Role of Chymase-Dependent Matrix Metalloproteinase-9 Activation in Mice with Dextran Sodium Sulfate-Induced Colitis

Kumi Ishida, Shinji Takai, Mitsuyuki Murano, Takashi Nishikawa, Takuya Inoue, Naoko Murano, Nao Inoue, Denan Jin, Eiji Umegaki, Kazuhide Higuchi, and Mizuo Miyazaki

Second Department of Internal Medicine (K.I., M.Mu., T.N., T.I., N.M., E.U., K.H.) and Department of Pharmacology (S.T., N.I., D.J., M.Mi.), Osaka Medical College, Takatsuki City, Osaka, Japan

Received September 21, 2007; accepted November 15, 2007

ABSTRACT

Matrix metalloproteinase (MMP)-9 plays an important role in the pathogenesis of colitis. Recent studies have demonstrated that chymase is involved in the conversion of promatrix metalloproteinase (proMMP)-9 to MMP-9. However, whether chymase contributes to the activation of proMMP-9 in colitis has remained unclear. In this study, we administered 5% dextran sodium sulfate (DSS) solution to mice for 7 days. At 7 days after starting administration, both chymase activity and MMP-9 activity were significantly increased. In extract from colitis in DSS-treated mice, MMP-9 activity was significantly increased after 8 h of incubation, but increased activity was almost completely suppressed in the presence of a chymase inhibitor, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[3,4-dioxo-1-phenyl-7-(2-pyridyloxy)]-2-heptyl acetamide (NK3201). At 7 days after starting administration, intestinal length was significantly shorter in DSS-treated mice than in normal mice, but these changes were significantly prevented by NK3201 (10 mg/kg per day i.p.). Disease activity index and histological damage score were also significantly reduced by NK3201. Thefiltrated neutrophil number was significantly decreased by NK3201. Furthermore, NK3201 significantly attenuated not only chymase activity but also MMP-9 activity in DSS-treated mice. These findings suggest that chymase plays an important role in the development of colitis via MMP-9 activation.

Ulcerative colitis is an inflammatory bowel disease (IBD) affecting the distal colon; the etiology remains unclear. In chronic tissues of patients with IBD, the synthesis and release of inflammatory cytokines and recruitment of inflammatory cells induce the degradation of extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are important enzymes for degrading ECM proteins, and MMPs are reportedly involved in the pathogenesis of IBD via ECM degradation (Medina and Radomski, 2006). MMPs are a family of zinc- and calcium-dependent endopeptidases. Among the MMPs, MMP-2 and MMP-9 are known as gelatinases and are consistently up-regulated during active flares of IBD in patients and animal models of colitis (Baugh et al., 1999; Tarlton et al., 2000). In particular, MMP-9 may play an important role in the degradation of ECM in ulcerative colitis. Oral administration of dextran sodium sulfate (DSS) is used to induce colitis in animal models, and the resulting colonic lesions resemble those observed in patients with ulcerative colitis (Elson et al., 1995). DSS-induced colitis is significantly attenuated in MMP-9-deficient mice but not in MMP-2-deficient mice (Castaneda et al., 2005; Garg et al., 2006). Thus, MMP-9 inhibition might offer a useful strategy for attenuating the development of colitis.

Chymase is a chymotrypsin-like serine protease located in the secretory granules of mast cells. Serine proteases, such as MMP-3, and trypsin are known to process promatrix metalloproteinase (proMMP)-9 to MMP-9 in vitro (Sang et al., 1995; Shapiro et al., 1995). Fang et al. (1996, 1997) reported that chymase can also convert proMMP-9 to MMP-9 in vitro. A recent article demonstrated that the conversion of proMMP-9 to MMP-9 in media from peritoneal cell cultures was significantly attenuated in chymase-deficient mice compared with wild-type mice (Tchougounova et al., 2005). However, whether chymase is involved in MMP-9 activation in colitis tissues has remained unclear.

The present study clarified whether chymase contributes to MMP-9 activation in colitis tissues in DSS-treated mice in

ABBREVIATIONS: IBD, inflammatory bowel disease; DAI, disease activity index; DSS, dextran sodium sulfate; ECM, extracellular matrix; HDS, histological damage score; MMP, matrix metalloproteinase; NK3201, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[3,4-dioxo-1-phenyl-7-(2-pyridyloxy)]-2-heptyl acetamide; proMMP, promatrix metalloproteinase.
vitro. Furthermore, we evaluated the effects of administering chymase inhibitor on the development of colitis in vivo.

**Materials and Methods**

**Drugs.** DSS with a molecular weight of 5000 was obtained from Meitou Sangyou (Osaka, Japan), and NK3201 was obtained from Nippon Kayaku (Tokyo, Japan) (Takai et al., 2001; Tsunemi et al., 2004).

**Animal Treatment.** Six-week-old BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). Mice were fed with regular mouse chow and were housed in a temperature-, humidity-, and light-controlled room. A mouse experimental model of colitis was induced using DSS in accordance with previous reports (Hirata et al., 2007; Inoue et al., 2007). For normal mice, each mouse was allowed ad libitum access to tap water for 7 days. For DSS-treated mice, each animal was allowed ad libitum access to 5% DSS solution, supplied as drinking water, for 7 days. To investigate the effect of chymase inhibitor, each mouse was intraperitoneally administered NK3201 (10 mg/kg per once a day) or placebo immediately after the start of DSS treatment. Experimental procedures for animals were conducted in accordance with the guidelines of Osaka Medical College.

**Tissue Extract.** Intestine was homogenized in 20 mM sodium phosphate buffer (pH 7.4) and separated by centrifugation at 10,000 rpm for 30 min. The supernatant was discarded, and then the pellet was resuspended and homogenized in 10 mM sodium phosphate buffer (pH 7.4) containing 2 M KCl and 0.1% Nonidet P-40. The homogenate was centrifuged at 10,000 rpm for 30 min, and then enzyme activities were measured in the supernatant.

**Chymase Activity.** Chymase activity was measured by incubating tissue extracts for 1 h at 37°C with 5 mM Suc-Ala-Ala-Pro-Phe-4-methylcoumaryl-7-amide (Peptide Institute, Osaka, Japan) as a substrate for the measurement of chymase activity in 100 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl (Kirimura et al., 2005). The enzyme reaction was terminated by the addition of 3% metaphosphoric acid. After centrifugation of the mixture, 7-amino-4-methylcoumarin was measured by fluorophotometry (excitation, 380 nm; emission, 460 nm). One unit of chymase activity was defined as the amount of enzyme cleaving 1 μmol of 7-amino-4-methylcoumarin/min. Protein concentration was assayed with BCA protein assay reagents (Pierce, Rockford, IL) using bovine serum albumin as a standard.

**Gelatin Zymography.** Equal volumes of tissue extract (80 μg of protein) were resolved by electrophoresis on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Furubayashi et al., 2007). Thereafter, gels were renatured in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 2.5% Triton X-100 for 90 min to remove SDS and then incubated with 50 mM Tris-HCl (pH 7.5) containing 10 mM CaCl₂ for 48 h. Gels were stained with Coomassie Brilliant Blue, and gelatinolytic activity was quantified using NIH Image1.61 software.

**Effect of Chymase Inhibitor on MMP-9 Activation in Colitis Tissue Extracts.** To investigate whether chymase could cleave proMMP-9 to MMP-9 in colitis, tissues from mice treated with DSS for 7 days were incubated for 8 h at 37°C before gelatin zymography. The effect of chymase inhibition was also examined after incubation with 1 μM of the specific chymase inhibitor, NK3201, for 8 h at 37°C.

**Evaluation of Colitis.** Disease activity index (DAI) was determined by two investigators blinded to the protocol by scoring the extent of body weight loss, stool consistency, and the presence of occult/gross bleeding in accordance with the method described by Murthy et al. (1993). In brief, no weight loss was considered as 0, weight loss of 1 to 5% was scored as 1, loss of 5 to 10% was scored as 2, loss of 10 to 15% was scored as 3, and loss of >15% was scored as 4. Stools were characterized as normal (score 0), pasty stools not sticking to the anus (score 2), or liquid stools sticking to the anus (score 4). For occult blood, no blood was scored as 0, positive occult blood was scored as 2, and gross bleeding was scored as 4. Total value of these scores was divided by three and was regarded as DAI ranging from 0 to 4. After inducing anesthesia with sodium pentobarbital (50 mg/kg i.p.), the large intestine was removed for measurement of intestine length and evaluation of intestinal shortening (Hirata et al., 2007).

**Histological Evaluation.** For histological damage score (HDS), the rectum was fixed in 10% neutral buffered formalin, and specimens (4-mm thick) were stained using hematoxylin and eosin. Randomly selected fields (n = 8) magnified 100-fold in each section were inspected and blindly graded by a pathologist using the protocol described by Murthy et al. (1993). In brief, normal colonic mucosa was considered as 0, loss of the bottom one-third of crypts was graded as 1, loss of the bottom two-thirds of crypts was graded as 2, loss of the entire crypt area with surface epithelium remaining intact was graded as 3, and loss of both the entire crypt area and surface epithelium (erosion) was graded as 4.

To elucidate the inflammatory process, neutrophils were immunostained with rat anti-mouse neutrophil antibody (Serotec Ltd., Oxfordshire, UK) (Furubayashi et al., 2007). The number of neutrophils was determined, using the computerized morphometry system, and was expressed as the number of stained cells per millimeter squared.

**Statistical Analysis.** Data are expressed as mean ± S.E.M. Significant differences between mean values of the two groups were evaluated using Student’s t test for unpaired data. Significant differences among mean values for multiple groups were evaluated using one-way analysis of variance followed by Fisher’s test. Values of P < 0.05 were considered statistically significant.

**Results**

**Chymase and MMP-9 Activities.** At 7 days after the start of DSS treatment, chymase activity was significantly higher in DSS-treated mice (0.131 ± 0.025 mU/mg protein) than in normal mice (0.064 ± 0.007 mU/mg protein; Fig. 1A).

MMP-9 level was also significantly higher in the DSS-treated group (217 ± 43.7%) than in the normal group (100 ± 25.4%; Fig. 1B).

**Effect of Chymase Inhibitor on MMP-9 Activation in Colitis Tissue Extracts.** Zymography showed that the MMP-9 band in colitis tissues from DSS-treated mice was significantly thinner with an 8-h incubation at 37°C than with nonincubation (Fig. 2A). Density of the MMP-9 band was significantly denser in the presence of a chymase inhibitor NK3201 at 37°C than in the absence and was the same as that seen with nonincubation (Fig. 2B).

**Effects of NK3201 on DAI Score and Length of Colon.** Body weights in placebo- and NK3201-treated groups were

![Fig. 1.](image-url)
17.8 ± 0.30 and 17.9 ± 0.36 g before the start of DSS treatment, respectively, and no differences were observed. Percentage loss of body weight 7 days after starting DSS treatment compared to baseline was significantly lower in the NK3201-treated group than in the placebo-treated group (Fig. 3A). Diarrhea score was 4 points in all placebo-treated mice, compared to 2 points in 7 mice and 1 point in 1 mouse in the NK-3201-treated group. Score of fecal blood was significantly lower in the NK3201-treated group than in the placebo-treated group (Fig. 3B). DAI score was significantly lower in the NK3201-treated group (1.63 ± 0.17 points) than in the placebo-treated group (3.16 ± 0.23 points; Fig. 3C). The intestinal length was significantly longer in the NK3201-treated group (9.03 ± 0.22 cm) than in the placebo-treated group (8.23 ± 0.30 cm; Fig. 3D).

**Histological Evaluation.** Figure 4A shows typical photographs of the rectum from placebo- and NK3201-treated mice. HDS for the rectum was 1.48 ± 0.20 in the placebo-treated group and 0.80 ± 0.23 in the NK3201-treated group, representing a significant difference (Fig. 4B).

Many neutrophils were detected in placebo-treated group, but there were few such cells in NK3201-treated group (Fig. 5A). The neutrophil number per millimeter squared was significantly lower in the NK3201-treated group than in the placebo-treated group (10.1 ± 1.11; Fig. 5B).

**Effects of NK3201 on Chymase and MMP-9 Activities.** Chymase activity was significantly lower in the NK3201-treated group (0.056 ± 0.007 mU/mg protein) than in the placebo-treated group (0.129 ± 0.035 mU/mg protein; Fig. 6A). MMP-9 level was also significantly lower in the NK3201-treated group (38.3 ± 15.4%) than in the placebo-treated group (100 ± 23.6%; Fig. 6B).

**Discussion**

The present study attempted to clarify two main issues: 1) whether chymase is involved in MMP-9 activation in colitis tissues in vitro and 2) whether chymase inhibitor NK3201 attenuates the development of colitis via inhibition of MMP-9 activation. In our model, MMP-9 activity in colitis tissues 7 days after starting DSS treatment was increased as seen in previous reports (Castaneda et al., 2005; Garg et al., 2006). We also observed significantly increased chymase activity in DSS-induced colitis tissues. To clarify the first issue, we used the specific chymase inhibitor NK3201 (Takai et al., 2001; Tsunemi et al., 2004). NK3201 inhibits human, dog, and hamster chymases with IC50 of 2.5, 1.2, and 28 nM, respectively (Takai and Miyazaki, 2003). We also confirmed that 1 µM NK3201 completely inhibited mouse chymase activity in colitis tissues (data not shown). Conversely, NK3201 displays no inhibitory activity on other serine proteases, tryptase, thrombin, elastase, plasmin, urokinase, or plasminogen activator, even at a concentration of 1 mM (Takai and Miyazaki, 2003). The present study demonstrated that NK3201 com-
were observed in the NK3201-treated group, resulting in mice. However, significant improvements in all parameters occult blood in stool or gross bleeding were observed in all group, body weight loss, liquid stools, and the presence of though DAI was 0 in all normal mice. In the placebo-treated after the start of DSS treatment in the present study, al-

duced by DSS in mice. As in our previous model (Hirata et al., 2007), DAI was markedly increased 7 days

2007; Inoue et al., 2007), DAI was markedly increased 7 days

chymase inhibitor NK3201 on the development of colitis in-

tissues in vitro. Therefore, we demonstrated the

importance of chymase-dependent MMP-9 activation in coli-

tissues. Thus, we confirmed the importance of

chymase-dependent MMP-9 activation in colitis tissues in vitro. Furthermore, MMP-9 level was significantly decreased by treatment with NK3201, whereas proMMP-9 level tended to be increased (placebo-treated group, 100 ± 6.6%; NK3201-treated group, 109.5 ± 7.9%). This finding suggests that chymase inhibi-
tion might induce the accumulation of proMMP-9 by inhibiting the conversion of proMMP-9 to MMP-9. The mechanism by which chymase inhibitor prevents aortic aneurysm might be thought to inhibit MMP-9 activation, but we have not confirmed whether chymase inhibition could inhibit MMP-9 activation in tissues of aneurismal aorta. In the present study, MMP-9 ac-

Figure 5. Typical photographs of the rectum slices stained with anti-neutrophil antibody obtained from placebo- and NK3201-treated mice at 7 days after the start of DSS treatment (A). Original magnification: 100x and 400x. The numbers of neutrophils in the placebo-treated and NK3201-treated groups at 7 days after the start of DSS treatment (B). Values represent mean ± S.E.M. (n = 8). *, P < 0.05 versus NK3201-treated group.

Figure 6. Effects of NK3201 on chymase activity in colitis tissues 7 days after starting DSS treatment (A). Effects of NK3201 on MMP-9 activity in colitis tissues 7 days after starting DSS treatment (B). Values represent mean ± S.E.M. (n = 8). *, P < 0.05 versus NK3201-treated group.

completely inhibited any increase in MMP-9 activity in colitis tissues after an 8-h incubation at 37°C. This finding shows that chymase might predominantly contribute to MMP-9 activ-
ation in colitis tissues. Therefore, we demonstrated the importance of chymase-dependent MMP-9 activation in coli-
tissues in vitro.

To clarify the second issue, we evaluated the effect of chymase inhibitor NK3201 on the development of colitis induced by DSS in mice. As in our previous model (Hirata et al., 2007; Inoue et al., 2007), DAI was markedly increased 7 days after the start of DSS treatment in the present study, although DAI was 0 in all normal mice. In the placebo-treated group, body weight loss, liquid stools, and the presence of occult blood in stool or gross bleeding were observed in all mice. However, significant improvements in all parameters were observed in the NK3201-treated group, resulting in

significantly reduced DAI score. Although we have observed intestinal shortening caused by intestinal inflammation in previous studies (Hirata et al., 2007; Inoue et al., 2007), the colon was significantly longer in the NK3201-treated group than in the placebo-treated group. HDS was improved in the NK3201-treated group compared with the placebo-treated group, and the infiltrated neutrophil number was also decreased in the NK3201-treated group. All of these findings strongly suggest that chymase inhibition might offer a useful strategy for preventing the development of colitis.

MMP-9 is involved in degradation and remodeling of the ECM and is thus involved not only in the pathogenesis of several inflammatory diseases, such as colitis, but also aneurysm. For example, MMP-9 expression is associated with the development of aortic aneurysms following elastic fiber disruption (Thompson et al., 1995). Moreover, targeted gene disruption of MMP-9 suppresses the development of aortic aneurysm in mice (Pyo et al., 2000). We recently demon-

strated that NK3201 significantly prevents the progression of abdominal aortic aneurysm in dogs (Furubayashi et al., 2007). In the dog aneurismal model, both chymase and MMP-9 activities were significantly increased in aneurismal aortas, but NK3201 significantly attenuated not only chymase activity but also MMP-9 activity. Chymase can also convert proMMP-9 to MMP-9 in vitro (Fang et al., 1996, 1997). Thus, the mechanism by which chymase inhibitor prevents aortic aneurysm might be thought to inhibit MMP-9 activation, but we have not confirmed whether chymase inhibition could inhibit MMP-9 activation in tissues of aneurismal aorta.

In our previous study, MMP-9 activity in colitis tissues from DSS-treated mice was significantly higher after incubation for 8 h than nonincubation; this sug-

gests that the extract included proMMP-9-activating enzymes. However, the incubated samples to which a specific chymase inhibitor, NK3201, was applied had significantly attenuated the MMP-9 activity. Thus, we confirmed the importance of chymase-dependent MMP-9 activation in colitis tissues in vitro. Furthermore, MMP-9 level was significantly decreased by treatment with NK3201, whereas proMMP-9 level tended to be increased (placebo-treated group, 100 ± 6.6%; NK3201-treated group, 109.5 ± 7.9%). This finding suggests that chymase inhibi-
tion might induce the accumulation of proMMP-9 by inhibiting the conversion of proMMP-9 to MMP-9. The mechanism by which chymase inhibitor prevents the development of colitis might thus depend on the inhibition of MMP-9 activation in colitis tissues.

MMP-9 is abundantly expressed in the colonic tissues of patients with ulcerative colitis compared with controls (Baugh et al., 1999). In mouse DSS-induced colitis, a significant in-

crease in MMP-9 activity has been observed, as observed in the present study (Castaneda et al., 2005; Santana et al., 2006). In MMP-9-deficient mice, loss of body weight, diarrhea, and fecal blood after DSS treatment was significantly improved compared with control mice (Castaneda et al., 2005). Thus, MMP-9 inhibition may represent a useful strategy for preventing the development of colitis. Although NK3201 could not inhibit MMP-9 activity (data not shown), this inhibitor could indirectly reduce MMP-9 activity in vivo and might result in the same effect as direct inhibition of MMP-9 activity.

Mast cells contain various chemical mediators, histamine, leukotriene, and platelet-activating factor, all of which play important roles in acute and chronic tissue injury (Matsuo et al., 2003; Barbara et al., 2006). Mast cells may play an
important role in the pathogenesis of IBD (McAuley and Sommers, 1961). For example, mast cell infiltration is increased in the colonic mucosa of active IBD (Nishida et al., 2002). Iba et al. (2002) reported that mast cell numbers in the rectum were significantly increased after DSS treatment in rats. In mast cell-deficient mice, macroscopic colonic damage was significantly suppressed compared with control mice after DSS treatment (Araki et al., 2000). Chymase is located in mast cell granules, and the mechanisms underlying reduced colonic damage in mast cell-deficient mice may include suppression of chymase levels.

In conclusion, we demonstrated the importance of chymase-dependent MMP-9 activation in colitis tissues in DSS-treated mice in vitro and the usefulness of chymase inhibition for preventing the development of colitis via inhibition of MMP-9 activation in vivo.

References

Address correspondence to: Dr. Shinji Takai, Department of Pharmacology, Osaka Medical College, Takatsuki City, Osaka 569-8686, Japan. E-mail: phas010@art.osaka-med.ac.jp