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Evaluation of damaged small intestine of mouse following methotrexate administration

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Abstract *Purpose*: Methotrexate (MTX) treatment causes damage to the small intestine, resulting in malabsorption and diarrhea. The active and passive transport capacities of the small intestine are decreased by the treatment. The purpose of this study was to evaluate the damage to the small intestine of mice caused by MTX administration by examining the permeability of the paracellular pathway of the small intestinal epithelium. *Methods*: MTX was administered orally to male ddY mice once daily for 1–6 days. The permeability of the small intestine to the nonabsorbable markers phenol red (PR) and fluorescein isothiocyanate (FITC) dextrans was examined using everted segments of the intestine. *Results*: PR and FITC dextran permeation through the small intestine increased significantly in parallel with changes in body weight of the mice, wet weight of the small intestine and chemical composition of the small intestinal epithelium. *Conclusions*: In addition to changes in permeation through the transcellular pathway reported previously, this study revealed that MTX treatment disorders the paracellular barrier function of the small intestinal epithelium, resulting in increased permeation of nonabsorbable markers via the paracellular pathway of the small intestinal mucosa. The present approach to the examination of the barrier function of the intestinal epithelium could be of great use in evaluating the damage to the small intestine and malabsorption.

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Introduction

Methotrexate (MTX) has been used extensively for the treatment of patients with osteosarcoma, and high-dose MTX therapy has been tried in recent years [1, 2]. Some side effects such as nausea, vomiting, diarrhea, stomatitis, gastrointestinal ulceration and mucositis are known often to accompany MTX chemotherapy [3, 4]. The antimitotic effect of MTX gives rise to the malabsorption syndrome which is manifested as poor nutrient absorption and diarrhea [5, 6]. The absorption of certain drugs by the small intestine is decreased as a result of MTX-induced damage to the small intestine [7–9]. MTX also inhibits the metabolic activity and active transport capacity of the intestinal mucosa [6, 10]. Damaged and shortened microvilli of the small intestine are observed following MTX treatment, resulting in a decreased surface area [5]. MTX treatment also decreases the amounts of proteins and lipids in the small intestinal mucosa and changes the physical structure of the brush border membranes [11, 12]. The chemical and morphological changes in the small intestine, which may possibly be triggered by damage to the crypt cells [5, 6, 13, 14], are considered to lead to a decrease in the intestinal absorption of nutrients and drugs. We have reported that the in vitro and in vivo absorption of glucose and amino acids by the small intestine is decreased in MTX-treated rats [15, 16].

Two major transport routes are thought to be active in the intestinal absorption of nutrients and drugs [17]. One is the transcellular pathway and the other is the paracellular pathway. Intestinal absorption in MTX-induced malabsorption has so far been studied in terms of absorption through the transcellular pathway, since nutrients are mostly absorbed via this pathway, and absorption through the paracellular pathway has been little studied. In this study, we investigated the effect of

MTX on intestinal absorption through the paracellular pathway by examining the permeation of the non absorbable markers phenol red (PR) and dextrans in everted segments of small intestine of mice treated orally with MTX.

Methods

Drugs and chemicals

MTX, fluorescein isothiocyanate (FITC) dextrans with average molecular weights of 4400 (FD-4K), 19 600 (FD-19K), 73 100 (FD-73K) and 148 300 (FD-148K) were purchased from Wako Pure Chemical Industry (Osaka, Japan). PR was from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were of analytical grade.

Animals

Male ddY mice at 8–10 weeks of age and 28–35 g body weight (Japan SLC, Shizuoka, Japan) were used. The mice were given food and water ad libitum. MTX dissolved in saline solution (15 mg/kg or 20 mg/kg; 5 ml/kg) or saline solution alone (5 ml/kg) as a control was administered orally to the mice once daily for 1–6 days.

Preparation of homogenates of small intestine

Small intestinal segments (10 cm) from the proximal end of the jejunum of treated mice were isolated. The lumen was washed with cold Tris-HCl buffer (0.1 m*M*, pH 8.0), the segment cut open and the adhering water blotted. The mucosa was scratched from the lumen with a slide glass and suspended in 0.1 m*M* Tris-HCl buffer (pH 8.0). The mixture was homogenized with a Teflon homogenizer and the lipid and protein contents of the homogenates were measured.

Determination of lipids

Lipids were extracted from the homogenates according to the method of Folch et al. [18] with slight modification. The homogenates were added to an organic solvent mixture (chloroform/methanol, 2:1 v/v) and homogenized with a Teflon homogenizer for 3 min. The mixture was then vigorously shaken for 3 min and centrifuged at 3000 *g* for 10 min. The chloroform phase was recovered and further chloroform/methanol solvent mixture was added to the residue. This procedure was repeated and the chloroform phase collected. The chloroform phase was washed with a quarter volume of 0.88% KCl solution and then evaporated completely to dryness in vacuo. The total lipids thus obtained from the homogenates were weighed.

Determination of proteins

The protein contents of the homogenates of the small intestine were determined by the method of Lowry et al. [19], using bovine serum albumin as standard.

Intestinal absorption experiment

The intestinal absorption was studied in vitro using everted segments of small intestine as described previously [16]. The treated mice were fasted overnight before experimental use. The mice were anesthetized with ethylether and the intestines were excised. Intestinal segments (12 cm) were cut off at a distance of 3 cm from the end of the duodenum. The segments were everted in saline solution on ice. An L-shaped glass cannula was inserted into each end (1 cm) of the everted segments and a 10-ml plastic syringe was attached to the exposed end of each cannula following the procedure described by Doluisio et al. [20]. The segments were then placed in 40 ml 0.05 *M* phosphate buffer 0.9% saline solution (pH 6.5) containing various concentrations of PR or FITC dextrans. Buffer solution (5 ml) was applied to the serosal side of the segments via the syringe. The plungers were gently moved up and down and the absorption experiments were started after incubating the intestinal segments for 7 min at 37 °C. Gas (95% O_2 5% CO_2) was gently bubbled through the mucosal side solution during the absorption experiments. At designated times after the start of the experiments, 0.3 ml of the solution was taken from the serosal side for determination of absorbed marker substance and at the same time, the same volume of buffer solution was added to maintain the volume. The permeation of marker substance through the segments was determined from its amount in the serosal solution. Further, 0.1 ml of the solution was taken from the mucosal solution to determine the concentration of marker substance in the mucosal solution.

Determination of FITC dextran

The sample solutions taken from the serosal and the mucosal sides were diluted with 0.05 *M* phosphate buffer 0.9% saline solution (pH 6.5) and the fluorescence intensity of FITC dextran in the sample solutions was determined at an excitation wavelength 495 nm and an emission wavelength 515 nm using a Hitachi fluorescence spectrophotometer F-2000.

Determination of PR

The concentration of PR in the sample solution was determined spectrophotometrically using a Hitachi 557 spectrophotometer at two wavelengths, 610 nm and 560 nm, immediately after alkalization by adding 1 *N* sodium hydroxide solution.

Data analysis

Absorption clearance was obtained as follows [21]:

$$\text{Absorption clearance} = \frac{\text{Absorption rate}}{\text{Marker substance concentration on mucosal side}}$$

$$\text{Absorption rate} = \frac{X_{t_2} - X_{t_1}}{t_2 - t_1}$$

where X_{t_1} and X_{t_2} are the amounts of marker substance transported to the serosal side for incubation times t_1 and t_2, respectively.

Statistical analysis was performed using Student's *t*-test. Results were considered significant at $P < 0.05$.

Results

Change in body weight of MTX-treated mice

MTX or saline solution was administered orally to mice once daily for 6 days and their body weights were measured daily. The body weight of MTX-treated mice was almost the same as that of the control mice for the first 3 days and then began to decrease significantly (Fig. 1).

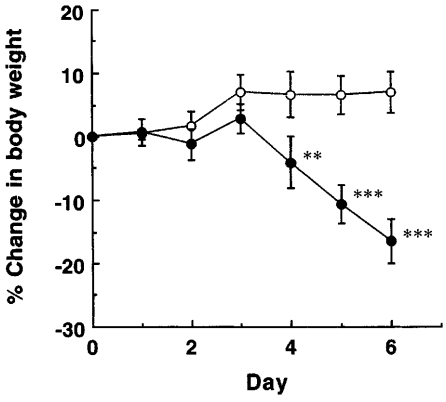

Fig. 1 Changes in mouse body weight during MTX treatment. MTX (20 mg/kg) or saline solution was administered orally to the mice once daily for 6 days. The data are expressed as the change in body weight on the designated days after starting MTX or saline solution administration in relation to the body weight at the start of treatment (○ control mice; ● MTX-treated mice). The values are means ± SD for four mice. $^{**}P < 0.01$, $^{***}P < 0.001$, vs control mice

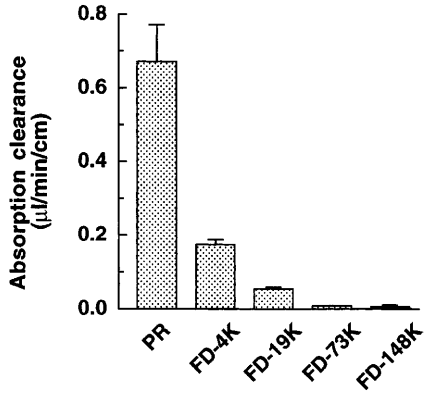

Fig. 3 PR and FITC dextran permeation through the small intestine of untreated mice. The permeation of the nonabsorbable markers indicated through the small intestine from the mucosal to the serosal side was examined using everted segments of small intestine. The results are expressed as the absorption clearance per centimeter of small intestine as described in Materials and methods. The values are the means ± SD for three or four mice

Change in wet weight and composition of the small intestine of MTX-treated mice

The wet weight of the small intestine of MTX-treated mice was significantly less than that of control mice (Fig. 2A). The protein content of the small intestine of MTX-treated mice was significantly, less than that of control mice (Fig. 2B). The lipid content of the small intestine showed the same trend, although it was not significant statistically (Fig. 2C).

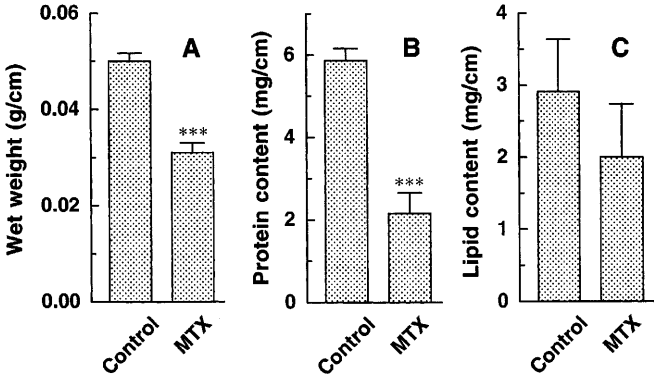

Fig. 2A–C Changes in weight and composition of mouse small intestine following MTX treatment. MTX (20 mg/kg) or saline solution was administered orally to mice once daily for 6 days (**A** wet weight of the small intestine; **B** total protein content of the homogenate of the small intestinal mucosa; **C** total lipid content of the homogenate of the small intestinal mucosa). The values are the means ± SD for three or four mice. $^{***}P < 0.001$, vs control mice

Permeation of nonabsorbable markers through the small intestine of mice

The permeation of the nonabsorbable markers, PR, FD-4K, FD-19K, FD-73K and FD-148K, through the small intestine of untreated mice was investigated using everted segments of small intestine. As shown by the low absorption clearance values in Fig. 3, the permeation of the markers through the small intestine was very low. The absorption clearance values of markers decreased with increase in molecular weight. The absorption clearances of FD-73 K and FD-148 K with higher molecular weights were much less than those of the other markers.

Effect of MTX treatment on the permeation through the small intestine of mice

The permeation of the nonabsorbable marker, PR, through the small intestine of mice treated with MTX (15 mg/kg per day) for 2 or 4 days was examined using everted segments of small intestine (Fig. 4). PR absorption clearance for the mice treated with MTX for 2 days was almost the same as that for untreated mice. PR absorption clearance for the mice treated with MTX for 4 days was significantly greater than that of the untreated mice.

Permeation of PR and FD-4K through the small intestine of mice treated with MTX

MTX (20 mg/kg per day) or saline solution was administered orally to mice for 6 days. The permeation of PR and FD-4K through the small intestine of the treated mice was examined using everted segments. PR

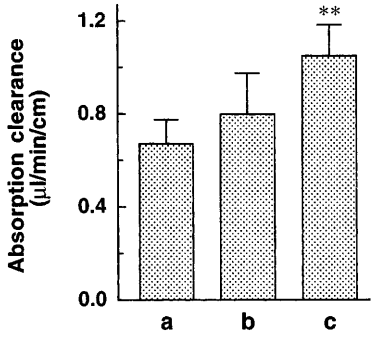

Fig. 4 Change in the small intestinal permeability of mice as a result of MTX treatment. Mice were treated orally with MTX (15 mg/kg) once daily for 2 or 4 days. The permeation of PR through the small intestine from the mucosal to the serosal side was examined using everted segments of small intestine (*a* untreated mice, *b* mice treated for 2 days, c mice treated for 4 days). The results are expressed as the absorption clearance per centimeter of small intestine as described in Materials and methods. The values are the means ± SD for four mice. $^{**}P < 0.01$, vs control (untreated) mice

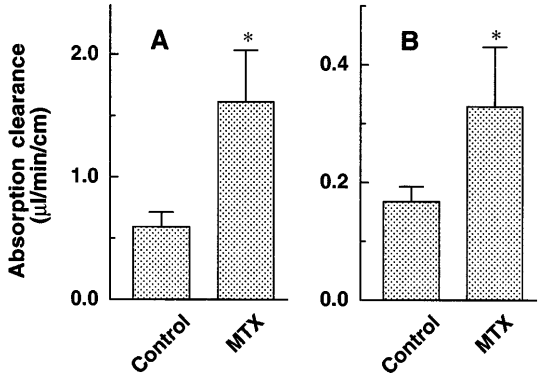

Fig. 5A,B Permeability of the small intestine of mice following MTX treatment. MTX (20 mg/kg) or saline solution as a control was administered orally to mice once daily for 6 days. The permeation of PR (**A**) and FD-4K (**B**) through the small intestine from the mucosal to the serosal side was examined using everted segments of small intestine. The results are expressed as the absorption clearance per centimeter of small intestine as described in Materials and methods. The values are the means ± SD for three or four mice. $^{*}P < 0.05$, vs control mice

absorption clearance was significantly greater than that of the control (saline-treated) mice (Fig. 5A). FD-4K absorption clearance was also significantly greater than that of the control mice (Fig. 5B).

Discussion

It is well known that some antitumor drugs decrease intestinal absorption. The absorption of D-glucose and other nutrients by the small intestine is decreased in 5-fluorouracil- and mitomycin C-treated rats and hamsters [10, 22, 23]. The absorption of D-glucose by the small intestine is also decreased by MTX-induced intestinal toxicity [6, 15, 16, 24]. The treatment of rats with MTX depresses not only the active transport capacity of the intestinal mucosa [6, 10], but also the passive transport of some acidic, basic and neutral drugs and mannitol [7–9].

PR and FITC dextrans are generally used as nonabsorbable markers in in vitro, in situ and in vivo absorption experiments. These nonabsorbable markers permeated the small intestine of untreated mice in the in vitro everted sac experiment, although the permeation was slight (Fig. 3). This suggests that these nonabsorbable markers can be used to assess MTX-induced damage to the small intestine, since they may permeate the damaged intestine more easily because of the disordered barrier function of the intestinal epithelium.

We have reported that MTX treatment of rats for 3 or 4 days decreases D-glucose permeation through the small intestine, although treatment for 1 or 2 days does not affect the permeation [16]. As shown in Fig. 4, PR permeation through the small intestine of the mice treated with MTX for 2 days was at the same level as that for the untreated mice, but was increased significantly in the mice treated with MTX for 4 days. This time-dependent change in the permeability of the small intestine following MTX treatment paralleled the change in body weight of the mice (Fig. 1). It should be noted that the time-dependence of the changes in intestinal absorption was consistent with the turnover rate of the small intestinal epithelial cells. Proliferation of small intestinal epithelial cells occurs in the crypt. The crypt cells, which rapidly generate, migrate to the villus tip, and replacement of the intestinal epithelium is complete in about 3 days in humans [25] and mice [26], and in about 2 days in rats [27].

The body weight of the mice treated with MTX for 6 days decreased markedly (Fig. 1). Simultaneously, the wet weight of the small intestine and the contents of proteins and lipids in the small intestinal mucosa were found to decrease, indicating that the small intestine was damaged (Fig. 2). In parallel with these changes, the permeability of the small intestine of the treated mice was affected. PR absorption clearance for the mice treated for 6 days was about three times larger than that for the control mice (Fig. 5A). FD-4K absorption clearance for the treated mice was about double that for the control mice (Fig. 5B). Thus, it was clearly shown that the permeation of nonabsorbable markers through the small intestine of mice was enhanced by MTX treatment. This is contrary to previously reported results on MTX-induced malabsorption indicating that MTX treatment decreases active and passive transport in the small intestine [6–10, 16, 24]. MTX treatment damages the small intestine morphologically, and this is accompanied by a decrease in the constituents of the small intestinal mucosa [12, 28]. This suggests that MTX treatment affects not only the transcellular pathway but also the paracellular pathway. As already mentioned, the absorption through the transcellular pathway including both active and passive transports is depressed by MTX treatment. The present results showed that the

permeation of nonabsorbable markers through the small intestine is promoted by MTX treatment, suggesting that the paracellular barrier function is disordered, resulting in enhanced permeation through the paracellular pathway.

In conclusion, the present approach for investigating the absorption of nonabsorbable markers was shown to be capable of assessing the barrier function of the small intestinal epithelium. The method should be of great use for the evaluation of MTX-induced damage to the small intestine and malabsorption and for developing the methods for protection against them.

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