Significance of Chymase-Dependent Matrix Metalloproteinase-9 Activation on Indomethacin-Induced Small Intestinal Damages in Rats

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ABSTRACT

The side effects of nonsteroidal anti-inflammatory drugs (NSAIDs) include gastrointestinal damage not only in the stomach but also in the small intestine. Chymase converts promatrix metalloproteinase-9 to matrix metalloproteinase (MMP)-9, which plays an important role in NSAID-induced gastric damage, but it has been unclear whether chymase-dependent MMP-9 activation is involved in the NSAID-induced small intestinal damage. To clarify the involvement of chymase-dependent MMP-9 activation on NSAID-induced small intestinal damage, the effect of a chymase inhibitor, 2-[[4-(5-fluoro-3-methylbenzo[b]thiophen-2-yl)sulfonylamido-3-methanesulfonyl(phenyl)thiazole-4-carboxylic acid (TY-51469), on indomethacin-induced small intestinal damage in rats was evaluated. Until 6 h after oral administration of indomethacin in rats, intestinal MMP-9 activity was unchanged compared with normal rats, but significant increases in MMP-9 activity were observed 12 and 24 h after indomethacin administration. Significant increases in the small intestinal damage score were also observed 12 and 24 h after indomethacin administration. In the extract from the small intestine 24 h after indomethacin administration, the MMP-9 activation was significantly attenuated by TY-51469. Intraperitoneal injection of TY-51469 (10 mg/kg) 3 h before indomethacin administration significantly attenuated the MMP-9 activity in the small intestine compared with placebo treatment. Myeloperoxidase activity, which indicates accumulation of neutrophils, was significantly increased in the small intestine in the placebo-treated rats, but its activity was significantly attenuated by TY-51469 treatment. The area of small intestinal damage was also significantly ameliorated by TY-51469 treatment. These findings suggest that chymase-dependent MMP-9 activation has a significant role in indomethacin-induced small intestinal damage in rats.

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have been widely used for their anti-inflammatory effects, but they are known to produce gastric damage. More recently, with the development of capsule endoscopy and double-balloon endoscopy, it has become possible to examine the small intestine endoscopically, and NSAID-induced damage in the small intestine has been clarified (Allison et al., 1992; Maiden et al., 2005). We recently proposed the possibilities of the therapeutic targets in NSAID-induced small intestinal damage (Higuchi et al., 2009). However, the mechanism of NSAID-induced small intestinal damage has been still unclear, and useful medications have not been identified.

Matrix metalloproteinases (MMPs) play a crucial role in physiological turnover of extracellular matrix with degradation and remodeling during inflammation. MMPs are a family of zinc- and calcium-dependent endopeptidases, divided into different subgroups: collagenases, gelatinases, stromelysins, membrane-type MMPs, and other MMPs. Among the MMPs, MMP-9 is known as a gelatinase, and it was recently documented that MMP-9 plays an important role in the development of gastric damage induced by an NSAID in rats (Ganguly et al., 2005). However, very little is known about the involvement of MMPs in NSAID-induced small intestinal enteritis.

Chymase is a chymotrypsin-like serine protease that is
contained 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), but chymase also converts proMMP-9 to MMP-9 in vitro (Fang et al., 1996; Furubayashi et al., 2008). We recently reported that chymase plays an important role in the development of colitis induced by dextran sodium sulfate via its MMP-9 activation in mice in vivo (Ishida et al., 2008). However, whether chymase is involved in MMP-9 activation in NSAID-induced small intestinal enteritis has remained unclear.

In the present study, the involvement of MMP-9 activation in indomethacin-induced small intestinal enteritis in rats was investigated, the contribution of chymase to MMP-9 activation was clarified, and the effect of a chymase inhibitor on the MMP-9 activity was measured in the supernatant (Ishida et al., 2008). Therefore, this compound was administered intraperitoneally in the present study. ONO-4817 has high inhibitory activity against MMP-2 and MMP-9; this inhibition is specific for MMPs, because ONO-4817 has almost no inhibitory activity against other proteases (Mori et al., 2001).

**Animal Treatment.** Male Sprague-Dawley rats (200–230 g; Japan SLC, Shizuoka, Japan) were used. Rats were fed with regular mouse chow and housed in a temperature-, humidity-, and light-controlled room. A rat experimental model of small intestinal damage was induced by oral administration of indomethacin (10 mg/kg) in accordance with previous reports (Tanaka et al., 2002; Takeuchi et al., 2006). For normal control rats, each rat was given water. Under pentobarbital anesthesia (50 mg/kg i.p.), the small intestine was removed; half of the small intestine was used for histological assessments of small intestinal damage, and the remaining tissue was used to measure enzyme activities. Either 10 mg/kg/day of TY-51469 (n = 6) or placebo (n = 6) was administered intraperitoneally 3 h before administration of indomethacin, and the effects of TY-51469 on enzyme activities and small intestinal damage were evaluated 24 h after administration of indomethacin. To study the effect of the MMP inhibitor ONO-4817 against MMP-9 activity and small intestinal damage 24 h after administration of indomethacin, 100 mg/kg ONO-4817 (n = 6) or placebo (n = 6) was administered intraperitoneally 3 h before administration of indomethacin. The dose of ONO-4817 was determined on the basis of previous reports (Okamoto et al., 2007; Takeuchi et al., 2007). The experimental procedures for the animals were conducted in accordance with the guidelines of Osaka Medical College.

**Evaluation of Small Intestinal Damage.** Under anesthesia, the small intestine was excised, treated with 2% formalin for 10 min for fixation of tissue walls, and opened along the antimesenteric attachment. The area of macroscopically visible damage (square millimeters) was measured under a dissecting microscope with square grids (×10), summed per small intestine, and used as a lesion score (Tanaka et al., 2002; Takeuchi et al., 2006). The observers were blinded, and the order in which the images were analyzed was also randomized.

**Tissue Extract.** Small intestine was homogenized in 20 mM 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; the MMP-9 activity was measured in the supernatant (Ishida et al., 2008).

**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 (Ishida et al., 2008). Thereafter, the 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. The gels were stained with Coomassie Brilliant Blue, and gelatinolytic activity was quantified using National Institutes of Health Image 1.61 software. Protein concentrations were assayed using BCA Protein Assay Reagents (Pierce Chemical, Rockford, IL), with bovine serum albumin as the standard.

**Effect of Chymase Inhibitor on MMP-9 Activation in Experiments of Small Intestine.** To investigate whether chymase could cleave proMMP-9 to MMP-9 in small intestine, tissues from rats 24 h after indomethacin administration were incubated for 8 h at 37°C before gelatin zymography (Ishida et al., 2008). The effect of chymase inhibition was also examined after incubation with a 1 µM concentration of the specific chymase inhibitor, TY-51469, for 8 h at 37°C.

**Myeloperoxidase Activity.** Myeloperoxidase (MPO) activity was measured according to the method of Mizoguchi et al. (2001). All blood was withdrawn from the heart by perfusion with saline, and the small intestine was excised and opened along the antimesenteric attachment. After the tissue was rinsed with cold saline, the mucosa was scraped with glass slides and homogenized in a 50 mM phosphate buffer containing 0.5% hexadecyl-trimethyl-ammonium bromide. The homogenized samples were centrifuged at 2000 rpm for 10 min. MPO activity in the supernatant was determined by addition of 1.5 M o-dianisidine hydrochloride containing 0.0005% w/v hydrogen peroxide. The changes in absorbance at 450 nm of each sample were recorded on a Hitachi spectrophotometer (U-2000; Hitachi, Ibaraki, Japan). The MPO activity was obtained from the slope of the reaction curve, based on the following equation: specific activity (micromoles of H2O2 per minute per milligrams of protein) = [(optical density per minute)/optical density/micromoles of H2O2 × milligram of protein).

**Histological Analysis.** The small intestine was removed and fixed for histological assessment with Carnoy’s fixative in 10% formalin overnight. Fixed tissues were embedded in paraffin and then cut from each block at a thickness of 3 µm. The mast cells were stained with 0.5% toluidine blue (Chroma-Gesellschaft, Stuttgart, Germany) at pH 4.8.

The expression of chymase in the small intestine was determined using immunohistochemical staining. For chymase staining, the sections were incubated with a 1:200 dilution of the anti-mouse chymase antibody (which was raised against rabbit by immunizing with EITTERGFTATC, which is a residue from position 19 to 30 of mouse mast cell protease-4; Sigma-Aldrich JAPAN, Tokyo, Japan) for 1 h at room temperature, followed by reaction with a labeled streptavidin-biotin peroxidase kit (LSAB kit; Dako, Carpinteria, CA) with 3-amin-9-ethylcarbazole color development. The sections were faintly counterstained with hematoxylin (Jin et al., 2001). The number of chymase-positive cells was determined, using the computerized morphometry system, and was expressed as the number of stained cells per square millimeter.

**Statistical Analysis.** The data are expressed as means ± 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. P < 0.05 was considered statistically significant.

**Results**

**Time Course of Small Intestinal Damage after Indomethacin Administration.** The single administration of indomethacin at a dose of 10 mg/kg gradually provoked se-
vere hemorrhagic lesions in the small intestine in a time-dependent manner, mainly in the jejunum and ileum, and the lesions showed significant increases 12 and 24 h after indomethacin administration compared with 6 h (Fig. 1).

**Time Course of MMP-9 Activity in the Small Intestine after Indomethacin Administration.** MMP-9 activity in the extract from small intestine was not significantly changed until 6 h after indomethacin administration (Fig. 2). However, MMP-9 activity was significantly increased to 5.0 and 6.1 times of the normal intestinal value 12 and 24 h after indomethacin administration, respectively (Fig. 2).

**Effect of Chymase Inhibitor on MMP-9 Activation in Small Intestine in Vitro.** Zymography showed that the MMP-9 band in extracts of small intestine from indomethacin-administered rats was more dense with an 8-h incubation than with nonincubation (Fig. 3). In contrast, the proMMP-9 band was obviously thinner with an 8-h incubation than with nonincubation (Fig. 3). The level of MMP-9 was significantly lower in the presence of a chymase inhibitor, TY-51469, than in its absence, but proMMP-9 was significantly higher (Fig. 3).

**Effect of Chymase Inhibitor on MMP-9 Activity in Small Intestine in Vivo.** The level of MMP-9 activity in extracts of small intestine from the placebo-treated group was significantly higher than that from the normal group 24 h after indomethacin administration (Fig. 4). The level of MMP-9 activity was significantly lower in the TY-51469-treated group than in the placebo-treated group (Fig. 4).

**Time Course of MPO Activity in the Small Intestine after Indomethacin Administration and Effect of TY-51469 on MPO Activity.** MPO activity in the extract from small intestine was not significantly changed until 6 h after indomethacin administration (Fig. 5). However, MPO activity was significantly increased 12 and 24 h after indomethacin administration compared with the normal level (Fig. 5). Twenty-four hours after indomethacin administration, the MPO activities in extracts of small intestine from normal and placebo-treated groups were 0.65 ± 0.04 and 2.29 ± 0.18 μmol of H_{2}O_{2}/mg protein, respectively; the difference was significant (Fig. 5). MPO activity was significantly attenuated by treatment with TY-51469 compared with the placebo-treated group, with a value of 0.81 ± 0.08 μmol of H_{2}O_{2}/mg protein (Fig. 5).

**Effect of TY-51469 on Small Intestinal Damage.** The lesion score of small intestinal damage was 209 ± 32.4 mm² in the placebo-treated group 24 h after indomethacin administration (Fig. 6). On the other hand, in the control rats, no
lesions were observed (data not shown). In the TY-51469-treated group, the lesion score was 33.4/7.0 mm² (Fig. 6).

**Effect of TY-51469 on Mast Cells and Chymase-Positive Cells.** Typical photographs of the specimens obtained from the small intestine in placebo-treated rats were stained with toluidine blue and immunostained with anti-chymase antibodies (Fig. 7). The chymase-positive cells were identified as positive cells stained with toluidine blue, which stains mast cells (Fig. 7). Both the mast cell and chymase-positive cell numbers were significantly higher in the placebo-treated group than in the normal group, but they were significantly attenuated in the TY-51469-treated group (Fig. 7).

**Effect of ONO-4817 on MMP-9 Activity and Small Intestinal Damage.** The level of MMP-9 activity was significantly reduced in the MMP inhibitor ONO-4817-treated group compared with the placebo-treated group 24 h after indomethacin administration (Fig. 8). Furthermore, the lesion of small intestinal damage was significantly lower in the ONO-4817-treated group than in the placebo-treated group 24 h after indomethacin administration (Fig. 8).

**Discussion**

In the present study we attempted to clarify two main issues: whether chymase-dependent MMP-9 activation is involved in small intestinal damage after administration of indomethacin and whether a chymase inhibitor, TY-51469, attenuates the development of small intestinal damage via inhibition of MMP-9 activation. In this study, we used the specific chymase inhibitor TY-51469 at a dose of 10 mg/kg/day. In a previous report, more than 1 mg/kg TY-51469 was observed (data not shown). In the TY-51469-treated group, the lesion score was 33.4/7.0 mm² (Fig. 6).

**Fig. 5.** A, MPO activities in small intestine in the indomethacin-administered rats at 3, 6, 12, and 24 h after indomethacin administration and in the normal rats. Values are shown as means ± S.E.M. (n = 6). **P < 0.01, versus normal after indomethacin administration. B, MPO activities in small intestine in the normal, placebo-treated and TY-51469-treated groups 24 h after administration of indomethacin. Values represent means ± S.E.M. (n = 6). *, ** < 0.05; **, ** < 0.01, versus placebo-treated group.

**Fig. 6.** Lesions of small intestinal damage in the placebo- and TY-51469-treated groups 24 h after administration of indomethacin. Values represent means ± S.E.M. (n = 6). **P < 0.01, versus placebo-treated group.

**Fig. 7.** Typical photographs of the small intestine sections stained with toluidine blue (A) and immunostained with anti-chymase antibodies (B) from a placebo-treated rat 24 h after administration of indomethacin. Arrows indicate toluidine blue-stained cells (A) and chymase-positive cells (B). Original magnification, 400×. Numbers of mast cells (C) and chymase-positive cells (D) in the small intestine sections from the normal (N), placebo-treated (P), and TY-51469-treated rats (TY). Values represent means ± S.E.M. *, **P < 0.05; **, **P < 0.01, versus placebo-treated group.

**Fig. 8.** A, MMP-9 levels in the small intestine in the normal, placebo-treated, and ONO-4817-treated groups 24 h after administration of indomethacin. Values represent means ± S.E.M. (n = 6). *, **P < 0.05; **, **P < 0.01, versus placebo-treated group. B, lesions of small intestinal damage in the placebo- and ONO-4817-treated groups 24 h after administration of indomethacin. Values represent means ± S.E.M. (n = 6). *, **P < 0.05; **P < 0.01, versus placebo-treated group.
significantly attenuated cardiac dysfunction in a mouse model, and we determined the dose of TY-51469 in this study. TY-51469 inhibits human chymase with an IC_{50} of 7 nM. Chymase is a chymotrypsin-like serine protease, but TY-51469 causes no inhibition of other chymotrypsin-like serine proteases, bovine chymotrypsin, and human cathepsin G, even at a concentration of 10 μM (Takai et al., 2009). Thus, TY-51469 has a high specificity for chymase. Indeed, in the present study, TY-51469 did not directly affect MMP-9 activity (data not shown). In the present study, MMP-9 activity in an extract of small intestine from indomethacin-administered rats was significantly higher after incubation for 8 h at 37°C than without incubation; this result suggests that the extract included proMMP-9-activating enzymes. However, the incubated samples to which TY-51469 was applied had significantly attenuated MMP-9 activity. On the other hand, the proMMP-9 level was significantly increased by treatment with TY-51469. This finding suggests that chymase inhibition may induce the accumulation of proMMP-9 by inhibiting the conversion of proMMP-9 to MMP-9. Thus, the importance of chymase-dependent MMP-9 activation in the damaged small intestine after indomethacin administration was confirmed in vitro.

To clarify the second issue, the effect of the chymase inhibitor TY-51469 on the development of small intestinal damage induced by indomethacin in rats was evaluated. In the present study, we observed a significant increase in the number of chymase-positive cells in the small intestine on 24 h after indomethacin administration. Indomethacin-induced prostaglandin deficiency may promote the mucosal dysfunction and accumulation of inflammatory cells, including not only neutrophils but also mast cells, which express chymase. Therefore, the increase in chymase-positive cells after indomethacin administration may be the result of increased inflammation. We also evaluated the effect of the chymase inhibitor on the development of small intestinal damage in an in vivo experiment. In our model, little change in the score of small intestinal damage was observed until 6 h after indomethacin administration, but the score was obviously augmented at 12 and 24 h. In previous reports, the small intestinal damage score was markedly increased 24 h after indomethacin administration, although small intestinal damage was not observed in all normal rats (Tanaka et al., 2002; Takeuchi et al., 2006). In the present study, the small intestinal damage score was high in the placebo-treated group, but a significant attenuation in the score was observed in the TY-51469-treated group. These findings suggest that chymase inhibition may offer a useful strategy for preventing the development of small intestinal damage after indomethacin administration.

MMP-9 is involved in degradation and remodeling of the extracellular matrix, inducing gut inflammation and damage. In a rat experimental model, up-regulation of MMP-9 induced intestinal barrier dysfunction and bacterial translocation (Mikami et al., 2009). On the other hand, reduction in MMP-9 activity resulted in attenuation of the development of indomethacin-induced gastric ulcers in rats (Ganguly et al., 2005). In a rat model with ethanol-induced gastric ulcer, MMP-9 down-regulation also prevented the development of gastric ulcer (Singh et al., 2007). These findings may suggest a strong relationship between MMP-9 up-regulation and the pathogenesis of indomethacin-induced gastric ulcer. In our model, MMP-9 activity in the small intestine was not changed until 6 h after administration of indomethacin, but the activity was significantly augmented at 12 and 24 h. The small intestinal damage score was also obviously increased 12 and 24 h after the administration of indomethacin. These findings may also suggest a relationship between MMP-9 activity and the degree of small intestinal damage. Furthermore, we demonstrated that the MMP inhibitor ONO-4817 significantly attenuated not only MMP-9 activity but also indomethacin-induced small intestinal damage. ONO-4817 specifically inhibited MMP-2 and MMP-9, but not other MMPs. In the present study, although the data are not presented, MMP-2 activity was not changed between normal and indomethacin-induced intestinal enteritis. The attenuation of intestinal enteritis may be dependent on the inhibition of MMP-9 by ONO-4817. These findings may suggest the importance of MMP-9 inhibition for attenuating indomethacin-induced intestinal enteritis. We previously demonstrated that MMP-9 activity was significantly augmented in extract of colitis from mice treated with dextran sodium sulfate (Ishida et al., 2008). MMP-9 activation in the extract after an 8-h incubation at 37°C was significantly attenuated by a chymase inhibitor in an in vitro experiment. Furthermore, chymase inhibition resulted in amelioration of the development of colitis along with the MMP-9 reduction in an in vivo experiment. Thus, a chymase inhibitor, TY-51469, significantly attenuated MMP-9 activity not only in an in vitro experiment but also in an in vivo experiment. Therefore, the mechanism by which the chymase inhibitor prevents the development of small intestinal damage may depend on the inhibition of MMP-9 activation in small intestinal damage after indomethacin administration.

We observed a significant increase in small intestinal MPO activity. MPO activity is thought to reflect the accumulation of neutrophils that accelerate small intestinal damage after indomethacin administration (Takeuchi et al., 2006). In the present study, MPO activity, like MMP-9 activity, in the small intestine was not changed until 6 h after administration of indomethacin, but the activity was significantly augmented at 12 and 24 h. As reported previously, MMP-9 expression may be expressed mainly in neutrophils (Medina et al., 2006; Mikami et al., 2009). In the TY-51469-treated group, the increased MPO activity after indomethacin administration was dramatically reduced to the normal level. This finding may show the attenuation of neutrophil accumulation in the damaged small intestine by treatment with chymase inhibition. He and Walls (1998) demonstrated the accumulation of neutrophils in the skin where purified human chymase was injected. Terakawa et al. (2006) also reported that chymase induced the accumulation of neutrophils after the injection of purified mouse chymase into mouse skin. These reports suggest that chymase may provide a potent stimulus for recruitment of neutrophils after mast cell activation. In contrast, chymase inhibition may result in attenuation of the accumulation of neutrophils. A chymase inhibitor, NK3201, significantly reduced the accumulation of neutrophils in colitis from mice treated with dextran sodium sulfate (Ishida et al., 2008). Therefore, the attenuation of neutrophil accumulation may be included in the mechanism of the prevention of small intestinal damage by the chymase inhibitor.
As a limitation, both MMP-9 activity and MPO activity, which is thought to reflect accumulation of neutrophils, in the small intestinal tract were simultaneously increased 12 and 24 h after indomethacin administration in the present study. Furthermore, the small intestinal damage score was also significantly increased 12 and 24 h after indomethacin administration. We showed that significant reductions in both MMP-9 and MPO activities by chymase inhibition may result in the attenuation of small intestinal damage. Therefore, we could not determine which is more important for attenuation of NSAID-induced small intestinal damage by chymase inhibition—the inhibition of MMP-9 activation or the attenuation of neutrophil accumulation. Further studies are needed to clarify the mechanism of action of the chymase inhibitor.

In conclusion, we demonstrated the importance of chymase-dependent MMP-9 activation in small intestinal damage in indomethacin-administered rats in vitro and the usefulness of chymase inhibition for preventing the development of small intestinal damage via inhibition of MMP-9 activation in vivo.

References


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