Thromboxane A$_2$ Up-Regulates Neutrophil Elastase Release in Syrian Hamsters With Trinitrobenzene Sulfonic Acid-Induced Colitis

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Abstract. Neutrophil elastase (NE) is a factor that aggravates colitis. We investigated the influence of thromboxane A$_2$ (TXA$_2$) and leukotriene B$_4$ (LTB$_4$) on NE release in Syrian hamsters with trinitrobenzene sulfonic acid-induced colitis. Colonic specimens with colitis were incubated with U-46619 (a TXA$_2$ analogue) or LTB$_4$ in vitro and NE release was examined. As a result, U-46619 increased NE release, while LTB$_4$ had no effect. The NE release induced by U-46619 was inhibited by a TP-receptor antagonist. To demonstrate that TXA$_2$ caused NE release in vivo as well, while LTB$_4$ did not, colitis animals were treated with nordihydroguaiaretic acid (NDGA), a dual inhibitor of cyclooxygenase/lipoxygenase; and colonic luminal TXB$_2$ and LTB$_4$ levels and NE activity were determined. The TXB$_2$ level was significantly correlated with NE activity, while no correlation was found between LTB$_4$ and NE activity. An inhibitory effect of NDGA on the ulcer area was also observed, and NE activity was significantly correlated with the ulcer area. The suppression of TXA$_2$ production by NDGA may result in the inhibition of NE release so that colonic tissue damage becomes less severe. Regulation of NE release is a new biological action of TXA$_2$ that has not been reported before.

Keywords: thromboxane A$_2$, up-regulation, neutrophil elastase release, trinitrobenzene sulfonic acid-induced colitis

Introduction

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 (1). In patients with active IBD, there is prominent neutrophil infiltration into the colonic mucosa and neutrophil elastase (NE) levels are increased (2 – 4).

NE is a protease that is known to have a broad spectrum of 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 of vascular permeability, causing tissue damage and organ failure (5 – 7). In IBD patients, the fecal NE level is increased, and it shows a correlation with both disease activity and the fecal hemoglobin level (2, 4).

Moreover, fecal NE in IBD patients does not form a complex with $\alpha$1-proteinase inhibitor (8), which is a major endogenous elastase inhibitor in serum, leading to NE/$\alpha$1-proteinase inhibitor imbalance (5 – 7). Consequently, NE activity may be excessively high in IBD patients, resulting in the aggravation of inflammation (2, 9). In Syrian hamsters with trinitrobenzene sulfonic acid (TNBS)-induced colitis, we recently demonstrated that the increase of colonic NE activity was well correlated with colonic ulceration and that administration of an NE inhibitor could decrease the ulcer area (10). Although these findings in IBD patients and Syrian hamsters suggest that NE plays an important role in the progression of IBD, the precise mechanism underlying the release of NE from neutrophils infiltrating the inflamed colon has not been clarified.

Thromboxane A$_2$ (TXA$_2$) and leukotriene B$_4$ (LTB$_4$) are inflammatory eicosanoids that are formed from arachidonic acid via the cyclooxygenase and lipoxygenase pathways, respectively. It has been reported that
TXA$_2$ and LTB$_4$ production is enhanced in the colonic mucosal tissue of patients with IBD so that intraluminal levels of TXA$_2$ and LTB$_4$ are elevated in these patients (11 – 13). Since prednisolone, a first-line drug for active IBD (14), inhibits the production of TXA$_2$ and LTB$_4$ in the colon, inhibition of these inflammatory eicosanoids is considered to partly explain the mechanism of action of anti-IBD drugs (11, 12). It has also been reported that expression of thromboxane synthetase in the colonic lamina propria is elevated in patients with active IBD (15) and that a thromboxane synthetase inhibitor or agonist, was used to investigate the role of TXA$_2$ in NE release in the colon. Tissue specimens of the colon from animals with experimental colitis were incubated with U-46619, thromboxane B$_2$ (TXB$_2$), or LTB$_4$, and NE release was examined. TXA$_2$ is unstable and is known to be rapidly hydrolyzed to TXB$_2$, a stable and inactive metabolite (20). Therefore, U-46619, a synthetic TP receptor (TXA$_2$ receptor) agonist, was used to investigate the role of TXA$_2$ in NE release (21). In addition, Syrian hamsters with TNBS-induced colitis were treated with nordihydroguaiaretic acid (NDGA: a dual inhibitor of cyclooxygenase/ lipoxigenase) (22), and the intraluminal levels of TXB$_2$ and LTB$_4$ were measured to investigate the correlation with NE activity. As a result, a difference was found in the role that these eicosanoids play with respect to NE release in the colon.

Materials and Methods

Animals

Male specific-pathogen-free Syrian hamsters (Japan SLC, Hamamatsu) weighing 90 – 130 g were used. The animals were maintained in a room ventilated with all fresh air at 15 ± 5 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 per plastic cage (345 wide × 403 long × 177 mm high), and dry autoclaved sawdust (Sankyo Labo Service, Tokyo) was used as the bedding. Standard pellet food for rodents, CRF-1 (Oriental Yeast Industry, Tokyo), and tap water were offered ad libitum.

This study was conducted after receiving the approval of the institutional animal care and use committee and was performed in compliance with the “Guidelines for Studies in Animals” produced by the Research Headquarters of Ono Pharmaceutical Co., Ltd., which are based on the “Guidelines for Animal Experimentation” of the Japanese Association for Laboratory Animal Science (23).

Reagents

TNBS was purchased from Tokyo Kasei Kogyo (Tokyo). Ethanol and aqueous hydrogen peroxide (H$_2$O$_2$) were obtained from Wako Pure Chemical Industries (Osaka). NDGA, N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroaniline (Suc-Ala-Ala-Pro-Val pNA), o-dianisidine, and cytochalasin B were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The TXB$_2$ and LTB$_4$ immunoassay kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). U-46619, TXB$_2$, LTB$_4$, and SQ29548 (a highly selective TP-receptor antagonist) were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

Induction of colitis

The day when colitis was induced was designated as Day 0. Colitis was induced by the method described previously (10). Briefly, under ether anesthesia, 0.1 ml of 90 mg/ml TNBS in 15 vol% ethanol/physiological saline (TNBS solution) was administered into the colon using a flexible tube with the tip advanced 1.5 cm beyond the anus, and then the anus was closed manually for 10 s. In the normal control group, physiological saline was administered intracolonically.

In vitro study of NE release from inflamed colonic tissue

Syrian hamsters were exsanguinated by incising the thoracic aorta under ether anesthesia on Day 5 after the induction of colitis and the colon was isolated. After opening the colon longitudinally and washing the lumen with physiological saline, colonic tissue specimens were cut from the sites of ulceration. The tissue specimens were cut into 1 × 3 mm pieces with a surgical knife.

Then the specimens were placed in 400 µl of Hanks’ balanced salt solution containing 5 µg/ml cytochalasin B (HBSS-C) and incubated under 5% CO$_2$ for 30 min at 37°C (pre-culture). Cytochalasin B is generally used to prime neutrophils for in vitro studies (24). Next, these tissue specimens were incubated for another 30 min with HBSS-C added with dimethyl sulfoxide (DMSO: vehicle control group), U-46619, TXB$_2$, or LTB$_4$, (treatment culture). U-46619, TXB$_2$, and LTB$_4$ were used at final concentrations of 10$^{-9}$ to 10$^{-6}$ M. NE activity in the pre-culture medium was measured and defined as basal elastase release (BER), while NE activity in the treatment culture medium was defined as
the post elastase release (PER). The relative value of PER (% basal value) was calculated by taking BER as 100%.

NE release from normal colonic tissue was also determined. Briefly, physiological saline was administered intracolonically instead of TNBS solution in Syrian hamsters. After pre-culture of normal colonic tissue specimens, treatment culture was done with DMSO, U-46619, TXB₂, or LTB₄ (all at a final concentration of 10⁻⁶ M). Then the BER and PER were determined.

Effect of a TP receptor antagonist on NE release induced by U-46619 (in vitro)

Because U-46619 increased NE release in the preceding experiment on the role of inflammatory eicosanoids in NE release from inflamed colonic tissue, the following experiment was conducted to determine whether or not NE release induced by U-46619 is mediated by the TP receptor.

Colitis tissue specimens were collected from the sites of ulceration as mentioned above. The tissue specimens were placed in 400 μl of HBSS-C containing DMSO (vehicle- and U-46619 control groups) or a TP-receptor antagonist (SQ29548) at concentrations of 10⁻⁹ to 10⁻⁶ M and were incubated under 5% CO₂ for 30 min at 37°C (pre-culture). Then the medium of the vehicle control group was replaced with HBSS-C containing DMSO, the medium of the U-46619 control group was replaced with HBSS-C containing both U-46619 (10⁻⁶ M) and DMSO, and the medium of the SQ29548-treated groups was replaced with HBSS-C containing both U-46619 (10⁻⁶ M) and SQ29548 (10⁻⁹ to 10⁻⁶ M). All of the tissue specimens were incubated for another 30 min (treatment culture).

BER and PER were measured in the same manner as in the preceding experiment, and a relative PER value (% basal value) was calculated for each specimen by taking BER as 100%.

Effect of NDGA on eicosanoid production and NE activity in TNBS-induced colitis (in vivo)

Colitis was induced in Syrian hamsters by the method described above. Animals in the normal control group were treated with physiological saline instead of TNBS solution. NDGA was suspended in 0.5 w/v% carboxymethyl cellulose sodium salt solution and was administered at a dose of 25 mg/kg using a flexible gavage tube once on the day before induction of colitis and twice daily from the day of induction until the day before autopsy. The normal control and colitis control groups were given the vehicle instead of NDGA.

Hamsters were exsanguinated by incising the thoracic aorta under ether anesthesia on Day 5, and then the colon and rectum up to 3 cm from the anus were removed. After opening each resected colon longitudinally and washing the lumen with 2 ml of physiological saline, a photograph was taken and was used to determine the ulcer area. After centrifugation of the bowel washings (4°C, 1710 × g, 20 min), the supernatant was stored at −80°C for measurement of the NE activity and TXB₂ and LTB₄ levels in the colonic lumen. The isolated colon was also stored at −80°C until measurement of tissue myeloperoxidase (MPO) activity.

Determination of the ulcer area

Photographic slides of the colon were scanned into a computer using a Minolta QuickScan 35 (Minolta, Osaka) and the data were processed with imaging software (Adobe PhotoShop (R) 4.0J; Adobe Systems, San Jose, CA, USA). The ulcerated region was copied with PhotoShop and the ulcer area (cm²) was measured using image analysis software (NIH Image 1.61/fat; NIH, Bethesda, MD, USA).

Assay of tissue MPO activity

As an index of the neutrophil count, tissue MPO activity in the colon was determined (25). Briefly, each isolated colon was placed in 3 ml of 0.05 M potassium phosphate buffer (pH 6.0) containing 0.5 w/v% 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% H₂O₂, 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 s (DU7400 SpectroPhotometer; Beckman, Toyonaka). 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 pNA, which is highly specific for NE, by the method described previously (26). 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, pNA release was measured spectrophotometrically (DU7400 SpectroPhotometer) at 405 nm as an indicator of NE activity.

Measurement of TXB₂ and LTB₄ in bowel washings

The TXB₂ and LTB₄ levels in bowel washings were measured with TXB₂ and LTB₄ immunoassay kits as
directed by the manufacturer.

Statistical analyses

Results are expressed as the mean ± S.E.M. For the in vitro study, the significance of differences between the vehicle control and the eicosanoid-treated groups was assessed by the two-tailed Dunnett’s t-test with significance assigned at P<0.05. The two-tailed Student’s t-test was used (with significance assigned at P<0.05) was used to compare the U-46619 control group with the vehicle control group in the study on the effect of the TP-receptor antagonist.

For the in vivo study, the significance of differences in each parameter was assessed by the two-tailed Student’s t-test with significance assigned at P<0.05. In addition, to investigate the correlations between luminal eicosanoid levels and NE activity and between NE activity and the colonic ulcer area, data obtained in the individual animals from the colitis control and NDGA-treated groups were plotted and Spearman’s rank-correlation coefficients were calculated.

Results

Effect of inflammatory eicosanoids on NE release from colitis tissue (in vitro)

The BER for normal colonic tissue specimens was 0 μM pNA release. The PER for normal colonic tissue specimens after treatment with DMSO, U-46619 (10−6 M), TXB2 (10−6 M), or LTB4 (10−6 M) was also 0 μM pNA release. On the other hand, the BER for colonic tissue specimens with TNBS-induced colitis was 18.0 ± 1.5 μM pNA release. These results provided evidence that NE is released from colitis tissue in vitro.

When colitis tissue was treated with U-46619, the % basal value increased significantly after treatment at concentrations of 10−7 M or more compared with that for the vehicle control group (122 ± 22% for vehicle control; 210 ± 23% at 10−7 M, P<0.05; 320 ± 28% at 10−6 M, P<0.001) (Fig. 1). NE release showed a 1.7-fold increase and 2.6-fold increase after treatment at concentrations of 10−7 and 10−6 M, respectively.

When colitis tissue was treated with TXB2 at the highest concentration of 10−6 M, the % basal value slightly tended to be elevated after treatment (10−6 M: 166 ± 26%), but the difference between the treated group and the vehicle control group (110 ± 10%) was not significant. When the concentration of TXB2 was 10−7 M or less, there was no difference in % basal value between the vehicle control and the treated groups.

When colitis tissue was treated with LTB4, there was no difference in % basal value between the vehicle control (84 ± 16%) and treated groups at any concentra-

Fig. 1. Effect of inflammatory eicosanoids on NE release from inflamed colonic tissue (in vitro). Tissue specimens from hamsters with TNBS-induced colitis were placed in Hanks’ balanced salt solution containing cytochalasin B (HBSS-C) and incubated for 30 min at 37°C (pre-culture). Then fresh HBSS-C containing DMSO (vehicle control), U-46619 (closed circle), TXB2 (closed square), or LTB4 (open circle) was prepared, and the colonic tissue specimens were incubated in the fresh solutions for another 30 min (treatment culture). NE activity in the pre-culture medium was defined as basal elastase release (BER), while NE activity in the treatment culture medium was defined as the post elastase release (PER). The relative value of PER (% basal value) was calculated by taking the BER as 100%. Each point represents the mean ± S.E.M. for six specimens. Values with asterisks are significantly higher than that for the vehicle control (*P<0.05; ***P<0.001).

tion of LTB4.

Effect of a TP-receptor antagonist on the U-46619-induced increase of NE release (in vitro)

The % basal value was 117 ± 14% for the vehicle control group and 266 ± 26% for the U-46619 control group (Fig. 2). Treatment with U-46619 caused a significant increase of NE release compared with that for the vehicle control group (P<0.001).

U-46619-incuced NE release was not inhibited by treatment with SQ29548 at a concentration of 10−9 or 10−8 M (284 ± 45% at 10−9 M and 289 ± 35% at 10−8 M), but was significantly inhibited after treatment with SQ29548 at a concentration of 10−7 or 10−6 M compared with that for the U-46619 control group (110 ± 18% at 10−7 M, P<0.01; 117 ± 11% at 10−6 M, P<0.01).

Effect of NDGA on TNBS-induced colitis

After colitis was induced using 90 mg/ml TNBS solution, the colonic ulcer area, tissue MPO activity, luminal NE activity, and luminal TXB2 and LTB4 levels were all significantly increased compared with those
for the normal control group (Figs. 3 and 4). In the NDGA-treated group, the colonic ulcer area was significantly decreased by 40% ($P < 0.05$), compared with that in the colitis control group. Tissue MPO activity was not inhibited by NDGA, with no significant difference between the colitis and NDGA-treated groups. Luminal NE activity was significantly inhibited by 55% in the NDGA-treated group ($P < 0.05$). The luminal TXB$_2$ and LTB$_4$ levels were also significantly reduced by NDGA, with the decrease being 73% and 89%, respectively ($P < 0.05$).

**Correlations between inflammatory eicosanoid levels and NE activity or NE activity and ulcer area in TNBS-induced colitis**

When luminal NE activity was plotted against the luminal TXB$_2$ level, there was a significant correlation between these parameters ($P < 0.001$, $r = 0.793$) (Fig. 5). When luminal NE activity was plotted against the luminal LTB$_4$ level, however, there was no correlation between them. When the ulcer area was plotted against luminal NE activity, there was a significant correlation between them ($P < 0.01$, $r = 0.684$).

**Discussion**

There have been many reports about enhanced production of inflammatory eicosanoids, such as TXA$_2$ and LTB$_4$ in human IBD or in a rat model of TNBS-induced colitis in the active phase (11 – 13, 27 – 29). TXA$_2$ and LTB$_4$ have been suggested as important aggravating factors for colitis (11 – 13, 16), but their role has not been clarified in detail. In the present study, TXA$_2$ was shown to enhance NE release in Syrian hamsters with TNBS-induced colitis and the pathophysiological role of TXA$_2$ in the development of colitis was partly elucidated. Furthermore, it was shown that the inhibition of TXA$_2$ production resulted in the inhibition of NE
release and lessened the destruction of colonic tissue.

In the rat model of TNBS-induced colitis, intraluminal NE activity is not increased despite prominent elevation of tissue MPO activity (i.e., massive neutrophils infiltration) and ulceration (10). Other tissue destructive factors from neutrophils, such as reactive oxygen species (28), may contribute to progress of inflammation rather than NE in the rat model. On the other hand, in Syrian hamsters with TNBS-induced colitis, the increase of luminal NE activity is well correlated with the colonic ulcer area, and inhibition of NE can prevent the progression of colonic ulceration (10). This suggests that, as in human IBD, NE acts as a colitis-aggravating factor in this hamster model of TNBS-induced colitis. This difference of NE activity among species may be accounted for by the fact that the anti-NE activity of serum (which contains endogenous proteinase inhibitors) is higher in rats than in humans or hamsters (30). In consideration of this species difference, a rat model of TNBS-induced colitis is inappropriate for investigating the relationship of inflammatory eicosanoids to NE release. In contrast, the TNBS-induced colitis model in Syrian hamsters seems to be useful for this purpose.

In the present study, colonic tissue specimens obtained from Syrian hamsters with TNBS-induced colitis were treated with a TXA$_2$ analogue (U-46619) (21), TXB$_2$, or LTB$_4$, and tissue NE release was deter-
mined. As a result, it was found that U-46619 enhanced NE release from colitis tissue, while TXB\(_2\) (an inactive metabolite of TXA\(_2\)) was far less effective than U-46619, and LTB\(_4\) had no influence on NE release. U-46619 is also known to bind to the FP receptor (prostaglandin F\(_{2\alpha}\) receptor), although its affinity for this receptor is about tenfold lower than for the TP receptor (21). To confirm involvement of the TP receptor in the enhancement of NE release by U-46619, it was determined whether or not a TP-receptor antagonist (SQ29548) could prevent the enhancement of NE release. SQ29548 exhibits highly selective antagonism for the TP receptor (21, 31, 32). In this study, SQ29548 was shown to inhibit the enhancement of NE release by U-46619. Taken together, these results suggest that TXA\(_2\) regulates NE release via the TP receptor in the Syrian hamster model of TNBS-induced colitis.

To obtain in vivo evidence that it was not LTB\(_4\) but TXA\(_2\) that causes NE release, NDGA was administered orally to Syrian hamsters with TNBS-induced colitis, and the correlation between intraluminal TXA\(_2\) or LTB\(_4\) levels and NE activity was investigated. Being a dual inhibitor of cyclooxygenase/lipoxygenase, NDGA allowed simultaneous determination of whether TXA\(_2\) or LTB\(_4\) was involved in NE release (22). Since TXA\(_2\) is unstable, the level of TXB\(_2\) was determined as an indicator of intraluminal TXA\(_2\) production. As in the in vitro study, there was no correlation between LTB\(_4\) and NE activity, but there was a significant in vivo correlation between TXB(A)\(_2\) and NE activity. This result again suggests that TXA\(_2\) regulates NE release. Platelet aggregation and vasoconstriction are well-known biological actions of TXA\(_2\) (20), but neutrophil degranulation has not been reported before. On the other hand, LTB\(_4\) is known to be a powerful biological agent causing neutrophil degranulation (24, 33). Therefore, we thought that LTB\(_4\) may also regulate NE release in this Syrian hamster model of TNBS-induced colitis, but we found that LTB\(_4\) had no such action and TXA\(_2\) enhanced NE release. Enhancement of NE release by TXA\(_2\) is a novel biological action that may be interesting in relation to studies of inflammatory eicosanoids. When, in the future, another TP-receptor antagonist with good properties and suitable for in vivo study is developed, our conclusion will be also supported by investigating its effect on NE release.

When TNBS-induced colitis was treated with NDGA, the colonic ulcer area decreased significantly, and there was a significant correlation between NE activity and the ulcer area. Since NDGA does not inhibit the proteolytic activity of NE (34), the decrease of the colonic ulcer area may be explained by the hypothesis that NDGA inhibits NE release induced by TXA\(_2\), thereby preventing colonic tissue destruction via the proteolytic activity of NE. This hypothesis is supported by the finding that intraluminal NE activity is significantly inhibited when Syrian hamsters with TNBS-induced colitis are treated with a specific NE inhibitor, ONO-6818, and there is a simultaneous decrease of colonic ulcer area (10).

On the other hand, a calculation of the correlation between LTB\(_4\) and colonic ulcer area with Spearman’s
rank-correlation coefficient failed to reveal any relationship (data not shown), so colonic tissue destruction cannot be ascribed to LTB₄ production. In addition, NDGA did not affect the MPO activity in this colitis model despite its ability to inhibit the production of LTB₄ which has a chemotactic activity (24). Therefore, it is also considered that LTB₄ is not related to neutrophil migration in this colitis model. Cytokines, such as IL-8 which is one of the strongest neutrophil chemotactic factors (35), may contribute to the regulation of neutrophil migration rather than LTB₄. Further studies are needed to clarify its exact mechanism.

Casellas and colleagues reported that 5-aminosalicylic acid and a thromboxane synthetase inhibitor (ridogrel) inhibited TXA₂ production in patients with IBD and that both agents had a similar beneficial effect on the inflammation (16). Finding that NE release was inhibited in the responders, the authors noted that NE activity of NE led to colonic tissue destruction, it remains possible that ridogrel or 5-aminosalicylic acid inhibited NE release induced by TXA₂ in patients with IBD, thereby improving the disease. To verify this hypothesis, clinical studies designed to demonstrate the effects of a specific NE inhibitor and ridogrel in patients with IBD are warranted. In addition, to know whether or not TXA₂ is related to NE release in human IBD, it would be necessary to demonstrate that NE is released when colonic tissue specimens obtained from patients with IBD are treated with U-46619 in vitro. This remains a subject for further inquiry.

In conclusion, TXA₂ enhanced NE release in a hamster model of colitis, while LTB₄ did not. Since NE causes the colonic tissue damage, TXA₂ seems to act as a proinflammatory eicosanoid during the development of colitis.

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