>46.2 ± 7.0***</td>
<td>0.87 ± 0.06***</td>
</tr>
<tr>
<td>Treatment with ONO-6818</td>
<td>10 mg/kg</td>
<td>0.15 ± 0.03</td>
<td>0.25 ± 0.03</td>
<td>12.1 ± 2.8***</td>
<td>0.78 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>30 mg/kg</td>
<td>0.09 ± 0.03*</td>
<td>0.16 ± 0.02*</td>
<td>10.5 ± 4.7***</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>100 mg/kg</td>
<td>0.09 ± 0.03*</td>
<td>0.15 ± 0.03**</td>
<td>4.4 ± 1.9***</td>
<td>0.68 ± 0.07</td>
</tr>
</tbody>
</table>

a) Intracolon administration of distilled water; b) Intracolon administration of 1% acetic acid.
c) ONO-6818 was administered orally at 18 and 1 hr before and 6 hr after induction of colitis. Values with sharps were significantly higher than those of Normal control (### p<0.001). Values with asterisks were significantly lower than those of Colitis control (* p<0.05, ** p<0.01, *** p<0.001).

DISCUSSION

A body of indirect evidence has accumulated in support of a role of NE in the progression of IBD, although the specific contribution of this protease to the disease pathogenesis remains unclear. In this study, we have shown that treatment with NE inhibitor can prevent the progression of colonic inflammation, and the results of this study support the hypothesis that the increase in NE activity in the colon contributes to the progression of inflammation [1].

It has been suggested that ROS contribute to aggravation of inflammation by a mechanism in which ROS inactivate endogenous protease inhibitors, thereby allowing NE to attack and degrade tissues [9, 18]. In this respect, selection of appropriate animal species may be important in studying the role of NE on colitis, as the anti-NE activity of protease inhibitors and the susceptibility of these inhibitors to ROS vary widely between species. It has been reported that the protease inhibitors of some rodents such as mice, rats and guinea pigs have 2- to 4-fold higher anti-NE activity than humans [16], and the endogenous protease inhibitors of rabbits are much more susceptible to ROS than those of humans [14]. In contrast, hamsters have been shown to have an anti-NE defence system similar to that of humans [14, 16]. It follows from these facts that a suitable animal in which to investigate the role of NE in colitis is the hamster.

We previously demonstrated that the ulcer area, colonic tissue MPO activity and NE activity were increased in a 1% AA-induced colitis model in Syrian hamsters [5]. These parameters peaked at 24 hr after induction of colitis, and acutely decreased toward the normal range by 48 hr. Since the duration of inflammation in this model is short, it was considered difficult to evaluate the effect of ONO-6818 by post-treatment after colitis induction. Therefore, ONO-6818 was administered before induction, and the efficacy was assessed at 24 hr when the inflammatory responses peaked. As shown in Table 1, although the tissue MPO activity was scarcely inhibited by the treatment with ONO-6818, the ulcer area and hemoglobin level were significantly
reduced at 30 and 100 mg/kg, and the colonic NE activity was also significantly inhibited. In addition, prednisolone, which is a glucocorticoid clinically used as a therapeutic agent for IBD [7], inhibits NE activity and the ulcer area in this colitis model, but has little effect on the tissue MPO activity [5]. These facts suggest that colonic tissue damage and hemorrhage were presumably inhibited because ONO-6818 or prednisolone blocked the NE activity. It is also considered that NE is not related to the process of neutrophil adhesion to the vascular wall/rolling or to the process of neutrophil infiltration into the colonic tissue in this colitis model because ONO-6818 did not inhibit the tissue MPO activity.

Although oral administration of ONO-6818 was effective on this colitis model, the possibility can’t be ruled out that such inhibition was caused by ONO-6818 which was unabsorbed and remained in the colonic lumen. Therefore, in order to clarify the effects of ONO-6818 that had been transported by the blood circulation to the colonic tissue, we assessed the efficacy of this drug after subcutaneous administration of ONO-6818 at 1,000 mg/kg. The plasma concentration of ONO-6818 at 1,000 mg/kg, s.c. was found to be almost the same as that at 30 mg/kg, p.o. As shown in Table 2, the ulcer area and hemoglobin level were significantly reduced in the ONO-6818 treated group, and the colonic NE activity was also significantly inhibited. In addition, there was significant correlation between the luminal NE activity and ulcer area of these colitis-induced animals in the subcutaneous dosing study (Fig. 2). These findings suggest that colonic tissue damage was presumably decreased because ONO-6818 transported via the blood into the colonic tissue inhibited the NE activity. On the other hand, the reason why oral treatment with ONO-6818 at 10 mg/kg had no significant inhibitory effects on ulcer area despite the significant inhibition of NE activity was presumably that unabsorbed ONO-6818 apparently inhibited the NE activity in the colonic lumen. ONO-6818 at 10 mg/kg was considered to be poorly effective on the ulcer area and hemorrhage level, because the plasma concentration did not attain the effective plasma concentration.

The inhibitory effect of ONO-6818 on the ulcer area and colonic hemorrhage level may be of great importance in the potential clinical application of NE inhibitor on IBD, since ulceration and intestinal bleeding is a major and important clinical symptom in patients with IBD [2]. Furthermore, an orally bioavailable compound such as ONO-6818 offers several advantages for the treatment of human IBD, the most important of which is the ability to inhibit NE activity in regions of the gastrointestinal tract that are not easily accessible by enema. For example, a compound that relies on topical activity for effectiveness would likely be of little value in the treatment of inflammation in the jejenum or ileum [17]. In addition to these facts, it is also important for clinical application of NE inhibitor that the inhibitory effects on colitis are shown by post-treatment after colitis induction. In the AA-induced colitis model in Syrian hamsters, as mentioned above, it is considered difficult to evaluate the effects of ONO-6818 by post-treatment. Nevertheless, with the TNBS-induced colitis model in Syr-

<table>
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<th>NE activity (µM pNA)</th>
<th>MPO activity (ΔOD/min)</th>
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<tbody>
<tr>
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<td>–</td>
<td>0.00 ± 0.00</td>
<td>0.02 ± 0.01</td>
<td>2.1 ± 0.3</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>Colitis control</td>
<td>–</td>
<td>0.30 ± 0.07**</td>
<td>0.50 ± 0.10***</td>
<td>73.3 ±12.6***</td>
<td>1.03 ± 0.12***</td>
</tr>
<tr>
<td>Treatment with ONO-6818</td>
<td>1,000 mg/kg</td>
<td>0.09 ± 0.03*</td>
<td>0.14 ± 0.04**</td>
<td>20.1 ± 4.0**</td>
<td>0.87 ± 0.08</td>
</tr>
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</table>

a) Intracolic administration of distilled water; b) Intracolic administration of 1% acetic acid. c) ONO-6818 was administered subcutaneously at 2 hr before the induction of colitis.

Values with sharps were significantly higher than those of Normal control (## p<0.01, ### p<0.001). Values with asterisks were significantly lower than those of Colitis control (* p<0.05, ** p<0.01).

Fig. 2. 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 (closed circle) and in ONO-6818 group treated by subcutaneous route (open circle).
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In conclusion, we demonstrated in the present study that a novel NE inhibitor, ONO-6818, could inhibit colonic ulceration and hemorrhage in the colitis model in Syrian hamsters. Therefore, it is considered that the inhibition of NE activity is important to prevent the progression of ulceration and hemorrhage in colitis. Appropriate clinical studies on the NE inhibitor are necessary to test the hypothesis that such agents will have beneficial effects in the treatment of IBD.

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REFERENCES


