Sodium Alginate Inhibits Methotrexate-Induced Gastrointestinal Mucositis in Rats

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Gastrointestinal mucositis is one of the most prevalent side effects of chemotherapy. Methotrexate is a pro-oxidant compound that depletes dihydrofolate pools and is widely used in the treatment of leukemia and other malignancies. Through its effects on normal tissues with high rates of proliferation, methotrexate treatment leads to gastrointestinal mucositis. In rats, methotrexate-induced gastrointestinal mucositis is histologically characterized by crypt loss, callus fusion and atrophy, capillary dilatation, and infiltration of mixed inflammatory cells. The water-soluble dietary fiber sodium alginate (AL-Na) is derived from seaweed and has demonstrated muco-protective and hemostatic effects on upper gastrointestinal ulcers. In the present study, we evaluated the effects of AL-Na on methotrexate-induced small intestinal mucositis in rats. Animals were subcutaneously administered methotrexate at a dosage of 2.5 mg/kg once daily for 3 d. Rats were treated with single oral doses of AL-Na 30 min before and 6 h after methotrexate administration. On the 4th day, small intestines were removed and weighed. Subsequently, tissues were stained with hematoxylin–eosin and bromodeoxyuridine. AL-Na significantly prevented methotrexate-induced small intestinal mucositis. Moreover, AL-Na prevented decreases in red blood cell numbers, hemoglobin levels, and hematocrit levels. These results suggest the potential of AL-Na as a therapy for methotrexate-induced small intestinal mucositis.

Key words sodium alginate; methotrexate; gastrointestinal mucositis; cell proliferation; hemoglobin

Sodium alginate (AL-Na) is a soluble dietary fiber comprising β-d-mannuronic and α-L-guluronic acids. AL-Na is widely used as a gastric coating agent for the treatment of gastric ulcers and esophageal reflux. In addition, AL-Na relieves lower gastrointestinal tract inflammation in experimental ulcerative colitis models and in radiation-induced colitis injury. Pharmacokinetics of AL-Na are characterized by poor absorption and excretion from the gastrointestinal tract. Considering these data, we hypothesized that AL-Na may affect the entire gastrointestinal tract.

Cytotoxic cancer chemotherapy and radiotherapy frequently cause gastrointestinal mucositis. This gastrointestinal toxicity is increasingly recognized as a consequence of all cancer chemotherapeutic and radiotherapy regimens. Methotrexate, a structural analogue of folic acid, is widely used in the treatment of leukemia and other malignancies. Methotrexate is often limited by severe side effects such as intestinal injury. Methotrexate-induced gastrointestinal mucositis results in malabsorption syndrome, leading to poor absorption of nutrients and diarrhea. In rats, methotrexate-induced gastrointestinal mucositis is characterized histologically by crypt loss, callus fusion and atrophy, capillary dilatation, and infiltration of mixed inflammatory cells.

These side effects necessitate dose reduction and discontinuation of selected cancer therapies, and increase healthcare costs, prolong hospital stays, increase re-admission rates, compromise patient nutritional status, impair patient quality of life, and are occasionally fatal. Thus, therapeutic agents that ameliorate chemotherapy-induced gastrointestinal mucositis are urgently required. In the present study, we examined the effects of AL-Na on methotrexate-induced gastrointestinal mucositis in rats.

MATERIALS AND METHODS

Animals Six-week-old male Sprague-Dawley rats (body weight, 160–200 g) were purchased from Japan SLC, Shizuoka, Japan. Animals were maintained in an air-conditioned room with controlled temperature (24±2°C) and humidity (55±15%) and were housed in steel cages with a 12h light/dark cycle (lights on from 0700 to 1900h) and freely available food and water. All procedures were approved by the Animal Care Committee of Sakai-Chemical Industry of Pharmaceutical Research Laboratories (Osaka, Japan).

Induction of Gastrointestinal Mucositis Methotrexate (Wako, Japan) was dissolved in distilled saline before administration. Methotrexate was administered to animals subcutaneously at a dose of 2.5 mg/kg once daily for 3 d. On the 4th day, rats were sacrificed under deep ether anesthesia, and stomachs, small intestines, and large intestines were removed and weighed. Blood samples were taken before sacrifice and blood cell numbers were estimated using an automated blood cell counter (KK-21NV; Sysmex, Japan). Body weight and food consumption were monitored every day. AL-Na was obtained from Kyosei Pharmaceutical (Japan), and was dissolved in distilled water and orally administered at a dose of 500 mg/kg to rats 30 min before and 6 h after methotrexate treatment.

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Control animals received distilled water instead of AL-Na.

**Evaluation of Morphological Changes** Rats were sacrificed, and both jejunal and ileal tissues were fixed immediately in 10% neutral buffered formalin. After fixing, materials were dehydrated with ethanol, cleared in xylene, and embedded in paraffin. From these specimens, 3-μm paraffin sections were prepared for staining with hematoxylin–eosin (HE). Villus heights and crypt depths were measured at three tissue levels in each animal, and the mean was calculated for each animal.

**Evaluation of Cell Proliferation** Bromodeoxyuridine (BrdU; Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) labeling was used as an index of cell proliferation in intestinal tissues. BrdU at a dose of 50 mg/kg was administered intraperitoneally 1 h before decapitation. Rats were sacrificed, and both jejunal and ileal tissues were removed. Three-micrometers paraffin sections of the tissues were prepared in the same way mentioned above. Immunostaining of BrdU was performed on 3-μm paraffin sections of the tissues. Deparaffinized sections were incubated for 20 min in ice-cold methanol containing 0.3% (w/v) H₂O₂ to quench endogenous peroxidase activity. Sections were then washed and treated with 1M HCl for 8 min at 60°C to partially denature double strand DNA. After blocking with 10% normal rabbit serum for 40 min in Tris-buffered saline (TBS), sections were incubated at room temperature for 1.5 h with a mouse anti-BrdU monoclonal immunoglobulin G (IgG) antibody (1:100 dilution; Dako, Denmark). Labeling was visualized using a rabbit anti-mouse biotinylated IgG, an avidin/biotinylated horseradish peroxidase reagent (Dako), and dimethylaminoazobenzene (DAB) substrate (Sigma). BrdU positive cells were counted in 5 well-oriented full crypts from each animal using a light microscope, and the mean was calculated for each animal.

**Statistical Analysis** All data are presented as the mean ± standard error of the mean (S.E.M.). Statistical analyses were performed using one-way analysis of variance (ANOVA) with Dunnett’s test, or Student’s unpaired t-test. Differences were considered significant when probability \( p \) values were less than 0.05.
RESULTS

The Effects of AL-Na on Body Weight and Food Intake

Methotrexate treatments caused loss of body weight, which became significant by the 4th day, but not in animals treated with AL-Na (Fig. 1A). In addition, compared with untreated

Fig. 3. Effect of AL-Na on Morphological Change
Small intestinal tissues were stained by HE. Jejunal tissues were obtained from normal rat (A), methotrexate treated rat (B) and methotrexate treated with AL-Na rat (C). Ileal tissues were obtained from normal rat (D), methotrexate treated rat (E) and methotrexate treated with AL-Na at 500mg/kg rat (F).

Fig. 4. Effect of AL-Na on Villus Height and Crypt Depth in Jejunum
Jejunal villus height (A) and crypt depth (B) were measured. The animals were treated with AL-Na at a dose of 500mg/kg. Each column and vertical bar represents the mean±S.E.M. (n=8). ***: Significantly different from the control group at p<0.05 and p<0.01, respectively (Student’s t-test). #: Significantly different from the methotrexate treated group at p<0.05 (Dunnett’s test).

Fig. 5. Effect of AL-Na on Villus Height and Crypt Depth in Ileum
Ileal villus height (A) and crypt depth (B) were measured. The animals were treated with AL-Na at a dose of 500mg/kg. Each column and vertical bar represents the mean±S.E.M. (n=8). ***: Significantly different from the control group at p<0.05 and p<0.01, respectively (Student’s t-test). #: Significantly different from the methotrexate treated group at p<0.05 (Dunnett’s test).
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rats, methotrexate caused significant decreases in daily food intake throughout the experiment (Fig. 1B). Administration of AL-Na to methotrexate treated rats alleviated this decrease in daily food intake.

The Effects of AL-Na on Gastrointestinal Organ Weights

Methotrexate caused significant weight loss from gastrointestinal organs. AL-Na administration alleviated this decrease in organ weights.

Fig. 6. Effect of AL-Na on Cell Proliferation

Small intestinal tissues were stained by BrdU. Jejunal tissues were obtained from normal rat (A), methotrexate treated rat (B) and methotrexate treated with AL-Na at 500mg/kg rat (C). Ileal tissues were obtained from normal rat (D), methotrexate treated rat (E) and methotrexate treated with AL-Na at 500mg/kg rat (F).

Fig. 7. Effect of AL-Na on Number of BrdU Labeling Cells

The number of BrdU labeling cell was counted in jejunum (A) and ileum (B). The animals were treated with AL-Na at a dose of 500mg/kg. Each column and vertical bar represents the mean±S.E.M. (n=8). *: Significantly different from the control group at p<0.05 (Student’s t-test). #: Significantly different from the methotrexate treated group at p<0.05 (Dunnett’s test).

Fig. 8. Effect of AL-Na on Blood Cell Loss

The number of erythrocyte (A), the concentration of hemoglobin (B) and hematocrit (C) were measured. The animals were treated with AL-Na at a dose of 500mg/kg. Each column and vertical bar represents the mean±S.E.M. (n=8). *: Significantly different from the control group at p<0.05 (Student’s t-test). #: Significantly different from the methotrexate treated group at p<0.05 (Dunnett’s test).
stomachs (Fig. 2A), small intestines (Fig. 2B), and large intestines (Fig. 2C) compared with those of untreated animals. A single oral dose of 500-mg/kg AL-Na significantly inhibited small intestinal weight loss compared with those of methotrexate-treated animals. Moreover, gastrointestinal bleeding was seen in some of the animals treated with methotrexate. In contrast, gastrointestinal bleeding was not observed in AL-Na treated animals.

The Effects of AL-Na on Small Intestinal Morphology
As shown in Fig. 3, methotrexate treatment markedly affected villous atrophy, and misaligned crypts in both jejunum (Fig. 3B) and ileal tissues (Fig. 3E) compared with those of untreated animals (Figs. 3A and D). Crypt damage was observed in both jejunum and ileum and was characterized by loss of crypt cells and flattening of the remaining epithelium. During villus atrophy, large numbers of epithelial cells were lost, and the remaining cells appeared flattened. This evidence of villus atrophy was alleviated by co-treatment with AL-Na (Figs. 3C and F). In the jejunum, methotrexate caused decreases in villous heights and crypt depths (Figs. 4A and B). Oral administration of AL-Na at a dose of 500mg/kg significantly prevented these villus and crypt damage. AL-Na also inhibited villous and crypt damage in the ileum (Figs. 5A and B).

The Effects of AL-Na on Cell Proliferation
Analyses of cell proliferation using BrdU labeling revealed inhibition of crypt cell proliferation in methotrexate treated jejunum (Fig. 6B) and ileum (Fig. 6E) compared with untreated animals (Figs. 6A and D). Co-treatment with AL-Na prevented this methotrexate-mediated decline in BrdU labeling of cells (Figs. 6C and F). Moreover, BrdU positive cell numbers per crypt were decreased in the jejunum (Fig. 7A) and ileum (Fig. 7B) of methotrexate-treated animals. Again, co-treatment with AL-Na ameliorated this deleterious effect.

The Effects of AL-Na on Blood Cell Loss
Figure 8 shows the effects of AL-Na on methotrexate-induced blood cell loss. Methotrexate significantly decreased the erythrocyte cell numbers, hemoglobin levels, and hematocrit levels compared with untreated animals (Figs. 8A, B, and C). Oral administration of AL-Na at a dose of 500mg/kg significantly inhibited these decreases in erythrocyte cell numbers, hemoglobin levels, and hematocrit levels.

DISCUSSION
In this study, we demonstrated the severe gastrointestinal toxicity of methotrexate. Treatment with methotrexate caused body weight loss and an anorexic decline in food intake. Moreover, weight of the stomach, small intestine, and large intestine was dramatically decreased in these animals. In particular, the loss of small intestine mass was due to inhibited cell proliferation in small intestinal mucosa. A previous report demonstrated that layers of 5% AL-Na solution can cover lesions, inhibit gastric injury, and protect mucosal surfaces of the upper gastrointestinal tract. AL-Na solution has very high viscosity, and the AL-Na solution of further density is inconvenient for experiments. Therefore, we tested the maximum dose 500mg/kg using 5% AL-Na solution. In this study, we examined the effects of AL-Na on the small intestines of methotrexate-treated rats and showed that daily co-administration of AL-Na prevented small intestinal weight loss in these animals. These data indicate that AL-Na acts as a muco-protective agent in the small intestinal tract. Subsequently, we evaluated morphology in both jejunum and ileum and confirmed that methotrexate causes marked changes to villous and crypt. In these experiments, loss of epithelial cells and shortened villous height and crypt depth were observed in both jejunum and ileum. Importantly, treatment of these animals with AL-Na ameliorated methotrexate-induced morphological changes.

In further experiments, we examined the effects of AL-Na on cell proliferation because reduced cell proliferation in the intestine is a common adverse effect of chemotherapy. BrdU is a synthetic nucleoside analogue of thymidine that is incorporated into DNA during the S-phase of the cell cycle, enabling investigation of cell proliferation in rats. Immunohistological studies revealed that AL-Na prevented inhibition of jejunal and ileum crypt cell proliferation by methotrexate. In agreement with these observations, treatment with AL-Na is popular for wounds and reportedly stimulates cell proliferation. Moreover, AL-Na-based gel matrices promoted collagen synthesis and stimulated transforming growth factor-beta (TGFβ1) secretion in skin wounds. As overexpression of TGFβ1 contributed to proliferation during wound healing, it was suggested that the effects of alginate hydrogel on collagen synthesis may primarily relate to increased TGFβ1 secretion. In addition, it is thought that AL-Na has no influence on anti-cancer action for leukemia and other malignancies because TGFβ that is highly produced in the wound area and cancer area may stimulate cell growth in the wound and cancer. Folic acid is essential to numerous biological functions. In particular, humans require folate for synthesis, repair, and methylation of DNA, and as a cofactor in numerous biological reactions. Folate is required for cell division and growth, and for the production of healthy red blood cells. As folic acid deficiency causes severe anemia and bleeding, we focused on the effects of methotrexate-induced hematological changes on gastrointestinal mucositis. Previously, methotrexate administration depleted red blood cells and hemoglobin levels, and increased cecal hemoglobin in rats. The present data also show depletion of red blood cells and hemoglobin levels in methotrexate-treated rats, suggesting that methotrexate may cause gastrointestinal bleeding. Importantly, AL-Na inhibited methotrexate depletion of red blood cell and hemoglobin levels and elicited a hemostatic effect on the stomach. Previous studies report that AL-Na precipitates fibrinogen, increases fibrin polymerization, and enhances aggregation of platelets. Thus, we suggest that these hemostatic effects of AL-Na may also be relevant to the small intestine.

As platelets contain tissue promoting factors such as TGFβ, vascular endothelial growth factor, and platelet factor-4, platelet aggregation is considered to play an important role in cell proliferation. TGFβ stimulates intestinal cell migration and proliferation, and platelets act as a delivery system for TGFβ that accelerates mucosal healing. We suggest that these processes may contribute to the effects of AL-Na on cell proliferation.

In conclusion, AL-Na prevents methotrexate-induced small intestinal mucositis and exerts a hemostatic effect on small intestinal bleeding. Therefore, AL-Na may be an effective agent for the treatment of chemotherapy-induced gastrointestinal mucositis.
REFERENCES


35) Daigo K, Yamada C, Yamaji M, Nakagiri N, Okada M, Komiya H,

