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[Original Article]

NEUTROPHIL ELASTASE INHIBITOR SUPPRESSES IL-17 BASED INFLAMMATION OF MURINE EXPERIMENTAL COLITIS

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Abstract : [Background] Neutrophil elastase (NE) is a proteinase in granulocytes and plays an important role in the pathogenesis of inflammatory disorders. It has been reported that NE activity is elevated in both colonic mucosa and blood in inflammatory bowel disease (IBD) patients, and that it can act as an aggravating factor in IBD. To develop novel therapies for IBD, we examined the effects of an NE inhibitor, Elaspor®, on murine experimental colitis. [Methods] Acute colitis was induced in BALB/c mice by administration of dextran sulfate sodium (DSS) in drinking water for 7 days. NE inhibitor was administered subcutaneously to mice prior to and during the induction of colitis. Disease activity index (DAI), colonic myeloperoxidase (MPO) activity, luminal NE activity, and mRNA expression in the colon were then investigated. [Results] Subcutaneous administration of NE inhibitor ameliorated the severity of DSS-induced colitis. NE activity was elevated in inflamed colon, and was reduced by NE inhibitor administration. mRNA expression levels of IL-17, a Th17-based inflammatory factor, was also decreased in the colon of NE inhibitor-administered mice. [Conclusion] These results suggest that NE inhibitor ameliorated colonic inflammation by decreasing both the activity of NE and the effects of cytokine balance. Clinically, NE inhibitor improves injuries associated with systemic inflammatory response syndrome. Similarly, clinical use of this inhibitor would further clarify its usefulness in clinical colonic inflammation.

Key words : inflammatory bowel disease, neutrophil elastase, experimental colitis

INTRODUCTION

Inflammatory bowel diseases (IBD), which include ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory disorders of the digestive tract. Although the exact immunopathogenesis of IBD remains unclear, it has been suggested that dysregulation of the balance between effector T cell subsets, such as Th17 cells, and $CD4^+$ T regulatory subsets can lead to inflammation and autoimmunity[1-3]. Both clinical studies of IBD and studies of animal colitis models have suggested that luminal bacteria are necessary for initiating and perpetuating intestinal inflammation[4,5]. The infiltration of inflammatory leukocytes, including polymorphonuclear neutrophils, is a characteristic histo-logical feature of mucosal lesions in IBD. Such cells produce several proteinases that participate in a number of events in the wound healing process[6].

Neutrophil elastase (NE) is a major secretory product from activated neutrophils and is a major contributor to tissue destruction in inflammatory diseases such as acute respiratory distress syndrome (ARDS), lung emphysema, and rheumatoid arthritis[7,8]. It has been reported that α_1-protease inhibitor (α_1-PI), an endogenous NE inhibitor, is inactivated by neutrophil-derived reactive oxygen species (ROS)[9], resulting in attenuation of the NE inhibitory activity of α_1-PI and a consequent increase in NE activity. Thus, NE may become capable of displaying its strong protease activity and degrading the main structural elements of connec-

tive tissue, such as elastin, collagen, and proteoglycans, at inflammatory sites.

Several findings have been obtained concerning the relationship between IBD and NE. Briefly, fecal NE levels are increased and show correlations with disease activity and with fecal hemoglobin levels in IBD patients[10,11]. Although these findings suggest that NE plays an important role in the progression of IBD, it has remained unclear how NE is related to the pathophysiology of IBD.

Elaspor®, sivelestat sodium hydrate (SNa), is a specific synthetic inhibitor of NE. In contrast to endogenous protease inhibitors, Elaspor® can even effectively inhibit NE at inflammatory sites, as it is not structurally inactivated by ROS[9]. In Japan, Elaspor® has already been used clinically in the treatment of patients with ARDS, which is characterized by the accumulation of numerous neutrophils in the lungs. Furthermore, Elaspor® has shown a protective effect against neutrophil-mediated tissue injury in some animal models, including lung injury and collagen-induced arthritis[12,13].

Here, we evaluated the therapeutic effects of NE inhibitor in colonic inflammation, and we showed that NE inhibitor is able to protect against inflammation based on IL-17.

MATERIALS AND METHODS

Reagents

Elaspor® was purchased from Ono Pharmaceutical (Osaka, Japan). The following materials were obtained from commercial sources : dextran sulfate sodium (DSS, molecular weight 36-50 kDa ; MP Biomedicals, Solon, OH, USA) ; Occult Blood Slide (Shionogi, Japan).

Mice

Seven- to 9-week-old female BALB/c mice were purchased from CLEA Japan (Kanagawa, Japan). Animals were housed under specific pathogen-free conditions. All experimental procedures were approved by the institutional committee for animal care and use of Fukushima Medical University.

Induction and evaluation of experimental colitis

Mice were given 7% DSS. DSS was dissolved in sterile, distilled water and was provided ad libitum for 7 days[14]. Mice were injected sterile saline or SNa at dose of 2 mg/body subcutaneously on 7 consecutive days. Disease activity index (DAI,

combined score of weight loss and bleeding) was determined as described previously[14,15]. Briefly, scores are defined as follows : for loss in body weight, 0=no loss, 1=5-10%, 2=10-15%, 3=15-20%, 4=over 20% ; for Occult Blood Slide, 0=no blood, 2=positive, and 4=gross blood.

Determination of MPO activity

Colon tissues were opened longitudinally and a 50-mg portion was homogenized in hexadecyltrimethyl-ammonium bromide (0.5%) in 50 mmol/l phosphate buffer, pH 6.0. Homogenates were sonicated for 10 s, frozen and thawed 3 times, and centrifuged for 15 min. An aliquot of supernatant was used to determine enzyme activity, as described previously[14-16].

Histological scoring

After 7 days of DSS administration, mice were sacrificed and the entire colon was excised, opened longitudinally, rolled onto a wooden stick, fixed with 10% Formaldehyde Solution (Wako Pure Chemical Industries Ltd., Osaka, Japan), and embedded in paraffin. Tissue sections were prepared, deparaffinized, and stained with hematoxylin and eosin. Histological scores were assigned by experimenters who were blinded to sample identity. Colonic epithelial damage was assigned scores as follows : 0= normal ; 1=hyperproliferation, irregular crypts, and goblet cell loss ; 2=mild to moderate crypt loss (10-50%) ; 3=severe crypt loss (50-90%) ; 4= complete crypt loss, surface epithelium intact ; 5 = small- to medium-sized ulcer (<10 crypt widths) ; 6=larger ulcer (≥10 crypt widths). Infiltration by inflammatory cells was assigned scores separately for mucosa (0=normal, 1=mild, 2=moderate, 3= severe), submucosa (0=normal, 1=mild to moderate, 2=severe), and muscle/serosa (0=normal, 1=moderate to severe). Scores for epithelial damage and inflammatory cell infiltration were added, resulting in a total scoring range of 0-12[15].

Determination of NE Activity

NE activity was determined using the synthetic substrate Suc-Ala-Ala-Pro-Val pNA, which is highly specific for NE, using a previously described method. Briefly, samples were incubated in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl and 1 mM substrate dissolved in 1-methyl-2-pyrrolidone for 24 hr at 37°C. After incubation, pNA release was measured spectrophotometrically at 405 nm and was considered to indicate NE activity[17].

Reverse Transcriptase–Polymerase Chain Reaction (RT–PCR)

Total RNAs were isolated with 100 μg of colonic sections of mice with DSS–induced colitis. The RNA was reverse transcribed to single–stranded cDNA using the Random Primer, dNTP Mixture (Takara Shuzo Co., Ltd., Shiga, Japan), and RNasin® Ribonuclease Inhibitor (Promega, Madison, WI, USA) according to the manufacturer's protocol. The cDNA was used for quantitative analysis by PCR.   The following were used as PCR primers.

KC (sense) 5´-CCACCCGCTCGCTTCTC-3´ (antisense) 5´-CACTGACAGCGCAGCTCATT-3´,

IL–1β (sense) 5´-GACGGCACACCCACCG-3´ (antisense) 5´-AAACCGTTTTTCCATCTTCTT CTTT-3´,

IL–17 (sense) 5´-CTGGAGGATAACACTGTG AGAGT-3´ (antisense) 5´-TGCTGAATGGCGACG GAGTTC-3´,

GAPDH (sense) 5´-CCAGTATGACTCCAC GACATACTCA-3´ (antisense) 5´-ATCAACGAC CCCTTCATTGACC-3´,

Quantitative real–time PCR (qPCR) was performed in a LightCycler 2.0 System (Roche Applied Science, Germany) using LightCycler DNA Master SYBR Green I (Roche Applied Science).   PCR mixtures contained 0.5 μM sense and antisense primers.   Samples were denatured at 95°C for 10 min, followed by 45 cycles of annealing and extension at 95°C for 15 s, 60°C for 5 s, and 72°C for 10 s. Melting curves were obtained at the end of amplification by cooling the samples to 65°C for 15 s, followed by further cooling to 40°C for 30 s.   Data were analyzed by the standard curve method for relative quantification using LightCycler analysis software.   For qRT–PCR, data were normalized against GAPDH[18].

Statistical analysis

Data are expressed as means±standard deviation (SD).   Statistical analysis for significant differences was performed according to the Student's *t* test for unpaired data.   *p* values of less than 0.05 were considered to be significant.

RESULTS

NE inhibitor administration protects mice from DSS–induced colitis

In order to evaluate the effective dose of DSS to obtain severe colonic inflammation, we titrated DSS concentrations and found that 7% DSS water induced severe colitis in BALB/c mice (Fig. 1A). We also initially determined the protective role of a single administration of SNa, and there were no inhibitory effects on DAI and MPO activity (data not shown).   Therefore, we evaluated the effective dose of SNa and found that daily subcutaneous administration of 2 mg/body SNa resulted in protection against DSS–induced colitis ; on the other hand, intraperitoneal injection resulted in inconsistent effects (Fig. 1B).   Repeated administration of SNa attenuated the severity of DSS–induced colitis, as reflected in DAI, colonic MPO activity and histological score (Fig. 2A).   On microscopy, extensive superficial ulceration with mucosal inflammatory reaction induced by DSS was abolished in mice treated with SNa (Fig. 2B).

NE inhibitor administration reduces NE activity in DSS–induced colitis

In order to confirm whether NE enzyme activity was inhibited by SNa in the DSS–induced colitis model, the effects of SNa on colonic tissue and in the feces was measured on day 7.   As shown in Fig. 3, administration of SNa was able to suppress the increase in NE enzyme activity in feces of DSS–induced colitis mice, but not on colonic tissue.   Due to the presence of α_1–PI in colonic tissue, NE enzyme activity on colonic tissue may result in no attenuation.   These results confirmed that SNa inhibited NE enzyme activity in the DSS–induced colitis locally and partially.

Inhibition of NE activity suppresses chemokines and proinflammatory cytokines

In order to further evaluate the potential effects of SNa on DSS–induced colitis, we used RT–qPCR to analyze the mRNA expression of chemokine and pro–inflammatory cytokines in the colon.   DSS–induced colitis colon showed significant increases in KC, a mouse homolog of human chemokines IL–8 and IL–1β.   In the SNa–treated group, mRNA expression of both chemokines was reduced, which correlated with *in vivo* data (Fig. 4).   Moreover, we evaluated IL–17 as a contributor to colonic inflammation, and found that SNa reduced IL–17 mRNA expression (Fig. 4).   Overall, these findings indicate that SNa suppressed cytokine–induced neutrophil chemoattractants (KC) and reduced the subsequent inflammatory reaction in the colon.
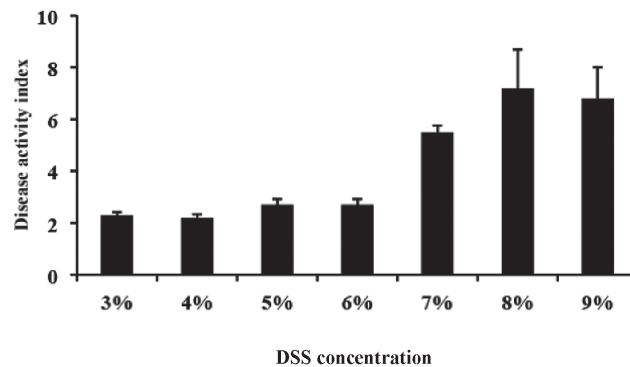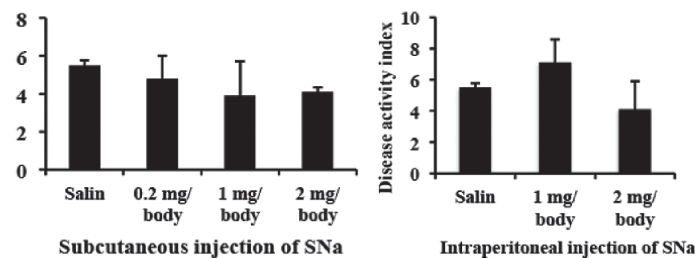
A.

B.

Fig. 1.   Evaluation of DSS concentration and SNa administration.
A : Mice were divided into 7 groups and given distilled drinking water containing 3%, 4%, 5%, 6%, 7%, 8% and 9% (wt/vol) synthetic DSS ad libitum.   Significant colitis occurred with 7%, 8% and 9% DSS (n=4 in each group).
B : We evaluated SNa administration methods and doses, and found that daily subcutaneous administration of 2 mg/body of SNa resulted in protection against DSS-induced colitis, as compared to the inconsistent results for intraperitoneal injection (n=4 in each group).

DISCUSSION

Our results provide evidence for the usefulness of NE inhibitors in clinical colonic inflammation. First, we showed that inhibition of NE ameliorated colonic inflammation by decreasing NE activity.   We then showed that this beneficial effect after NE inhibitor treatment was accompanied by diminished local neutrophil infiltration and reduced expression of pro-inflammatory cytokines and chemokines. Furthermore, we showed that NE inhibitor suppressed the expression of IL-17 that would lead to Th17-based colonic inflammation.   Hence, we conclude that targeting NE represents a useful approach to treatment of IBD.

Increased numbers of polymorphonuclear neutrophils have been observed in IBD[19], and these cells are believed to be involved in the pathogenesis of IBD through their potential to release multiple microbial products, including reactive oxygen species, cationic peptides, eicosanoids, and proteolytic enzymes[9].   Although NE is produced by neutrophils upon activation, and normally serve in host defense against invading microbial pathogens, this cytotoxic product may lead to impairment in tissue repair when released in an unregulated manner[20]. The protective effects of NE inhibitors were first documented in acute lung injury associated with systemic inflammation response, which may occur after infection, surgical intervention, or traumatic or burn injury[21].   Although NE activity was elevated in colon and serum in IBD patients[10,11], and some have shown the benefits of targeting NE in colitis models[22,23], the details remain unclear.   Therefore, we used a commercially available NE inhibitor, SNa, in DSS-induced colitis in an attempt to assess the mechanisms of inflammation inhibition.

Neutrophils can be triggered to express a number of mediators that influence local inflammatory and immune responses.   These include ROS, complement components, and proteases, as well as a variety of cytokines and chemokines[24].   The

A.

Disease activity index **MPO activity** **Histological score**

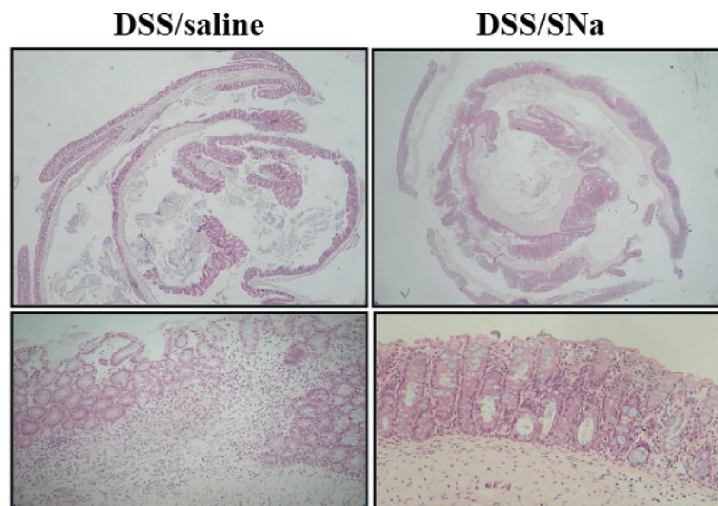
B.

DSS/saline   DSS/SNa
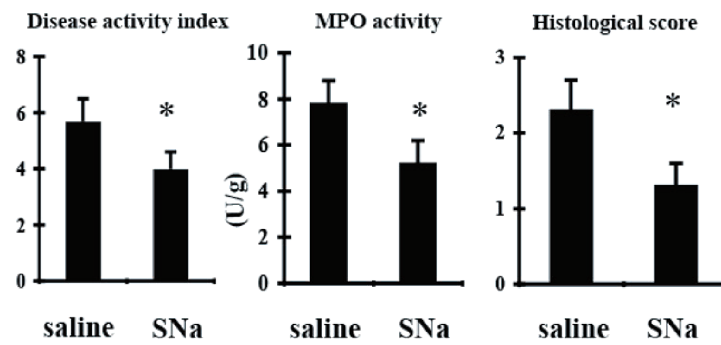
Fig. 2.   Inflammation of DSS colitis was suppressed by administration of SNa.
   A : Administration of SNa significantly suppressed colonic inflammation of DSS-induced colitis, showed lower
   DAI, MPO activity and Histological score than saline-administered mice ($n=8$ in each group.   *$p<0.05$).
   B : Mucosal inflammatory reaction, as assessed by colonic wall thickness and accumulation of inflammatory cells,
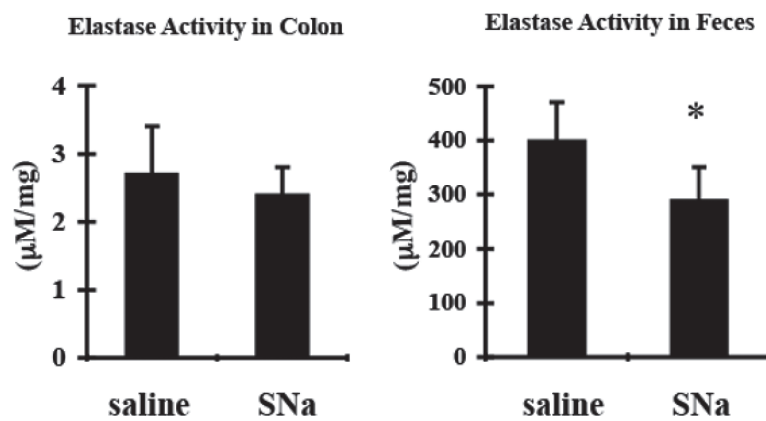   induced by DSS was abolished in mice treated with SNa.

Elastase Activity in Colon   **Elastase Activity in Feces**

Fig. 3.   NE enzyme activity was inhibited by SNa in feces, but not in colon, of the DSS-induced colitis model ($n=8$ in
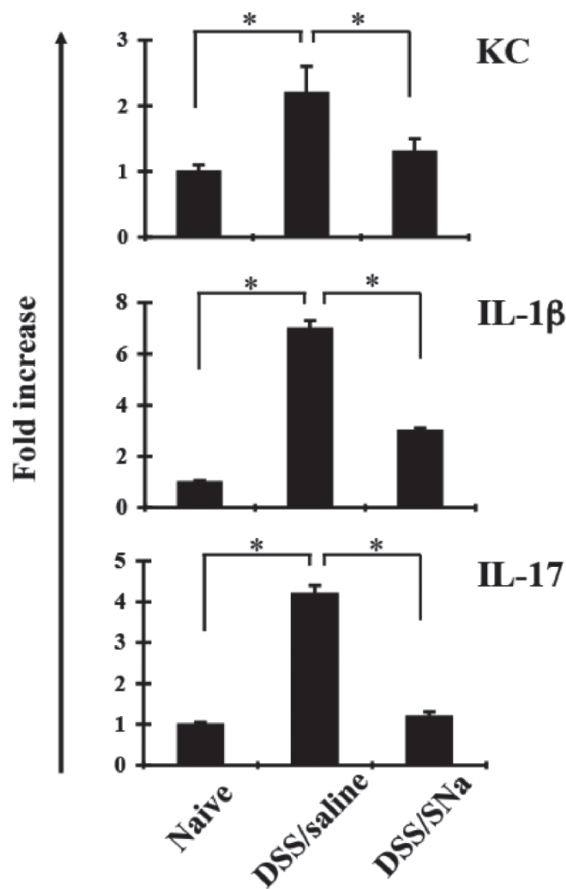   each group.   *$p<0.05$).

Fig. 4. mRNA expression of chemokines and cyto-
kines in colon was analyzed by qRT-PCR. KC,
IL-1b and IL-17 mRNA expression was reduced
by SNa administration (*$p < 0.05$).

C-X-C chemokines CXCL-1 (KC) and CXCL-2
(MIP-2) are potent neutrophil chemoattractants that
are important in colonic inflammation. Murine KC
is a chemokine considered to be functionally analo-
gous to human IL-8 and rat neutrophil chemoattrac-
tant[25] and is primarily induced by pro-inflammatory
cytokines such as TNF-α[26], IL-6, and IL-1β.
Moreover, some studies have revealed several func-
tions of NE in inflammation ; for example, NE stim-
ulates the production of proinflammatory cytokines
such as cytokine-induced neutrophil chemoattrac-
tant, macrophage inflammatory protein-1, and
IL-1β[27]. Here, we have shown that administration
of NE inhibitor suppresses expression of IL-1β and
KC in the colon. These effects of NE inhibitor
were correlated with colonic injury by DSS-induced
inflammation, and represent one of the mechanisms
of the anti-inflammatory effects.

Although the importance of the IL-17 cytokine
family in various immune-inflammatory diseases
and antibacterial responses has been known for sev-
eral years, it was only recently that IL-17-producing

CD4^{+}T cells were recognized to constitute a sepa-
rate Th cell subset, termed Th17 cells[28,29]. Th17
cells appear to be predominant in the intestine of
patients with IBD[2,30]. In IBD, Th17-associated
cytokines may play a decisive role in the production
of chemokines, which facilitate the recruitment of
neutrophils into inflamed intestine, and they could
coordinate intestinal inflammation by inducing the
synthesis of other inflammatory molecules, includ-
ing TNF-α, IL-6, and IL-1β[29,31]. Accumulating evi-
dence also suggests that Th17 cells have a patho-
genic role in experimental models of IBD. For
example, up-regulation of IL-17 occurs in mice with
acute and relapsing TNBS colitis, and blockade of
IL-17R signals protects animals against the develop-
ment of acute TNBS colitis[32,33]. We have also
shown that administration of NE inhibitor sup-
presses expression of IL-17 in the colon. Suppres-
sed expression of chemokines by SNa might also
lead to the accumulation of Th17 cells in inflamed
intestine, resulting in IL-17 suppression and attenu-
ation of colitis. This would also be one of the
mechanisms of its anti-inflammatory effects.

In conclusion, we demonstrate for the first time
that colonic IL-17 expression in a DSS-induced coli-
tis model was suppressed by NE inhibitor. NE
inhibitor also ameliorated DSS-induced colitis due
to suppression of pro-inflammatory cytokines and
chemokines. These findings suggest that NE
inhibitor may actually have potential as a new thera-
peutic approach for patients with IBD.

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