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ORIGINAL ARTICLE

Zonula Occludens-1 alterations and enhanced intestinal permeability in methotrexate-treated rats

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Abstract
Purpose The molecular mechanisms that underlie the methotrexate (MTX)-mediated disruption of intestinal barrier function have not been fully characterized. Epithelial barrier function is determined in large part by a multiprotein complex located at the most apical part of the lateral membrane, which is referred to as a tight junction (TJ). In the present study, we examined the alteration of zonula occludens-1 (ZO-1), which is a scaffolding protein that plays a pivotal role in the formation of TJs, to identify an additional molecular mechanism for epithelial barrier dysfunction.
Methods Male Wistar rats were administered MTX (15 mg kg^{-1}) orally once daily for 3–5 days. Intestinal mucosal permeability was determined using the in vitro everted intestinal sac technique. Mucosal inflammation was assessed by myeloperoxidase activity and production of reactive oxygen species. Altered expression, tyrosine phosphorylation, and localization of ZO-1 were evaluated by RT–PCR, Western blotting, immunoprecipitation, and immunohistochemistry.
Results A barrier function study revealed increased intestinal permeability in rats treated with MTX for 4 days, as indicated by enhanced fluorescein isothiocyanate-dextran flux. In addition, mucosal inflammation was linked to enhanced intestinal permeability. Quantitative analysis of ZO-1 expression showed the absence of significant differences in MTX-treated rats, whereas tyrosine dephosphorylation of ZO-1 was observed. Moreover, we also detected an obvious reduction of ZO-1 immunostaining along the apical membrane of intestinal villi.
Conclusions These results indicate that, in MTX-treated rats, ZO-1 alterations may contribute to disturbance of the TJ barrier, which leads to enhanced intestinal permeability.

Keywords Intestinal permeability · ZO-1 · Methotrexate · Tight junction · Reactive oxygen species

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Introduction

The intestinal mucosa is an essential barrier that separates host tissues from toxic macromolecules of bacterial, viral, and host origin that are present in the intestinal lumen. Disruption of its barrier function increases intestinal permeability to these toxic molecules, which possibly leads to intestinal inflammation, life-threatening sepsis, or multiple organ dysfunction [1, 2]. Methotrexate (MTX), which exerts anti-inflammatory and immunosuppressive effects, is widely used in clinical practice for the treatment of rheumatoid arthritis and for cancer chemotherapy, while its adverse effects, such as gastrointestinal injury and liver dysfunction, are recognized as the major factors limiting its use [3]. A five-phase model has been suggested to explain the pathophysiology of chemotherapy-induced intestinal mucosal injury: (1) initiation, (2) upregulation and message generation, (3) signaling and amplification, (4) ulceration, and (5) healing [4]. Each phase can be a therapeutic target for intestinal mucosal injury. Although prevention and treatment of MTX-induced intestinal mucosal injury based on biological mechanisms are required, the molecular mechanisms underlying the alteration of intestinal

epithelial cells in response to several inflammatory signaling stimuli and the damage to intestinal epithelial barrier function have not been completely described for the model currently available. Characterization of additional mechanisms involved in intestinal barrier dysfunction may lead to the establishment of the safe application of MTX in clinical practice.

The maintenance of intestinal barrier function is highly dependent on epithelial cell-to-cell adhesion, which is indispensable for intestinal architecture [5]. Tight junctions (TJs) are intercellular junctional complexes that act as a primary barrier to the diffusion of solutes through the paracellular pathway [6]. TJs are located at the most apical part of the lateral membranes of epithelial and endothelial cells and comprise various molecules, such as the transmembrane proteins occludin [7], claudins [8], and tricellulin [9], and the peripheral membrane proteins zonula occludens (ZOs).

Zonula Occludens, which contain three PSD95/Dlg/ZO-1 (PDZ) domains, act as scaffolding proteins and interact with many TJ proteins [10]. ZOs bind to the cytoplasmic tail of claudins, which determine the properties of the cell barrier [11], at their first PDZ domain and connect claudins to underlying actin filaments at their C-terminal region [12]. Thus, the localization of claudins in TJs is regulated by ZOs, which are thought to play an important role in the maintenance and formation of TJs. So far, although three ZO isoforms (ZO-1, ZO-2, and ZO-3 [13–15]) have been identified, functional analyses indicate that ZO-1 has its own specific function and is a particularly important molecule regarding the formation of TJs [16, 17]. Furthermore, the physiological significance of ZO-1 was recently demonstrated in knockout mice [18].

We reported previously on the increased intestinal permeability, reactive oxygen species (ROS) production, neutrophil infiltration, and downregulation of intracellular glutathione in the intestine of MTX-treated rats [19, 20]. In addition, we also reported that *N*-acetylcysteine (NAC), which is a thiol antioxidant, supplementation not only prevents ROS production and neutrophil infiltration, but also prevents increase of intestinal permeability in MTX-treated rats [20, 21]. However, the molecular mechanisms by which MTX induces intestinal barrier dysfunction remain undefined. A variety of inflammatory mediators, such as ROS and inflammatory cytokines, including TNF-alfa and IFN-gamma, disrupt the TJ barrier and alter the localization and phosphorylation status of ZO-1 [22–24]. Therefore, these MTX-mediated alterations of ZO-1 may be associated with disruption of the TJ barrier, leading to enhanced intestinal permeability. In the present study, we focused on the disturbance of the intestinal TJ barrier, especially on ZO-1 alterations, to clarify the mechanisms of MTX-induced intestinal barrier dysfunction.

Materials and methods

Chemicals

MTX was purchased from Wako Pure Chemical Industries (Osaka, Japan). Fluorescein isothiocyanate-dextran (average molecular weight, 4,400 Da; FD-4) was obtained from Sigma Chemical Company (St Louis, MO, USA). Protein A-Sepharose was purchased from Pierce Biotechnology (Rockford, IL, USA). Human myeloperoxidase (MPO) was obtained from Alexis Biochemical, Inc. (San Diego, CA, USA). Other chemicals and solvents were of analytical grade.

Antibodies

Rabbit polyclonal anti-ZO-1 antibody and horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG was from Vector Laboratories, Inc. (Burlingame, CA, USA). Rabbit polyclonal anti-phosphotyrosine (*p*-Tyr) antibody was obtained from Transduction Laboratories (San Diego, CA, USA). Mouse monoclonal anti-β-actin antibody was purchased from Sigma Chemical Company (St Louis, MO, USA).

Animals and drug treatment

Male Wistar rats (7–8 weeks old; Japan SLC Inc., Shizuoka, Japan) were used throughout the experiments. Food (MF diet, standard laboratory diet available commercially; Oriental Yeast Co., Ltd, Tokyo, Japan) and water were provided to animals ad libitum. MTX was administered to rats (15 mg kg^{-1}, dissolved in saline solution, p.o.) once daily for 3–5 days. For the studies described below, the rat intestine (20 cm from the end of the duodenum) was collected 24 h after the final MTX administration. All animal experiments were approved by the animal care committee of Chiba University and were performed using the criteria for humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Assessment of intestinal permeability

Intestinal mucosal permeability to FD-4 was determined using in vitro everted intestinal sac experiments, as described previously [19]. Briefly, the everted intestinal segments were placed in incubation medium (modified Krebs–Ringer bicarbonate-phosphate buffer, pH 7.4) containing FD-4 (1 μM) at 37°C. At designated times after the

onset of the experiments, the solution was taken from the serosal side to examine the permeation of FD-4. The fluorescence intensity of FD-4 in sample solutions and permeation clearance of FD-4 were determined as described previously [19].

Luminol-enhanced chemiluminescence study

Luminol-enhanced chemiluminescence was used to estimate the levels of ROS generation, as described previously [20], with a slight modification. Briefly, chemiluminescence was measured using a single photoelectron counting system (Tohoku Electronic Industries Co., Ltd, Sendai, Japan). The intestinal mucosa was homogenized in ice-cold oxygenated carbonate buffer (50 mM, pH 8.7) and the homogenate was then centrifuged at 20,600×*g* for 2 min. The supernatant was mixed with incubation mixture (5 mM glucose and 400 μM luminol in phosphate-buffered saline (PBS) solution) in test tubes and placed in the chemiluminescence spectrophotometer, where light production was measured for 5 min. Chemiluminescence intensity was expressed as counts/mg protein by subtracting the chemiluminescence of the incubation mixture alone.

MPO activity

To evaluate the association between increased intestinal permeability and inflammation, we examined tissue MPO activity using the *o*-dianisidine method [25], with a slight modification. Briefly, the obtained intestinal mucosa was homogenized in phosphate buffer (0.5% hexadecyltrimethylammonium bromide, 50 mM, pH 6.0). The homogenate was sonicated twice for 15 s and was then centrifuged at 20,600×*g* for 15 min at 4°C. The supernatant was added to phosphate buffer (0.5% hexadecyltrimethylammonium bromide, 0.4 mg ml^{-1} *o*-dianisidine dihydrochloride, 0.0005% H_2O_2, 50 mM, pH 6.0). Changes in absorbance at 450 nm were recorded for 6 min. A standard curve of MPO activity was determined using human leukocyte-derived MPO. MPO activity was expressed as MPO units/mg protein.

RNA extraction and semiquantitative RT–PCR

Total RNA from rat intestinal mucosa was isolated using the RNA-Solv Reagent according to the manufacturer's instructions (Omega Bio-tek, Inc., Doraville, GA, USA). Reverse transcription was performed using the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (Takarabio Co., Ltd, Shiga, Japan). PCR was carried out using KOD plus polymerase (TOYOBO Co., Ltd, Osaka, Japan). The primers used were: ZO-1-F (5′-ATTCAGTTCGCTCCCATGAC-3′) and ZO-1-R (5′-GCTGTGGAGACTGTGTGGAA-3′); and GAPDH-F (5′-GTTACCAGGGCTGCCTTCTC-3′) and GAPDH-R (5′-GGGTTTCCCGTTGATGACC-3′). Reactions were performed at 94°C for 2 min, followed by 26 cycles (ZO-1) or 23 cycles (GAPDH) of 94°C for 15 s, 59°C for 30 s, and 68°C for 1 min. The number of cycles and amount of template were optimized to fall within a linear amplification range. PCR products were separated on 8% polyacrylamide gels, followed by staining with SYBR Green I Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) and detection using LAS-1000 plus (Fuji Photo Film Co., Ltd, Tokyo, Japan).

Immunoprecipitation of phosphotyrosine

For immunoprecipitation, intestinal mucosa was homogenized in lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM *β*-glycerophosphate, 1 mM Na_3VO_4, 1 mM Na_2 EDTA, 1 mM EGTA, 5 μg ml^{-1} leupeptin, 2 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} pepstatin, 5 μg ml^{-1} aprotinin, 150 mM NaCl, and 20 mM Tris–HCl, pH 7.5). After 30 min incubation at 4°C, the lysate was sonicated three times for 15 s and centrifuged at 15,600×*g* for 30 min at 4°C. Protein concentration in the supernatant was determined using the Pierce bicinchoninic acid (BCA) Protein Assay Reagent kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol, and 5 mg of protein from each lysate was incubated with 3 μg of polyclonal anti-*p*-Tyr antibody overnight at 4°C with gentle rocking. The remaining lysate was directly immunoblotted for total ZO-1 and *β*-actin. Fifty microliters of protein A-Sepharose beads was then added to each lysate and incubated with rocking for 90 min at 4°C. The beads were then pelleted by centrifugation at 5,800×*g* and washed three times with 400 μl lysis buffer (not containing sodium deoxycholate and SDS). Washed beads were suspended in elution buffer (3% SDS, 10% glycerol, 5% *β*-mercaptoethanol, 8 M urea, 0.001% bromophenol blue, and 10 mM Tris–HCl, pH 6.5) and heated at 95°C for 5 min. Extracts were immunoblotted for ZO-1.

Immunoblotting

Tissue lysates or immunoprecipitates were separated using SDS–PAGE. Proteins were transferred onto a PVDF membrane (Immobilon P; Millipore Corp.). The membrane was blocked for 90 min in TTBS (0.05% (v/v) Tween 20, 150 mM NaCl, and 10 mM Tris–HCl, pH 8.0) containing 3% (w/v) bovine serum albumin (BSA) and was then incubated with primary antibodies overnight at 4°C, followed by incubation with the appropriate secondary antibodies for 90 min. The dilutions of the primary antibodies were as follows: ZO-1, 1:500; anti-*β*-actin, 1:10,000. Immunoreactive

bands were detected using enhanced chemiluminescence (ECL Plus, Amersham Bioscience Corp., Piscataway, NJ, USA), according to the manufacturer's instructions.

Immunohistochemistry

Cryosections (6 μm) of rat intestine were fixed in acetone for 10 min at room temperature. After hydration with PBS, the sections were incubated for 1 h at room temperature with the primary antibody (ZO-1, 1:50). The sections were then rinsed with PBS and incubated for 1 h at room temperature with FITC-conjugated rabbit IgG (1:100). The samples were mounted in VECTASHIELD with propidium iodide (Vector Laboratories, Burlingame, CA, USA), which was used to counterstain nuclei. Samples were examined using a confocal laser scanning microscope (LSM 510; Zeiss, Jena, Germany).

Statistical analyses

Statistical analyses were performed using one-way ANOVA followed by Bonferroni's test or Student's *t* test. Differences were considered significant at $P < 0.05$.

Results

Evaluation of barrier function in the small intestine of MTX-treated rats

FD-4 is a poorly absorbable compound that is widely used as a paracellular marker, although it permeates, to some extent, the intestine of untreated rats [21, 26]. A time-course analysis of FD-4 permeation through the intestinal mucosa was examined in rats given MTX (15 mg kg^{-1} day^{-1}) for 3–5 days (Fig. 1a). The intestinal permeability of FD-4 was significantly increased in rats given MTX for 4 days, and it was further exacerbated by 5-day treatment. Figure 1b shows the permeation clearance of FD-4. A significant increase in the permeation clearance of FD-4 was observed in rats treated with MTX for 4 and 5 days compared with MTX-untreated control rats (control, 0.130 ± 0.010; 4 days, 0.242 ± 0.039; 5 days, 0.297 ± 0.016 μl min^{-1} cm^{-1}). Although the underlying mechanism may be different in rats treated with MTX for a longer period, we chose the shorter 4-day treatment to cause intestinal barrier dysfunction via treatment with MTX in all further studies aimed at characterizing the mechanism of MTX-induced intestinal dysfunction.

ROS production and neutrophil infiltration in the small intestine of MTX-treated rats

To confirm the association between increased intestinal permeability and intestinal mucosal inflammation, ROS production and MPO activity were examined in the intestine of MTX-treated rats. Luminol-enhanced chemiluminescence from the intestinal mucosa was measured for 5 min (Fig. 2a). Figure 2b shows the total counts of chemiluminescence. MTX treatment significantly increased chemiluminescence compared with control rats (Fig. 2b). In addition, MTX treatment increased MPO activity in the rat intestine compared with control rats (Fig. 2c).

Effect of MTX treatment on the expression level and tyrosine phosphorylation of ZO-1 in the small intestine of rats

To better understand the molecular mechanisms underlying increased intestinal permeability after MTX treatment, we performed a quantitative analysis of the TJ protein ZO-1 in rat intestine using RT–PCR and Western blotting. As shown in Fig. 3, there were no apparent differences in ZO-1 mRNA or protein expression levels in MTX-treated versus control rats. Variation in ZO-1 tyrosine phosphorylation, which could lead to a disturbance of the TJ barrier, was also examined. Immunoprecipitation analysis showed that MTX treatment significantly decreased the level of tyrosine phosphorylation of ZO-1 in rat intestine ($66.9 \pm 4.6\%$ of the control) (Fig. 3c).

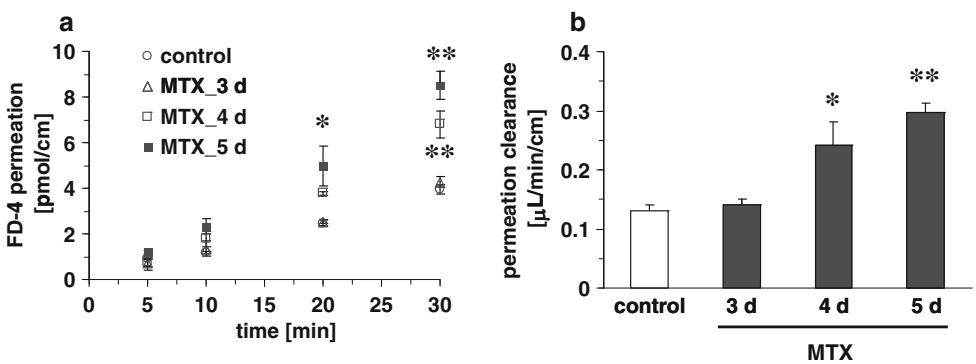

Fig. 1 Effect of MTX treatment on intestinal permeability in rats. **a** Time-course analyses of FD-4 permeation and **b** permeation clearance of FD-4 were performed using in vitro everted sac experiments, as described in the "Materials and methods" section. Data represent the means ± SEM ($n = 4$). $^*P < 0.05$ and $^{**}P < 0.01$, significantly different from control rats

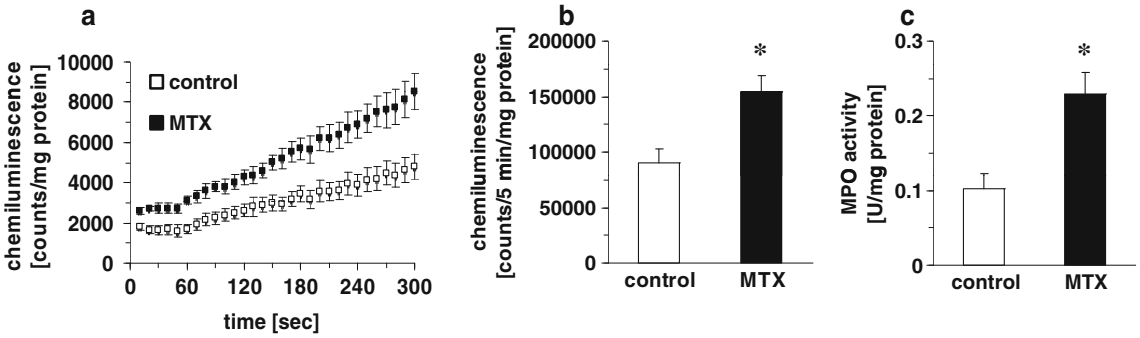

Fig. 2 MTX-induced increase in ROS production (**a**, **b**) and MPO activity (**c**) in the rat intestine. **a**, **b** Luminol-enhanced chemiluminescence and **c** MPO activity were measured in the intestinal mucosa as described in the "Materials and methods" section. Data represent the means ± SEM ($n = 4$). *$P < 0.05$, significantly different from control rats

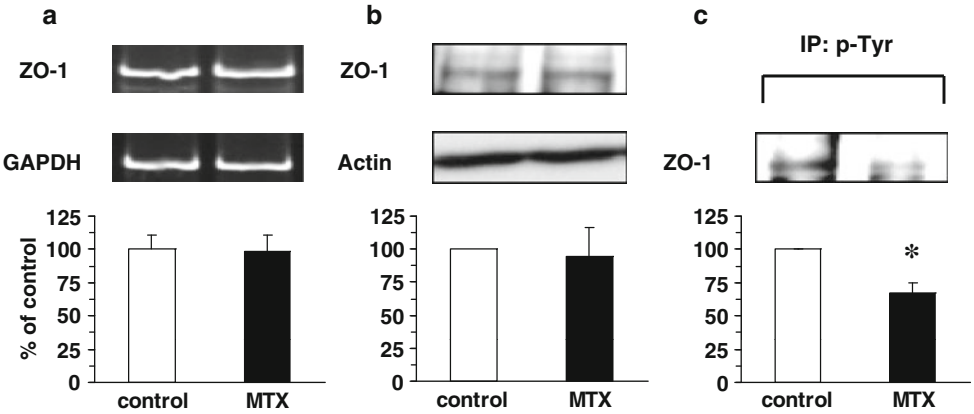

Fig. 3 Effect of MTX on the expression levels and tyrosine phosphorylation of ZO-1 in the rat intestine. The expression levels of intestinal mucosa-derived ZO-1 were detected using RT–PCR (**a**) and Western blot analysis (**b**). **a** Quantization of ZO-1 mRNA-specific signals is shown as the percentage of expression compared with control rats after normalization to GAPDH expression. **b** Tissue extracts were directly immunoblotted for total ZO-1 and β-actin. **c** Protein extracts were subjected to immunoprecipitation (under denaturing conditions) of p-Tyr, followed by immunoblotting for ZO-1. Data represent the means ± SD ($n = 3$). *$P < 0.05$, significantly different from control rats

Immunohistochemical localization of ZO-1 in the small intestine

We then evaluated the localization of ZO-1 in frozen sections of rat intestine. Immunohistochemical analysis of control rat tissue revealed that ZO-1 was predominantly localized along the apical membrane of intestinal villi (Fig. 4a). In contrast, MTX-treated rats exhibited a significant decrease in ZO-1 immunostaining along the apical membrane of intestinal villi (Fig. 4b). Finally, the mucosal architecture appeared to be altered in MTX-treated rats compared with control animals.

Discussion

To identify an additional molecular mechanism for MTX-induced intestinal barrier dysfunction, here we investigated various alterations of ZO-1 in the intestine of rats treated with MTX. Oral administration of MTX to rats resulted in a time-dependent increase in intestinal permeability, as indicated by enhanced FD-4 flux, which suggests that MTX disturbed intestinal barrier function. Although an increase in intestinal permeability was observed at 48 h after a single intravenous injection of MTX [21], our present data showed an increase in intestinal permeability after 4 days of oral MTX treatment. This different kinetics may be explained by the difference of administration route, i.e., intravenous versus oral. In addition, the rate of intestinal epithelial renewal is considered as an important determinant of the kinetics of intestinal mucosal injury during chemotherapy [4]. Our results were partly consistent with this idea, as the turnover rate of intestinal epithelia is reportedly about 24–96 h in mammals [27].

We reported previously that neutrophil infiltration and increase in permeability were observed in the intestine of MTX-treated rats after ROS production [20, 21]. These alterations were significantly prevented by NAC supplementation, which suggests that MTX-induced ROS production is an important initiation factor that leads to neutrophil

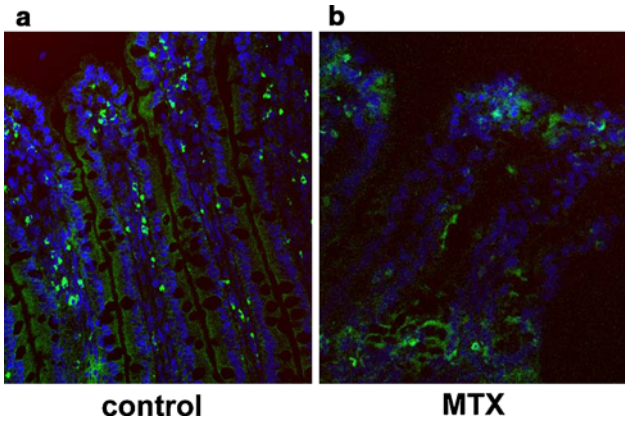

Fig. 4 Immunohistochemical localization of ZO-1 in the rat intestine. ZO-1 localization in villus epithelia of the rat jejunum was evaluated using immunohistochemical analysis. Cryosections (6 μm) of rat intestine were stained with the primary antibody for ZO-1, followed by incubation with a secondary antibody conjugated with FITC. ZO-1 and nuclei are shown in *green* and *blue*, respectively. Immunohistochemical analysis revealed that ZO-1 was predominantly localized along the apical membrane of intestinal villi in control rats (**a**). In contrast, a significant reduction in ZO-1 immunostaining was observed along the apical membrane of intestinal villi in MTX-treated rats (**b**)

migration and intestinal barrier dysfunction. In the present study, the intestinal mucosa of MTX-treated rats also exhibited an increase in MPO activity and chemiluminescence, which indicates that inflammation and neutrophil infiltration, as well as ROS production, are linked to increased intestinal permeability. Allopurinol has been used to demonstrate the ROS-dependent expression of the macrophage inflammatory protein-2 (MIP-2) and of the cytokine-induced neutrophil chemoattractant (KC), which are CXC chemokines that are major neutrophil chemoattractants, in an ischemia/reperfusion model using the intestine [28]. Moreover, inhibition of MIP-2 and KC by specific antibodies reduced neutrophil adhesion and rolling in the intestine. Our data were partly consistent with these findings. ROS-dependent intestinal injury seems to be mediated by neutrophil infiltration, which is an important step in the initiation of inflammatory responses. Increased inflammatory cytokines, such as TNF-alfa and IL-1beta, have been suggested to be involved in MTX-induced intestinal mucositis [29, 30], although the mechanism underlying signaling initiation remains unclear. Taking these findings into consideration, the increased intestinal permeability observed in MTX-treated rats may be induced mainly by inflammatory mediators, which are mediated by ROS-dependent neutrophil infiltration. However, the possibility that ROS caused intestinal barrier dysfunction directly was not excluded.

Increased intestinal permeability to FD-4 reflects the disturbance of the intestinal TJ barrier. To better understand its molecular mechanisms, we examined the expression levels and tyrosine phosphorylation status of ZO-1 in the intestine of MTX-treated rats. Quantitative analysis of ZO-1 using RT–PCR and Western blot analysis revealed the absence of significant differences between MTX-treated and untreated rats, whereas immunoprecipitation analysis showed that MTX treatment significantly decreased ZO-1 tyrosine phosphorylation. Although the effect of tyrosine phosphorylation of ZO-1 on the TJ barrier is not fully defined, there are several evidences for the association between changes in tyrosine phosphorylation of ZO-1 and TJ barrier alterations [31]. Interestingly, the role of tyrosine phosphorylation of ZO-1 in the TJ barrier seems to be cell type dependent; for example, oxidative stress increases paracellular permeability in polarized Caco-2 cells, in which tyrosine phosphorylation of ZO-1 is increased [22], which is in contrast with our results. In addition, the tyrosine residues of ZO-1 are phosphorylated during the assembly of TJs in Ras-transformed MDCK cells and subconfluent A431 cells [31, 32]. In contrast, tyrosine phosphorylation of ZO-1 is decreased in the disrupted intestinal TJ barrier in mice [33] and humans [34]. Thus, although it is unclear why tyrosine phosphorylation of ZO-1 exerts different effects on the TJ barrier, several evidences indicate that changes in ZO-1 tyrosine phosphorylation are associated with the processes of TJ assembly and disruption. Interestingly, and consistently with previous physiological studies, our results demonstrated that tyrosine phosphorylation of ZO-1 was decreased in animals that exhibited intestinal barrier dysfunction. Disruption of TJ integrity and ZO-1 localization in TJs were recently reported in Caco-2 cells after inhibition of nPKC [35]. However, unlike what was observed for oxidative stress, a significant change in tyrosine phosphorylation of ZO-1 was not observed. The various effects of tyrosine phosphorylation of ZO-1 on the TJ barrier may be partly explained by the different kinases that interact with TJ components.

Given the observed association between increased intestinal permeability and tyrosine dephosphorylation of ZO-1, we then examined the effect of this post-translational modification on the physiological TJ barrier in frozen sections of rat intestine. In control rats, ZO-1 was predominantly detected along the apical surface of intestinal villi. In contrast, an obvious decrease in ZO-1 immunostaining was observed along the apical surface of intestinal villi in MTX-treated rats, which indicates that the localization of ZO-1 to TJs was disturbed. TJ barrier function is reported as mainly determined by polymerization of claudins, which are TJ integral membrane proteins [36, 37]. Importantly, their polymerization requires direct interaction with ZOs [17]. Therefore, increased intestinal permeability may be caused by ZO-1 alterations in MTX-treated rats, at least partly, as the disturbance of ZO-1 localization to TJs probably prevents its interaction with claudins. Recently, it was reported that immunohistochemical alteration of ZO-1 is closely

associated with increased intestinal permeability in patients with nonalcoholic fatty liver diseases [38]. Thus, the role of ZO-1 in the function of the intestinal TJ barrier appears to be important.

In the present study, we demonstrated that MTX treatment caused ZO-1 tyrosine dephosphorylation and altered the localization of ZO-1 in the small intestine of rats. These molecular events observed in the small intestine of MTX-treated rats may contribute to the disturbance of the TJ barrier and lead to enhanced intestinal permeability.

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