Regular Article

Rebamipide Attenuates 5-Fluorouracil-Induced Small Intestinal Mucositis in a Mouse Model

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5-Fluorouracil (5-FU)-induced intestinal mucositis is one of the most common morbidities in chemotherapy and involves the reactive oxygen species (ROS) system, apoptosis, and inflammatory cytokines. Rebamipide exerts a mucosal-protective effect, mediated through several mechanisms. The aim of this study was to evaluate the effects of rebamipide in 5-FU-induced mouse small-intestinal mucositis. BALB/c mice were assigned randomly to four groups; (1) control group (n=10; receiving saline orally for 6 d), (2) rebamipide group (n=10; 150 mg/kg rebamipide for 6 d orally), (3) 5-FU group (n=10; 30 mg/kg 5-FU for 5 d, intraperitoneally (i.p.)), and (4) rebamipide +5-FU group (n=10; 150 mg/kg rebamipide for 6 d orally and 30 mg/kg 5-FU for 5 d, i.p.). Body weights and diarrhea scales were assessed. At day 5, the mice were sacrificed. Small intestinal tissue was used for: (1) hematoxylin and eosin (HE) staining for determination of small intestinal villi height, (2) terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay, (3) immunohistochemistry for inducible nitric oxide synthase (iNOS), F4/80, and transforming growth factor (TGF)-β1, (4) measurement of serum and tissue GSH levels, and (5) measurement of serum tumor necrosis factor (TNF)-α levels. Rebamipide attenuated the severity of mucosal injury reflected by body weight changes, degrees of diarrhea, and heights of villi. Rebamipide reduced the expression of iNOS and TGF-β1, apoptosis, macrophage accumulation, serum TNF-α levels, and prevented reductions in serum and tissue glutathione (GSH) levels by 5-FU administration. These results suggest that rebamipide promotes several mechanisms of mucosal protection and attenuated the 5-FU-induced mucosal injury. In conclusion, administration of rebamipide may have significant protective effects against 5-FU-induced intestinal mucositis.

Key words mucositis; 5-fluorouracil; small intestine; rebamipide

5-Fluorouracil (5-FU) is a cytostatic agent that has been used widely in the treatment of various solid tumors for more than 20 years. It is still considered to be among the most effective anti-neoplastic agents in advanced colorectal cancer and malignancies of the head and neck.1) Many non-chromatographic and chromatographic methods for the quantitation of 5-FU, related pro-drugs, and metabolites in biological matrices have been developed over the last 30 years to support preclinical and clinical studies.2) Nevertheless, 50–80% of patients who undergo 5-FU chemotherapy show clinical manifestations of mucositis, symptoms of which include severe diarrhea.2) Its incidence in patients who underwent standard-dose chemotherapy was 40%, while it approached 100% in patients treated with high-dose chemotherapy.3)

The pathophysiological mechanism of 5-FU-induced mucositis comprises five phases4): (1) initiation, signaling, amplification, ulceration, and healing. In the first, initiation, chemotherapy causes DNA damage in basal epithelial cells and generates reactive oxygen species (ROS), which further causes damage to cells and blood vessels in the submucosa.5) In the second phase, signaling, chemotherapy and ROS induce apoptosis and upregulate inflammatory cytokines in cells.6) In the third phase, amplification, inflammatory cytokines result in further tissue damage, amplifying signaling cascades and the injury process.6) The fourth phase is ulceration; loss of mucosal integrity produces extremely painful lesions, providing portals of entry of bacteria, viruses, and fungi.7) The last phase is healing; proliferation, differentiation, and migration of epithelial cells occur, restoring the integrity of the mucosa.8) Therefore, the ROS system, apoptosis, and inflammatory cytokines are involved in 5-FU induced mucositis.

Rebamipide (2-(4-chlorobenzylamino)-3-[2(1H)-quinolinon-4-yl]propionic acid) is an anti-gastric ulcer and gastritis agent. It is known to have preventative effects against various acute experimental gastric mucosal lesions and to accelerate the healing of gastric ulcers. Increased mucous secretion,9) enhanced generation of endogenous prostaglandins,9) suppression of neutrophil function,9) inhibition of inflammatory cytokines,10) and scavenging of oxygen free radicals11) are known to be important effects of rebamipide in the stomach. Likewise, protective effects of rebamipide in the mucosa of the small intestine have been reported. Banan et al.12) demonstrated that rebamipide prevented the oxidation of actin, leading to protection of the actin cytoskeleton, using in vitro human intestinal cell monolayers. Accordingly, they suggested that this agent prevented intestinal hyperpermeability and stabilized

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human intestinal barrier function. Mizoguchi et al.\textsuperscript{13} reported protective effects of rebamipide against indomethacin-induced intestinal damage in rats, and suggested that the mechanism underlying the preventative action involved the scavenging of oxygen free radicals.\textsuperscript{14}

The present study aimed to investigate the protective effects of rebamipide on 5-FU-induced small intestinal mucositis. Basically, we assumed that the degree of tissue inflammation was positively correlated with functional and structural tissue injury by 5-FU and this injury was abrogated by rebamipide.

MATERIALS AND METHODS

Experimental Design and Measurements of Serum Tumor Necrosis Factor (TNF-\(\alpha\), Serum Glutathione (GSH), and Tissue GSH Levels This study was conducted using male BALB/c mice (20–25 g; Koatech Animals Inc., Poengtaek, Gyeonggi-Do, Korea). All animals were housed in temperature-controlled conditions under a light/dark photocycle with food and water supplied ad libitum. The experiments were performed according to Gyeongsang National University Animal Care and Use Committee guidelines (GLA-110324-R0022).

Mice were dehydrated for 16 h before 5-FU (Sigma-Aldrich, MO, U.S.A.) injection. The mice were randomly assigned to four groups: (1) mice receiving saline for 6 d (0–5 d) orally (control group; Con, \(n=10\)), (2) a group receiving 150 mg/kg rebamipide for 6 d (0–5 d) orally (rebamipide group; Rab, \(n=10\)), (3) a group receiving 30 mg/kg 5-FU for 5 d (1–5 d) intraperitoneally (5-FU group; 5-FU, \(n=10\)), and (4) a group receiving 150 mg/kg rebamipide for 6 d (0–5 d) orally and 30 mg/kg 5-FU for 5 d (1–5 d) intraperitoneally (rebamipide+5-FU group; Rab+5-FU, \(n=10\)).

At day 5 after 5-FU injection, mice were anesthetized with zoletil (Virbac Korea, Seoul, Korea) and sacrificed. Small intestines were removed from the animals through a midline abdominal incision. Sera samples were taken for cytokine analysis (TNF-\(\alpha\), SinglePlex system, Bio-Rad).

Body Weight and Diarrhea Assessment Each mouse was checked per day to record body weight and diarrhea score. The severity of diarrhea was scored using the following scale: 0, normal (normal stool or absent), 1, slight (slightly wet and soft stool), 2, moderate (wet and unformed stool with moderate perianal staining of the coat), and 3, severe (watery stool with severe perianal staining of the coat).\textsuperscript{15} The incidence of diarrhea scores of 0 to 3 and average diarrhea score were used to evaluate the severity of diarrhea. The first day that the severity of diarrhea was observed was regarded as “onset of diarrhea.”

Small Intestinal Histology: Height of Villi Segments of ileum were collected. Analyses were performed on 5-\(\mu\)m-thick sections of paraformaldehyde-fixed and paraffin wax-embedded tissue. Sections were stained with hematoxylin and eosin (HE). Measurements of villus height (from the top of the villus to the villus-crypt junction) were performed by light microscopy using a calibrated micrometer (\(\times 200\)). Ten intact and well-oriented villi and crypts were measured and averaged for each sample.\textsuperscript{15}

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay The degree of apoptosis was assessed by TUNEL assay. Detection of DNA fragmentation was performed using a kit from Roche Applied Sciences (Indianapolis, IN, U.S.A.). A semiquantitative analysis was performed by counting the number of TUNEL-positive cells per field, in the small intestine, at \(\times 400\) magnification. At least 10 areas in the cortex per slide were selected randomly. The mean number of brown-colored cells in these selected fields was expressed as the number of TUNEL-positive cells.

Immunohistochemistry: Inducible Nitric Oxide Synthase (iNOS), F4/80, and Transforming Growth Factor (TGF)-\(\beta\)\textsuperscript{1} Immunohistochemical studies using the Vectastain ABC kit (Vector Laboratories, CA, U.S.A.) were performed on 5-\(\mu\)m-thick sections of paraformaldehyde-fixed and paraffin wax-embedded tissue. Sections were blocked with 1% normal goat serum and then treated with anti-rabbit iNOS (1 : 100, Santa Cruz), anti-rat F4/80 (1 : 100, bsience), and anti-rabbit TGF-\(\beta\)1 (1 : 100, Santa Cruz) antibodies at 4°C overnight in a humidified chamber. Then, phosphate buffered saline (PBS)-washed tissue sections were incubated for 90 min at room temperature with the secondary antibody. Finally, the sections were incubated with avidin-biotinylated-horseradish peroxidase (HRP)-complex for 60 min at room temperature, rinsed in PBS, and developed using dimethyldiaminoazobenzene (DAB) with hydrogen peroxidase. The density and number of TUNEL, iNOS and TGF-\(\beta\)1-positive cells were analyzed by blinded observer using NIS Elements BR3.1 (Nikon, Japan) software in ten randomly selected fields.

GSH Level Measurement The small intestine and serum glutathione content was measured using Glutathione Assay kits (Sigma, St. Louis, MO, U.S.A.) according to the manufacturer’s instructions. Briefly, 100 mg of each tissue was homogenized in 0.5 mL of glutathione reaction buffer containing 0.1 mL of 5% sulfoalicylic acid. To generate nicotinamide adenine dinucleotide phosphate (NADPH), 20 \(\mu\)L of NADPH Generation Mix and 140 \(\mu\)L of glutathione reaction buffer was mixed and incubated at room temperature for 10 min. Then, 20 \(\mu\)L of either the GSH standard solution or the sample solution was added, followed by incubation at room temperature for 5 to 10 min and a further addition of 20 \(\mu\)L of substrate solution. A microplate reader was used to measure the absorbance at 405 nm (Molecular Devices Corp., Sunnyvale, CA, U.S.A.).

Statistical Analysis Data are expressed as means±S.E. (\(n=10\)). Statistical analysis was conducted using the Sigma Plot 7.0 software (SPSS Inc., Chicago, IL, U.S.A.). Differences between the each group were evaluated using one-way ANOVA. The \(p\) values <0.05 (control vs. 5-FU or 5-FU vs. Reb+5-FU) were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Rebamipide Improved Clinical Symptoms The clinical severity of 5-FU-induced mucositis was monitored in terms of daily body weight and diarrhea score. No clinically significant weight loss, death, or diarrhea was observed in the control group. 5-FU injection induced markedly decreased body weight (average, 17.0±5.2% weight loss; \(p<0.05\)), earlier death, and advanced onset of diarrhea. The rebamipide +5-FU group showed a moderate degree of weight loss (average, 13.7±1.8%; \(p<0.05\)) and delayed death and development of
diarrhea induced by 5-FU administration (Fig. 1). Thus, this result suggests that rebamipide apparently protected against mucosal injury, so diarrhea and weight loss hardly occurred.

**Rebamipide Protected against Serum TNF-α Elevation**

The serum TNF-α and serum and tissue GSH levels indicate the degree of systemic inflammation and stimulate the acute phase reaction. Serum TNF-α levels and serum and tissue GSH levels were calibrated as a percentage of the control. In the 5-FU group, serum TNF-α levels were increased markedly (2.85±1.29; p<0.05). This level was decreased significantly in the rebamipide +5-FU group (1.27±0.64, p<0.05; Fig. 2A). Serum and tissue GSH level was decreased significantly in the 5-FU group (0.41±0.05, p<0.05, Fig. 2B and 0.62±0.19, p<0.05, Fig. 2C, respectively). However, in the rebamipide +5-FU group, there was a tendency towards increased serum and tissue GSH levels compared with 5-FU group, but this was not significant (0.81±0.03 and 0.85±0.04, respectively, Figs. 2B, C). This result may support why rebamipide administration improved survival and diarrheal scale scores.

**Rebamipide Protected against 5-FU-Induced Small Intestinal Mucosal Injury**

Measurement for the height of the villi can reflect a direct degree of overall mucosal damage. Histological changes were evaluated by measurements of small intestinal villi height. The control group showed
tall and well-arranged mucosal epithelial cells, but 5-FU injection resulted in severe mucosal damage. Villi height decreased significantly in the 5-FU group compared with the control (212.1±90.3 µm vs. 188.3±64.4 µm; p<0.005). The villi height in the rebamipide+5-FU group was significantly decreased less than that in the 5-FU group (p<0.05, Fig. 3). These results suggest that rebamipide protects the small intestinal mucosa against 5-FU, as it did the gastric mucosa.  

Rebamipide Decreased iNOS Expression in the Small Intestine

iNOS was predominantly expressed in the edge of villi and also strongly in the muscularis externa (ME) and serosa (S) (A). Whereas, F4/80-positive cells were localized at the small intestinal mucosa and submucosa (B). TGF-β1 was expressed in small intestinal mucosa and also detected in the muscularis externa and serosa like iNOS-positive cells (C). Data means±S.E.; **p<0.005 indicate differences between 5-FU and Reb+5-FU group. All experiments were performed in repeated three times.
Attenuated the acute inflammatory process and macrophage increased in small intestinal mucosa with 5-FU administration. TGF-β expression, one of inflammatory cytokines, increased markedly in 5-FU administered small intestinal mucosa and submucosa (9.7 ± 3.8; p < 0.005, data not shown). Some positive signals were observed in the submucosal glands of rebamipide + 5-FU group. The rebamipide + 5-FU group showed significantly repressed macrophage accumulation in the small intestinal mucosa (5.2 ± 0.9; p < 0.005, data not shown). The density of F4/80-positive cells showed a significantly decreased in rebamipide + 5-FU group compared with 5-FU-induced increase in density of F4/80-positive cells (p < 0.005, Fig. 5). Thus, rebamipide apparently attenuated the acute inflammatory process and macrophage accumulation in mice small intestine.

Rebamipide Reduced Macrophage Accumulation Macrophages are produced by the differentiation of monocytes in tissues. They are associated with both innate immunity and adaptive immunity in vertebrates and accumulate at sites of acute inflammation. F4/80-positive cell number, which reflects macrophage accumulation, increased markedly in 5-FU treated small intestinal mucosa and submucosa (9.7 ± 3.8; p < 0.005, data not shown). Some positive signals were observed in the submucosal glands of rebamipide + 5-FU group. The rebamipide + 5-FU group showed significantly repressed macrophage accumulation in the small intestinal mucosa (5.2 ± 0.9; p < 0.005, data not shown). The density of F4/80-positive cells showed a significantly decreased in rebamipide + 5-FU group compared with 5-FU-induced increase in density of F4/80-positive cells (p < 0.005, Fig. 5). Thus, rebamipide apparently attenuated the acute inflammatory process and macrophage accumulation in mice small intestine.

Rebamipide Prevents TGF-β1 Expression TGF-β1 is one of the best-known inflammatory cytokines involved in immune system regulation by T cells. Increased expression of TGF-β1 can lead to development of a stronger inflammatory process. TGF-β1 expression, one of inflammatory cytokines, increased in small intestinal mucosa with 5-FU administration (1423.0 ± 126.9; p < 0.05) versus the control (685.2 ± 112.1). TGF-β1-positive cells were also detected in the muscularis externa and serosa like iNOS-positive cells. Rebamipide administration prevented most of the increase in TGF-β1 expression (889.6 ± 266.1; p < 0.05, Fig. 5). Thus, rebamipide apparently decreased the secretion of inflammatory cytokines by 5-FU in the mouse small intestine.

We investigated the various steps in the pathophysiology of mucositis and evaluated the inflammatory response in each. In this study, we demonstrated that rebamipide had clinical and histological mucosal protective effects and also a preventative effect against apoptosis and decreased the expression of inflammatory cytokines, macrophage accumulation, and diarrhea. In conclusion, although few clinical data on the effects of rebamipide on small intestinal mucosal injury are available, we suggest that rebamipide might exert a protective effect against 5-FU-induced mucositis in humans.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES