Potential role of the NADPH oxidase NOX1 in the pathogenesis of 5-fluorouracil-induced intestinal mucositis in mice

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Yasuda M, Kato S, Yamanaka N, Imori M, Utsumi D, Kitahara Y, Iwata K, Matsuno K, Amagase K, Yabe-Nishimura C, Takeuchi K. Potential role of the NADPH oxidase NOX1 in the pathogenesis of 5-fluorouracil-induced intestinal mucositis in mice. Am J Physiol Gastrointest Liver Physiol. 2012;302(2):G1133–G1142. First published March 7, 2012; doi:10.1152/ajpgi.00535.2011.—Although NADPH oxidase 1 (NOX1) has been shown to be highly expressed in the gastrointestinal tract, the physiological and pathophysiological roles of this enzyme are not yet fully understood. In the present study, we investigated the role of NOX1 in the pathogenesis of intestinal mucositis induced by the cancer chemotherapeutic agent 5-fluorouracil (5-FU) in mice. Intestinal mucositis was induced in Nox1 knockout (Nox1KO) and littermate wild-type (WT) mice via single, daily administration of 5-FU for 5 days. In WT mice, 5-FU caused severe intestinal mucositis characterized by a shortening of villus height, a disruption of crypts, a loss of body weight, and diarrhea. In Nox1KO mice, however, the severity of mucositis was significantly reduced, particularly with respect to crypt disruption. The numbers of apoptotic caspase-3- and caspase-8-activated cells in the intestinal crypt increased 24 h after the first 5-FU administration but were overall significantly lower in Nox1KO than in WT mice. Furthermore, the 5-FU-mediated upregulation of TNF-α, IL-1β, and NOX1 and the production of reactive oxygen species were significantly attenuated in Nox1KO mice compared with that in WT mice. These findings suggest that NOX1 plays an important role in the pathogenesis of 5-FU-induced intestinal mucositis. NOX1-derived ROS production following administration of 5-FU may promote the apoptotic response through upregulation of inflammatory cytokines.

Nox1 knock-out mice; apoptosis; inflammatory cytokines; caspase; reactive oxygen species

INTESTINAL MUCOSITIS IS A common side effect experienced by cancer patients undergoing clinical chemotherapy treatment (3, 43). The antimitabolite anticancer agent 5-fluorouracil (5-FU) is widely used to treat malignant tumors due to its ability to improve tumor-free status and survival rates (27). However, 50%–80% of patients who undergo 5-FU chemotherapy display clinical manifestations of mucositis, the symptoms of which include severe diarrhea (3, 41, 43). These serious side effects often necessitate a diminution of drug doses or even a discontinuation of the treatment, thereby hampering the success of the cancer chemotherapy.

Gastrointestinal mucositis induced by 5-FU chemotherapy is a consequence of various processes including apoptosis, hyperproliferation, and abnormal inflammatory responses leading to intestinal malabsorption and dysfunctions (6, 10). Apoptosis is considered to play a critical role in the occurrence of intestinal mucositis induced by 5-FU chemotherapy. Indeed, a large number of apoptotic cells where found in mouse intestinal crypt prior to serious mucosal destruction (1, 16) and in intestinal biopsy specimens of patients undergoing chemotherapy (19). The mechanisms involved in chemotherapy-mediated induction of small intestinal cell apoptosis are still poorly understood but may correlate with the production of reactive oxygen species (ROS) and proinflammatory cytokines such as TNF-α and IL-1β (13, 24, 40).

NADPH oxidase (NOX) was originally identified as a major source of ROS in phagocytes and has recently been shown to play a key role in cellular signaling (6). NOX is a catalytic subunit of NADPH oxidase, a multisubunit enzyme composed of membrane-bound NOX and p22phox, associated with several cytosolic regulatory subunits including p47phox (2). Multiple homologues of NOX have recently been identified, such as NOX2 (gp91phox) and NADPH oxidase 1 (NOX1), which are respectively phagocytic and nonphagocytic forms of the enzyme (37). NOX1 was recently shown to be implicated in the pathogenesis of hypertension (28), inflammatory pain (14), and liver fibrosis (9, 38). In addition, NOX1 is highly expressed in the gastrointestinal tract and has been suggested to play a role in local innate immune and inflammatory responses (17, 38).

In the present study, we investigated the role of NOX1 in the pathogenesis of intestinal mucositis induced by 5-FU chemotherapy in Nox1-deficient mice. We focused primarily on the association of NOX1 with apoptosis in the intestinal crypt preceding intestinal mucosal atrophy and hypoplasia.

MATERIALS AND METHODS

Animals. Nox1-deficient (Nox1KO) (28) and control littermate wild-type (WT) mice were acclimated to standard laboratory conditions (12:12-h light-dark cycle, temperature: 22 ± 1°C). Experiments were carried out using six to eight mice per group under unanesthetized conditions. All experimental procedures were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Induction of intestinal mucositis. Intestinal mucositis was induced in Nox1KO and WT mice via single daily administration of 50 mg/kg 5-FU (Sigma-Aldrich, St. Louis, MO) over the course of 5 days (days 0 to 4), with saline (vehicle) used as control. Disease severity was assessed daily by measuring body weight and scoring the stool consistency: 0, normal stool; 1, slight diarrhea (slightly wet and soft stool); 2, moderate diarrhea (watery stool with some perianal staining); and 3, severe diarrhea (watery stool with severe perianal staining) as previously described (19).
Assessment of intestinal mucositis. Twenty-four hours after the final 5-FU injection (day 5), animals were killed under deep ether anesthesia, and the jejunum was removed and immersed overnight in 10% neutralized formalin. Tissue samples were excised, embedded in paraffin, and cut into 4-μm-thick sections. Hematoxylin and eosin staining was subsequently performed. Measurement of the villus height (from the top of the villi to the villus-crypt junction) and crypt damage (surviving crypt number per millimeter and surviving crypt cells per crypt) were performed under light microscope at magnifications of ×100 and ×1,000, respectively (model BX-50; Olympus, Tokyo, Japan). Five intact, well-oriented villi and crypts were measured and averaged for each sample.

Apoptosis analysis. Animals were killed 24 and 72 h after initial 5-FU administration (days 1 and 3, respectively), and jejunum samples were fixed with 10% neutralized formalin, embedded in paraffin, and cut into 4-μm-thick sections. Apoptosis of enterocytes in the small intestine was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using an in situ Apoptosis Detection Kit (Takara, Shiga, Japan) according to the manufacturer’s instructions. For each sample, the number of TUNEL-positive apoptotic cells from 10 crypts was counted and averaged at a magnification of ×1,000 under a light microscope (model BX-50; Olympus).

Determination of caspase-3, caspase-8 activation, and cell proliferation. Animals were killed 24 h after initial 5-FU administration (day 1), and the jejunum samples were fixed in 10% neutralized formalin, embedded in paraffin, and cut into 4-μm-thick sections. Caspase-3, caspase-8 activations, and cell proliferation were determined immunohistochemically after activation with HistoVT One (Nacalai Tesque, Kyoto, Japan) using rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), rabbit anti-cleaved caspase-8 (Imgenex, San Diego, CA), and rabbit anti-Ki-67 (Novus Biologicals, Littleton, CO) antibodies, respectively. The immunocomplex was visualized by the avidin-biotin-peroxidase method using the Vectastain Elite ABC Rabbit IgG kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Sections were counterstained with hematoxylin. The number of cleaved caspase-3- and caspase-8-positive cells in 10 cells was counted under a light microscope at a magnification of ×1,000 (model BX-50; Olympus) and averaged for each sample.

Determination of proinflammatory cytokines expression by real-time RT-PCR. Animals were killed under deep ether anesthesia at 12, 24 (day 1), and 72 h (day 3) after initial 5-FU administration, and the jejunum was removed, washed with cold PBS, and stored in RNA later (Ambion, Austin, TX) at 4°C until use. Total RNA was extracted from the whole jejunum layer using Separase RNA-1 Supper G (Nacalai Tesque) following the manufacturer’s instructions. RT was performed using RevaTra Ace-α with random hexamers (Toyobo, Osaka, Japan). Real-time PCR amplification was performed using SYBR Premix ExTaq (Takara, Shiga, Japan) with specific primers sets, prepared using the Perfect real-time supporting system (Takara) for β-actin (primer set ID: MA050368), TNF-α (primer set ID: MA097070), IL-1β (primer set ID: MA025939), and NOX1 (primer set ID: MA051645) using an ABI PRISM 7500 real-time PCR System (Applied Biosystems, Foster City, CA). Expression level of each mRNA was standardized to that of β-actin mRNA, and expressed as the ratio to the mean value for control (saline alone) or 0 h (before 5-FU injection) in WT mice at each time point.

Determination of ROS production. Animals were killed 12 h after the first 5-FU administration and the jejunum was removed, washed with cold PBS, and stored at −80°C until use. Tissue samples were homogenized in ice-cold Krebs-HEPES buffer (in mmol/l: 20 HEPES, 9.9 NaCl, 4.7 KCl, 1.2 MgSO4, 1 K2HPO4, 1.9 CaCl2, 25 NaHCO3, 11.1 glucose) containing a cocktail of protease inhibitors (Complete mini; Roche, Mannheim, Germany), and then centrifuged at 1,100 g at 4°C for 15 min to remove unbroken cells and nuclei. Protein concentration was determined using a BCA Protein Assay kit (Pierce, Rockford, IL). Generation of O2·− was measured via a lucigenin chemiluminescence assay. Briefly, the supernatant (100 μl) was transferred into 900 μl of Krebs-HEPES buffer containing 100 μmol/l NADPH, 50 μmol/l lucigenin (Tokyo Chemical Industry, Tokyo, Japan). After 10-min incubation at 37°C, chemiluminescence was measured for 1 min using a luminometer (model Lumat LB9507; Berthold, Wildbad, Germany). The production of ROS was expressed as arbitrary light units per minute per milligram protein.

Fluorescent immunohistochemical study for TNF-α and macrophages. Animals were killed under deep ether anesthesia 24 h (day 1) after the first 5-FU administration. Jejunum were removed, washed with cold PBS, and immersed in 4% paraformaldehyde for 48 h at 4°C. After treatment in 10% and 20% sucrose solutions, tissue samples were embedded in optimum cutting temperature compound (Miles, Elkhart, IN) and frozen rapidly in CO2 gas. Cryostat sections (cat. no. CM1510; Leica, Wetzlar, Germany) were cut serially into 10-μm-thick sections and mounted on silane-coated slides (Matsunami, Osaka, Japan). The slides were incubated for 48 h at 4°C with a rabbit anti-human TNF-α polyclonal antibody (Abbiotec, San Diego, CA) and a rat anti-mouse CD68 monoclonal antibody (Serotec, Oxford, UK) and for 2 h at room temperature with an Alexa Fluor 546-labeled donkey polyclonal anti-rabbit IgG antibody and a 488-labeled donkey polyclonal anti-rat IgG antibody (Molecular Probes, Eugene, OR), respectively. Immunofluorescence was observed using a confocal laser scanning microscope (model LSM-510; Carl Zeiss Microimage, Thornwood, NY).

Statistical analysis. Data are presented as means ± SE from 6–8 mice per group. Statistical analyses were performed using a two-tailed Student’s t-test or Dunnett’s multiple comparison test with P values < 0.05 regarded as significant.

RESULTS

Loss of body weight and severity of diarrhea induced by 5-FU treatment were reduced in Nox1KO compared with WT mice. Repeated administration of 5-FU caused body weight loss and diarrhea in both WT and Nox1KO mice, and these changes were particularly prominent 3 days after the initial 5-FU treatment. The body weight on day 5 in 5-FU-treated WT and Nox1KO mice decreased to 80.6 ± 1.3% and 80.0 ± 1.2% of initial body weights, respectively; but these changes were not statistically significant. Although an equal body weight loss was observed in both WT and Nox1KO mice, the severity of diarrhea was significantly lower in the latter. The diarrhea scores on day 5 in 5-FU-treated WT and Nox1KO mice were 1.7 ± 0.6 and 0.8 ± 0.3, respectively.

5-FU-induced intestinal mucositis was attenuated in Nox1KO compared with WT mice. The heights of intestinal villi in the control (5-FU-untreated) WT and Nox1KO mice were not statistically different (344.0 ± 16.5 and 345.0 ± 11.6 mm, respectively) (Fig. 1, A and B). In WT mice, repeated administration of 5-FU caused shortening of intestinal villi to an average height of 134.3 ± 5.1 mm on day 5, but this reduction was significantly lower in Nox1KO mice (228.8 ± 14.3 mm). Repeated administration of 5-FU also caused severe crypt damage in the small intestine, characterized by a decrease in numbers of surviving crypt and crypt cells in both WT and Nox1KO mice, but these changes were significantly lower in Nox1KO mice on day 5 (Fig. 2A). In WT mice, the average number of surviving crypts decreased from 30.6 ± 3.1 to 13.4 ± 1.3 crypt/mm (Fig. 2B) and the number of surviving crypt cells decreased from 18.1 ± 1.2 to 3.9 ± 1.2 cells/crypt.

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while in Nox1KO mice, these numbers decreased from 29.8 ± 1.2 to 26.4 ± 3.7 crypt/mm and 18.4 ± 0.7 to 10.4 ± 0.7 cells/crypt, respectively.

Apoptotic response to 5-FU was reduced in Nox1KO compared with WT mice. Very few TUNEL-positive apoptotic cells were found in the intestinal crypt of controls in both WT and Nox1KO mice (Fig. 3A). The administration of 5-FU markedly increased the number of apoptotic cells in the intestinal crypt of WT mice on days 1 and 3, this response being more evident on day 1 than day 3 (86.0 ± 7.1 and 21.8 ± 1.1 cells/mm, respectively) (Fig. 3, A and B). In contrast, the increase in apoptosis induced by 5-FU was significantly lower in Nox1KO mice both on days 1 and 3 (21.8 ± 1.1 and 5.3 ± 0.4 cells/mm, respectively).

To investigate the mechanisms of 5-FU-induced apoptosis, we immunohistochemically determined the extent of caspase-3 and caspase-8 activation using anti-cleaved caspase-3 and caspase-8 antibodies, respectively. The administration of 5-FU caused a 5.3-fold increase in the number of caspase-3-activated cells 24 h after initial inoculation in WT mice, but this increase was significantly attenuated in Nox1KO mice, with inhibition of 72.5% (Fig. 4, A and D). Likewise, although 5-FU caused a 4.5-fold increase in the number of caspase-8-activated cells in WT mice, it failed to trigger such a dramatic increase in activity in Nox1KO mice, with inhibition of 93.2% (Fig. 4, B and E).

Anti-proliferative effect of 5-FU in intestinal crypt cells was equally observed in WT and Nox1KO mice. To confirm the effect of 5-FU on proliferation in intestinal crypt cells of WT and Nox1KO mice, we immunohistochemically determined the proliferative activity using anti-Ki-67 antibody. There was no significant difference in the number of Ki-67-positive proliferative cells between control (5-FU-untreated) WT and Nox1KO mice (Fig. 4, C and F). The administration of 5-FU reduced the number of Ki-67-positive cells in both WT and Nox1KO mice, the inhibition being 67.7% and 66.3, respectively, but these changes were not statistically significant.

(Fig. 2C), while in Nox1KO mice, these numbers decreased from 29.8 ± 1.2 to 26.4 ± 3.7 crypt/mm and 18.4 ± 0.7 to 10.4 ± 0.7 cells/crypt, respectively.
Increases in NOX1 expression and ROS production was attenuated in Nox1KO compared with WT mice. NOX1 expression in the small intestine of 5-FU-treated WT mice relative to that of controls increased significantly as early as 12 h, reaching a level of 3.1 ± 0.9 times at 24 h after the first 5-FU administration. However, NOX1 mRNA levels were undetectable in the small intestine of Nox1KO mice with or without 5-FU treatment (Fig. 5A).

ROS production tended to be lower, albeit not significantly, in Nox1KO mice than in WT mice (average of 1.5 ± 0.3 and 3.0 ± 1.9 light units·min⁻¹·mg protein⁻¹, respectively). The administration of 5-FU enhanced ROS production by 5.2-fold at 12 h following the first administration of 5-FU in WT mice, but this response was almost totally absent in Nox1KO mice (Fig. 5B).

Upregulation of TNF-α and IL-1β was prevented in Nox1KO compared with WT mice. In WT mice, TNF-α was upregulated in response to 5-FU as early as 12 h after, and levels reached 5.2 ± 1.2 times those in the control animals at 24 h after initial administration (day 1) (Fig. 5C). Although IL-1β expression also increased, this response was apparently delayed with only a slight increase observed in the first 24 h after the onset of 5-FU treatment (1.9 ± 0.4 times greater than that of control) (Fig. 5D). The expression of both TNF-α and IL-1β did not change significantly in Nox1KO mice at any time point.

Expression of TNF-α protein induced by 5-FU was enhanced in macrophages located in the intestinal mucosa. TNF-α protein expression was not detected in the intestinal mucosa of control WT mice, but it was mostly detected after 5-FU treatment (day 1) in the lamina propria of intestinal mucosa (Fig. 6A). The administration of 5-FU did not enhance TNF-α expression in Nox1KO mice. On the other hand, CD68-positive staining (macrophages) was broadly distributed in the lamina propria of intestinal mucosa in both WT and Nox1KO mice, and this was not affected by the administration of 5-FU. Moreover, a merged image showed that most TNF-α-positive staining in 5-FU-treated WT mice was colocalized with CD68-positive staining (Fig. 6B).

DISCUSSION

Our study demonstrated that NOX1 plays a critical role in the pathogenesis of 5-FU-induced intestinal mucositis. Repeated administration of 5-FU caused severe intestinal mucositis in WT mice, but to a significantly lesser degree than in Nox1KO mice. The hallmarks of the apoptotic response in the intestinal crypt induced by 5-FU were also significantly attenuated in Nox1KO mice. Nox1KO mice indeed displayed lower overexpression of TNF-α, and IL-1β, and lesser ROS production than did WT mice following treatment with 5-FU. This suggests that NOX1-derived ROS possibly promote apoptotic responses in the intestinal crypt via upregulation of inflammatory cytokines after administration of 5-FU. In the gastrointestinal tract, the role of NOX1 has been extensively studied in the colon (12, 17, 18, 23, 34, 37) but not in the small intestine. This is probably due to its much lower expression in the small intestine than colon (12). It is interesting therefore that NOX1
is upregulated and plays a pathogenic role in certain diseases in the small intestine.

Several studies have demonstrated that treatment with chemotherapeutic agents causes intestinal mucositis, characterized histologically by a shortening of intestinal villi and the disruption of intestinal crypts, along with clinical manifestations that include diarrhea and body weight loss (3, 41, 43). Although the pathogenesis of chemotherapy-induced intestinal mucositis is not yet fully understood, it is considered to be a consequence of various responses such as apoptosis and abnormal inflammation in addition to hypoproliferation (9, 10). Coant et al. (7) recently demonstrated that the number of S-phase proliferating cells was reduced and goblet cell proliferation was increased in the colon of Nox1KO mice. In the present study, however, we could not find a significant difference in control (5-FU-un-treated) cell proliferation of small intestinal crypts between WT and Nox1KO mice. Furthermore, the anti-proliferative effect of 5-FU was equally observed in both WT and Nox1KO mice. It is thus unlikely that the Nox1KO mouse may be preferentially resistant to anti-metabolite anti-cancer agents such as 5-FU.

Apoptosis has been shown to be a critical factor for intestinal mucositis induced by chemotherapeutic agents such as 5-FU (1, 16, 19, 36). In the present study, we observed a shortening of villi and a disruption of crypts in the small intestine 5 days after onset of 5-FU treatment. Furthermore, the marked increase in number of apoptotic cells was observed in the intestinal crypt 24 h after the first 5-FU administration (day 1) even though there were obvious histological changes. We therefore believe that 5-FU chemotherapy caused apoptosis of stem cells in the intestinal crypt, adversely affecting the cell metabolism leading to morphological changes such as intestinal villus atrophy and crypt hypoplasia. Interestingly, the increased number of apoptotic cells was significantly lower on day 3 following the onset of 5-FU treatment than on day 1. This observation is consistent with findings from a previous study by Inomata et al. (16) and suggests that the apoptosis triggered within 24 h after the first administration of 5-FU may be important in the occurrence of intestinal mucositis.

In addition to reduced villi shortening and crypt disruption in the small intestine, 5-FU-treated Nox1KO mice suffered considerably less severe diarrhea than did WT mice. Since the occurrence of diarrhea is considered to be closely linked to the severity of intestinal mucositis (41), these findings strongly support the involvement of NOX1 in the pathogenesis of 5-FU-induced intestinal mucositis. In contrast, the loss of body weight induced by 5-FU treatment was comparable in WT and Nox1KO mice. Oral mucositis, similar to intestinal mucositis, is a common side effect during the course of cancer chemotherapy (40) and may be a major cause of body weight loss.
Therefore, the loss of body weight is likely to result from other toxicities of 5-FU but not from the intestinal dysfunction that it caused. Our study also showed that the increase in the number of apoptotic cells induced by 5-FU was significantly reduced in Nox1KO mice compared with WT mice. The production of ROS was markedly enhanced by the upregulation of NOX1 as early as 12 h after the first administration of 5-FU. Taken together, these findings suggest that the augmentation of NOX1-derived ROS production induced by 5-FU is involved in intestinal crypt cell apoptosis followed by the morphological abnormality and dysfunction of the small intestine.

NOX2, the phagocytic form of NOX (37), is believed to be responsible for the pathogenesis of various diseases. Indeed, Paik et al. (33) recently reported upregulation of both NOX1 and NOX2, with both performing important roles in the pathogenesis of hepatic fibrosis induced by bile duct ligation. It is therefore possible that other types of NOX homologs may also be associated with 5-FU-induced intestinal mucositis. However, given our observation that increased ROS production in response to 5-FU was almost totally prevented in Nox1KO mice, NOX1 is likely the predominant homolog in the ROS generation occurring in 5-FU-induced intestinal mucositis.

While the mechanisms of intestinal cell apoptosis induced by chemotherapeutic agents remain unclear, they may involve proinflammatory cytokines such as TNF-α and IL-1β (26, 29, 39). Caspase-3, the main downstream effector, cleaves the majority of cellular substrates in apoptotic cells (21, 35). Several studies showed that caspase-3 activity was increased in the process of intestinal apoptosis during chemotherapy, including 5-FU treatment (5, 25, 44). We observed a marked increase in immunopositive cells for cleaved caspase-3, the active form of caspase-3, which localized to the intestinal crypt in a manner consistent with the localization of apoptotic cells.
in WT mice. The activation of caspase-3 was significantly lower in Nox1KO than in WT mice. Interestingly, WT mice also displayed significant activation of caspase-8, an initiator caspase that promotes caspase-3 activation, in the intestinal crypt after treatment with 5-FU, while Nox1KO displayed no such increase. These findings suggest that under 5-FU treatment, NOX1-derived ROS causes apoptosis via activation of caspase-8, followed by that of caspase-3 in the intestinal crypt.

Caspase-8 is an essential component of the extrinsic cell death pathways initiated by the TNF-family members through recruitment of the death-inducing signaling complex via binding to the adaptor protein FADD (4, 31). We noted that administration of 5-FU caused upregulation of both TNF-α and IL-1β in the small intestine. Upregulation of TNF-α preceded that of IL-1β and correlated with the presence of apoptosis hallmarks, i.e., NOX1 expression and activation of caspase-3 and caspase-8. Recently, ROS have been recognized as important signaling molecules that modulate the transcription of various genes via activation of redox-sensitive protein kinases and transcription factors (6). Indeed, ROS have been shown to promote the upregulation of proinflammatory cytokines, such as TNF-α via activation of various transcriptional factors including NF-κB and p38 MAPK (30, 32, 39). Impellizzeri et al. (15) recently reported that NOX-derived ROS increased the expression of inflammatory cytokines, such as TNF-α, via activation of NF-κB and MAPKs. We therefore suggest that ROS derived from NOX1 in response to 5-FU may promote apoptotic response, including caspase-3 and caspase-8 activation via upregulation of proinflammatory cytokines, especially TNF-α. Further studies are required to confirm the molecular mechanism of NOX1/ROS-induced apoptotic responses via upregulation of inflammatory cytokines.
Kamizato et al. (17) showed that various inflammatory cytokines including TNF-α/H9251 upregulated the expression of NOX1 in human colonic epithelial cells and mouse colons. It is possible thus that the upregulation of these cytokines in response to NOX1-derived ROS may further enhance NOX1 expression. Several studies have demonstrated that in addition to caspase-3 activation, 5-FU-induced intestinal cell apoptosis was accompanied by an alteration in the expression of Bax and Bcl-2 (16, 44), but a preliminary experiment performed in our lab showed that 5-FU failed to affect the expression of Bax or Bcl-2 under these conditions (data not shown). The dose of 5-FU used in the present study (50 mg/kg ip) was lower than those used in previous studies (130 mg/kg iv and 200 mg/kg ip) (16, 44). It is therefore possible that higher 5-FU doses may induce apoptosis via other apoptosis-related factors in addition to those detected herein (TNF-α/caspase-8/caspase-3 pathways). Further investigations will therefore be required to elucidate this point.

Immunohistochemical studies showed that the expression of TNF-α protein following the administration of 5-FU in WT

Fig. 6. Fluorescence immunohistochemical study for TNF-α and CD68-positive macrophages in the small intestine of WT and Nox1KO mice with or without 5-FU treatment. Animals were given 5-FU (50 mg/kg ip) and were killed 24 h (1 day) later. The expression of TNF-α (red) and CD68-positive macrophages (green) was detected by fluorescence immunohistochemically using anti-TNF-α and CD68 antibodies (A, ×200; B, ×400). Fluorescence overlaps for TNF-α and CD68 (yellow) are indicated with arrows (B).

A

![Fluorescence immunohistochemical study](image_url)

B

![Fluorescence immunohistochemical study](image_url)
mice was located in the lamina propria of intestinal mucosa. In contrast, the expression of TNF-α was not detected in Nox1KO mice similarly to control (5-FU-untreated) WT mice. This result supports the assumption that TNF-α is upregulated in response to 5-FU not only at the mRNA, but also at the protein level. We further observed that the expression of TNF-α was mostly colocalized with CD68-positive cells (macrophages) located in lamina propria of intestinal mucosa. This finding suggests that NOX1-derived production of ROS induced by 5-FU causes the upregulation of TNF-α in macrophages located in the lamina propria of intestinal mucosa, consequently triggering apoptotic mechanisms, including the activation of caspase-3 and caspase-8 in the intestinal crypt. While several studies have demonstrated that NOX1 is expressed in intestinal epithelial cells (18, 42), expression of both NOX1 and NOX2 was recently observed in the murine monocyte/macrophage cell line RAW264.7 (11, 20). Interestingly, we also detected NOX1 mRNA expression in isolated peritoneal macrophages, and this expression was upregulated by lipopolysaccharide (data not shown), suggesting a possibility that NOX1 expressed in inflammatory cells may be involved in the pathogenesis of intestinal mucositis. Further studies are certainly needed to clarify the localization of NOX1 involved in the apoptotic response and intestinal mucositis.

Taken together, our results show the important role of NOX1 in the pathogenesis of 5-FU-induced intestinal mucositis. We suggest that NOX1-derived ROS production caused by 5-FU chemotherapy induces apoptosis in the intestinal crypt cell through activation of caspase-8 and caspase-3 via upregulation of inflammatory cytokines. NOX1 may therefore represent a novel and useful target for prevention of chemotherapy-induced intestinal mucositis.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES
26. Logan RM, Stringer AM, Bowen JM, Gibson RJ, Sonis ST, Keefe DM. Is the pathobiology of chemotherapy-induced alimentary tract mucositis...


