Rebamipide ameliorates radiation-induced intestinal injury in a mouse model

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Radiation-induced enteritis is a major side effect in cancer patients undergoing abdominopelvic radiotherapy. Radiation exposure produces an uncontrolled inflammatory cascade and epithelial cell loss leading to impaired epithelial barrier function. The goal of this study was to determine the effect of rebamipide on regeneration of the intestinal epithelia after radiation injury. The abdomens of C57BL/6 mice were exposed to 13 Gy of irradiation (IR) and then the mice were treated with rebamipide. Upon IR, intestinal epithelia were destroyed structurally at the microscopic level and bacterial translocation was increased. The intestinal damage reached a maximum level on day 6 post-IR and intestinal regeneration occurred thereafter. We found that rebamipide significantly ameliorated radiation-induced intestinal injury. In mice treated with rebamipide after IR, intestinal barrier function recovered and expression of the tight junction components of the intestinal barrier were upregulated. Rebamipide administration reduced radiation-induced intestinal mucosal injury. The levels of proinflammatory cytokines and matrix metallopeptidase 9 (MMP9) were significantly reduced upon rebamipide administration. Intestinal cell proliferation and β-catenin expression also increased upon rebamipide administration. These data demonstrate that rebamipide reverses impairment of the intestinal barrier by increasing intestinal cell proliferation and attenuating the inflammatory response by inhibiting MMP9 and proinflammatory cytokine expression in a murine model of radiation-induced enteritis.

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1. Introduction

The treatment of malignant tumors with radiation therapy also affects surrounding healthy tissues (Haydott et al., 2007). The gastrointestinal tract, especially the small intestine, is particularly sensitive to radiation, which renders it vulnerable to collateral radiation from radiotherapeutic treatments for abdominal and pelvic cancers (Bachmann et al., 2015). Radiation-induced enteritis reduces patients’ qualities of life and increases treatment and social health care costs (Fyles et al., 1992; Abayomi et al., 2009). Although many studies have examined the radioprotective effects of various agents, there are no effective clinical treatments for radiation-induced intestinal injury. Therefore, the development of effective therapeutic treatments to improve the outcomes of radiation-induced enteritis is urgently needed.

Radiation-induced gastrointestinal injury is described as destruction of crypt cells, a decrease in villous height and number, and impaired epithelial barrier function (Atasoy et al., 2010). Impaired intestinal barrier function has been observed during the early stages of radiation-induced gastrointestinal injury (Touchefeu et al., 2014). The intestinal epithelial barrier regulates the penetration of substances, such as macromolecules, bacteria, and other intralumen toxins (Deitch, 1990). Bacterial penetration potentiates the development of septicemia, which is one of several causes of death following radiation exposure. Thus, the epithelial barrier of the small intestine has promise as a therapeutic target.

The intestinal epithelium is a single layer of columnar epithelial cells that separates the intestinal lumen from the underlying lamina propria. These epithelial cells are tightly bound together by intercellular junctional complexes that regulate paracellular permeability and that are crucial for the integrity of the epithelial barrier (Ulluwishewa et al., 2011). Proinflammatory cytokines are major inducers of matrix metalloproteinase 9 (MMP9) production in intestinal inflammation models (Gan et al., 2001; Sternlicht and Werb, 2001; Castaneda et al., 2005). MMP9 release during inflammation may lead to tight junction degradation and loss of mucosal integrity (Naito and Yoshikawa, 2005; Kofla-Dlubacz and Iwanczak, 2010). Tight junctions, the major components of junctional complexes, seal paracellular spaces between epithelial cells and prevent paracellular diffusion of microorganisms and other antigens across the epithelium (Assimakopoulos et al., 2004; Garg
et al., 2014; Shim et al., 2015). Therefore, during intestinal epithelial cell loss and inflammation, intestinal junctional complexes are destroyed (Dublineau et al., 2004; Prasad et al., 2005; Uluwisheva et al., 2011; Wells et al., 2011; Miner-Williams and Moughan, 2016).

Rebamipide is a gastroprotective agent that is already in clinical use for the treatment of gastric ulcers and gastritis (Han et al., 2015; Kamada et al., 2015). Rebamipide has been shown to suppress immune responses (Byun et al., 2014; Yamane et al., 2015) and regulate inflammatory cell activation (Aihara et al., 1998; Nagano et al., 2001) in various inflammatory disease models. Numerous studies using a nonsteroidal anti-inflammatory drug (NSAID)-induced intestinal inflammation model have shown that rebamipide affects the release of inflammatory cytokines and growth factors (Lai et al., 2015; Watanabe et al., 2015). Because abnormal inflammatory responses lead to impaired epithelial barrier function in radiation-induced enteritis, these findings suggest that rebamipide may be a promising therapeutic agent for radiation-induced enteritis. The aim of this research was to investigate the effect of rebamipide on intestinal barrier function in a mouse model of radiation-induced enteritis.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice aged 7 weeks were obtained from Harlan Laboratories (IN, USA). The mice were kept under controlled conditions, including constant temperature, and were allowed free access to regular chow and 3-stage filtered water. The Animal Investigation Committee of the Korea Institute of Radiological and Medical Sciences approved all of the animal experiments.

2.2. Irradiation and administration of rebamipide

Animals were anesthetized by intraperitoneal injection of 85 mg/kg of alfaxalone (Alfaxan®; Carexide, Republic of Korea) and 10 mg/kg of xylazine (Rompun®, Bayer Korea, Republic of Korea) and underwent whole abdominal irradiation with 13 Gy of radiation at a dose rate of 2 Gy/min using an X-RAD 320 X-ray irradiator (Softex, Republic of Korea). The irradiated dose and dose rate were measured with an UNIDOS® E dosimeter (PTW-Freiburg, Germany). After radiation exposure, animals were treated orally with 200 mg/kg/day (IR + Rb200) or 400 mg/kg/day (IR + Rb400) of rebamipide (Mucosta®, Otsuka, Republic of Korea) for the duration of the experiment.

2.3. Bacterial translocation assay

The presence of viable bacteria in mesenteric lymph nodes (MLNs), which were harvested under sterile conditions, represented bacterial translocation from the lumen of the intestine. Equal aliquots of homogenates were plated onto MacConkey agar (Becton Dickson, Franklin Lakes, NJ), were incubated at 37 °C for 24 h, and the numbers of colonies on the plates were counted.

2.4. Intestinal permeability assay

Intestinal permeability to 4 kDa FITC-dextran (Sigma-Aldrich, St. Louis, MO) was measured 6 days post-IR (n = 5 animals per group). Animals were anesthetized with alfaxalone and xylazine, a midline laparotomy incision was made, and the small intestine was exposed. A 5 cm segment of distal small intestine was isolated between bulldog clamps and was injected with 12.5 μl of FITC-dextran that had been dissolved in 100 μl of phosphate buffered saline (PBS). Animals were kept under anesthesia for 30 min, and then blood was obtained via cardiac puncture. Blood samples were placed into serum separating tubes and centrifuged at 1000g for 15 min to obtain sera. FITC-dextran concentrations were measured using a fluorescence spectrophotometer (BioTek microplate reader) at excitation and emission wavelengths of 495 nm and 520 nm, respectively.

2.5. Histological examination of the intestine

Terminal ileum samples were fixed with a 10% formalin solution, embedded in paraffin wax, and sectioned at a thickness of 4 μm. Sections were stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, sections were treated with 0.3% hydrogen peroxide in methyl alcohol for 20 min to block endogenous peroxidase activity. After three washes with PBS, sections were blocked with 10% normal goat serum (Vector ABC Elite Kit, Vector Laboratories, Burlingame, CA) and incubated with antibodies to claudin-3 (Invitrogen, Carlsbad, CA), occludin (Santa Cruz, CA), β-catenin (BD Bioscience, NJ), and Ki-67 (Acris Antibodies, Germany). After three subsequent washes with PBS, sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Carpinteria, CA). Detection was performed using a diaminobenzidine substrate (Dako) according to the manufacturer’s instructions. Quantitative assessment of immunoreactivity was performed with i-solution software.

2.6. RNA extraction, reverse transcription, and real-time PCR quantification

Total RNA was extracted from small intestines using TRizol Reagent (Invitrogen). Total RNA samples (1 μg) were reverse transcribed into cDNA using oligo (dT) 18 primers and Accu-Power RT PreMix (Bioneer, Daejon, Korea). SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany) was used for real-time RT-PCR reactions. The primers sequences were as follows: IL-1β sense 5′-GAACGTTCCTGAACCTCA-3′ and antisense 5′-CTTGGACTATGCTGTGTA-3′, and antisense 5′-TGCAAGTGCATCCTGCTG-3′, and antisense 5′-CCGGATTITGGAAAGCTC-3′, and antisense 5′-GAAAACCCGAAAGACTCCTC-3′, and antisense 5′-GCTTCCTCTACTTCCCTCCT-3′, and antisense 5′-ACATGTGGTGTGTCTGAGCA-3′, and antisense 5′-GCCCTGGAACACTCAACGACA-3′, and antisense 5′-TTGGAACAAGCAGCAGA-3′, and antisense 5′-ATCCATATCCCAAAGGACA-3′, and antisense 5′-AACCGATATCCCAAAGGACA-3′, and antisense 5′-ACTGCTGAGCTCTC-3′ and antisense 5′-TGATGGCGTAAAGCAC-3′, and antisense 5′-TGGGAAAAAGCATTACG-3′, and antisense 5′-AAAGCAGAATGGAAAGAACG-3′, and antisense 5′-AGGACAAGAAGACTGTG-3′, and antisense 5′-AAGCTACTCTTCGATTACG-3′, and antisense 5′-ACTCGGACACACTGACTT-3′, and antisense 5′-ACACGGTCTACTGAGGAAAC-3′, and antisense 5′-GTGGACAGGCAAGCCTGA-3′, and antisense 5′-AGCAGTGGTGTGTGAGCC-3′, and antisense 5′-GACGGTTACTGTTGAGAAC-3′, and antisense 5′-ACAGGGAAGAAAAAGCACA-3′, and antisense 5′-AGAGGTGAGCTTGTGCTCG-3′, and antisense 5′-CTCTGGAGAGACTATGTA-3′, and antisense 5′-CGATAAAGAGGCTCTGAAA-3′. All of the reactions were performed in duplicate with 2 μl of cDNA on a LightCycler 480 II Real-time PCR platform (Roche). Relative mRNA levels were determined using the 2ΔΔCt method with β-actin as the internal control. +1.5 fold cut off is considered for upregulation in mRNA expression levels.

2.7. Enzyme-linked immunosorbent assay

After general anesthesia, blood samples were collected from the inferior vena cava, placed in heparin tubes, and centrifuged at 2000g for 15 min.
20 min at 4 °C. Plasma supernatants were collected and stored at −80 °C. Plasma diamine oxidase (DAO) concentrations were quantified by sandwich enzyme immunoassay using the Quantikine ELISA kit (Cusabio, MD, USA) according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader (BioTek microplate reader).

2.8. Statistical analysis

All of the data were expressed as mean ± SD, statistical significances were evaluated by Student’s t-test, and p values < 0.05 were considered statistically significant.

3. Results

3.1. Enteritis was induced by exposing mice abdomens to radiation

Histopathological changes and bacterial translocation were examined in MLNs on days 4, 6, and 10 after IR (Fig. 1). Epithelial loss, shortening of villi, and fewer crypts were observed in the small intestine on day 4, and these changes were at their maximum on day 6 (Fig. 1A). The irradiated group (IR) presented elevated bacterial translocation on day 4 when compared with the control group, and the maximum level of bacterial translocation was seen on day 6 (p < 0.05, Fig. 1B). The crypt-villi structure and the intestinal barrier were regenerated by day 10 post-IR. These data indicate that irradiation damaged the small intestinal mucosal barrier, which resulted in loss of intestinal epithelium integrity. In further experiments, we analyzed the effects of rebamipide on radiation-induced intestinal injury on day 6 post-IR.

3.2. Rebamipide ameliorated radiation-induced intestinal injury

To examine the putative role of rebamipide in protection from radiation-induced intestinal injury, we compared histologically the small intestines of irradiated mice with and without rebamipide treatment. On day 6 post-IR, mucosal structural damage in the small intestine of irradiated mice was reversed upon treatment with rebamipide (Fig. 2A). The heights of villi and numbers of crypts per mm were measured on day 6 post-IR. The heights of villi in the IR group were lower than the heights of villi in the control group. However, treatment with rebamipide after irradiation attenuated the reduction in the heights of the villi (control: 324.2 ± 27.6 μm; IR: 216.9 ± 50.4 μm; IR + Rb200: 300.4 ± 59.3 μm; IR + Rb400: 324.8 ± 45.8 μm; p < 0.05; Fig. 2B). In addition, the number of crypts in the rebamipide treatment groups (IR + Rb200: 8.8 ± 2.0, IR + Rb400: 9.8 ± 3.7) were greater than in the IR group (3.9 ± 1.2; P < 0.05; Fig. 2C).

3.3. Rebamipide reversed radiation-induced damage to the intestinal barrier

Next, to determine whether rebamipide treatment after IR improved epithelial barrier function, we assessed intestinal barrier function on day 6 post-IR using bacterial translocation and FITC-dextran absorption assays. In the bacterial translocation assay, bacterial colony counts in MLNs from the IR group were higher than in the control group (p < 0.05, Fig. 3A). However, bacterial colony counts reduced significantly on day 6 post-IR, mucosal structural damage in the small intestine of irradiated mice was reversed upon treatment with rebamipide (Fig. 2A). The heights of villi and numbers of crypts per mm were measured on day 6 post-IR. The heights of villi in the IR group were lower than the heights of villi in the control group. However, treatment with rebamipide after irradiation attenuated the reduction in the heights of the villi (control: 324.2 ± 27.6 μm; IR: 216.9 ± 50.4 μm; IR + Rb200: 300.4 ± 59.3 μm; IR + Rb400: 324.8 ± 45.8 μm; p < 0.05; Fig. 2B). In addition, the number of crypts in the rebamipide treatment groups (IR + Rb200: 8.8 ± 2.0, IR + Rb400: 9.8 ± 3.7) were greater than in the IR group (3.9 ± 1.2; P < 0.05; Fig. 2C).

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Fig. 1. Time course of radiation-induced enteritis. C57BL/6 mice were exposed abdominally to 13 Gy of IR. (A) H&E staining of the small intestine on day 4, 6, and 10 post-IR. (B) MLNs collected 4, 6, and 10 days after IR were analyzed for bacterial translocation (5 mice per condition). The bars represent mean values and the error bars show standard deviations. *p < 0.05 versus control group.

Fig. 2. Effects of rebamipide on radiation-induced structural damage in the intestine. Tissues from the small intestines of C57BL/6 mice exposed abdominally to 13 Gy of IR and treated with saline or rebamipide (Rb) were collected on day 6 post-IR (5 mice per condition) and H&E stained (A) for villus height (B) and crypt number (C). The bars represent mean values and the error bars show standard deviations. *p < 0.05 versus control group; +p < 0.05 versus IR group.
and in a dose-dependent manner with rebamipide treatment. In addition, the concentration of FITC-dextran in sera was lower in the rebamipide treatment group than in the IR group ($p < 0.05$, Fig. 3B).

Because paracellular diffusion of microorganisms across the epithelium is prevented by tight junctions that seal the paracellular spaces between epithelial cells, we analyzed the mRNA levels of tight junctional molecules in the small intestine on day 6 post-IR (Fig. 4A–D). mRNA levels of claudin-3, claudin-4, and occludin, which are molecules within paracellular junctional complexes in intestinal epithelial cells (Lee, 2015), were lower in the IR group than in the control group ($p < 0.05$, Fig. 4A–C). However, the level of ZO-1, intracellular junction complexes of intestinal epithelial cells, was not significantly changed among the experimental groups (Fig. 4D). The claudin-3 and occludin mRNA levels were higher in the rebamipide treatment groups than in the IR group ($p$.}

Fig. 3. Effects of rebamipide on radiation-induced impairment of the intestinal barrier. Samples from C57BL/6 mice exposed abdominally to 13 Gy of IR and treated with saline or rebamipide (Rb) were collected on day 6 days post-IR (5 mice per condition). (A) MLNs were collected and evaluated for bacterial translocation. (B) Sera were collected and evaluated for FITC concentration. The bars represent mean values and the error bars show standard deviations. *$p < 0.05$ versus control group; +$p < 0.05$ versus IR group; +++$p < 0.05$ versus IR + Rb200 group.

Fig. 4. Effects of rebamipide on intestinal junctional complexes. Tissues from the small intestines of C57BL/6 mice exposed abdominally to 13 Gy of IR and treated with saline or rebamipide (Rb) were collected on day 6 post-IR (5 mice per condition). Total RNA was isolated and quantitative real-time PCR was performed to measure expression of claudin-3 (A), claudin-4 (B), occludin (C), and zona occuldens-1 (ZO-1) (D). The bars represent mean values and the error bars show standard deviations. *$p < 0.05$ versus control group; +$p < 0.05$ versus IR group. (E, F) Immunohistochemical staining for claudin-3 (E) and occludin (F) protein expression on day 6 post-IR. Nuclei, purple; labeled cells, brown.
Furthermore, claudin-3 and occludin immunostaining was performed to examine protein expression of these intestinal barrier molecules in the small intestine. Claudin-3 and occludin expression were clearly observed throughout the membranes of epithelial cells with surface villi (Fig. 4E, F). Thus, IR reduced expression of junctional proteins in epithelial cells with villi, and this reduced expression was attenuated with rebamipide treatment. These data suggest that rebamipide treatment reverses intestinal barrier dysfunction by accelerating synthesis of tight junctional molecules during radiation-induced enteritis.

3.4. Rebamipide suppressed MMP-9 expression in the irradiated small intestine

MMP-9, which is released from intestinal epithelial cells in response to proinflammatory cytokines (Naito and Yoshikawa, 2005; Kofia-Dlubacz and Iwanczak, 2010), degrades junctional molecules, and leads to loss of mucosal integrity. Because rebamipide has been shown to function as an anti-inflammatory agent in various intestinal inflammation models (Aihara et al., 1998; Byun et al., 2014; Han et al., 2015; Kamada et al., 2015; Lai et al., 2015), we analyzed the effect of rebamipide on MMP9 expression in our abdominally irradiated mouse model (Fig. 5).

We measured mRNA levels of the proinflammatory cytokines TNF-α, IL-6, IL-1β, TGF-β1, and IL-4 in tissues isolated from the small intestines of our experimental groups (Fig. 5A-E). The mRNA levels of the proinflammatory cytokines were higher in the IR group than in the control group. This irradiation-induced increase in proinflammatory cytokine mRNA expression was reversed in mice treated with rebamipide. Moreover, MMP-9 expression, which has been shown to be involved in the inflammatory response to irradiation (Hosgorler et al., 2016), was suppressed in the small intestines of the groups treated with rebamipide (Fig. 5F). These data suggest that rebamipide suppresses MMP9 expression during radiation-induced intestinal injury by inhibiting the production of proinflammatory cytokines.

3.5. Rebamipide improved proliferation of intestinal epithelial cells in the irradiated small intestine

Immunostaining of Ki-67, a cellular marker of proliferation, was performed to assess whether rebamipide induces proliferation of intestinal cells in the irradiated intestine (Fig. 6A). We counted 40.88 ± 5.66 Ki-67-positive cells per field (1 × 10^4 μm^2) in control mice, whereas we counted only 21.04 ± 3.49 Ki-67-positive cells per field in irradiated mice. We counted 30.49 ± 6.45 Ki-67-positive cells per field in the 200 mg/kg/day rebamipide-treated group (IR + Rb200) and 36.99 ± 7.56 Ki67-positive cells per field in the 400 mg/kg/day rebamipide-treated group (IR + Rb400; p < 0.05; Fig. 6B).

Because high concentrations of DAO have been found in epithelial cells of the small intestine (Bieganski et al., 1983) and because plasma DAO concentrations have been shown to decrease upon IR-induced intestinal injury (Ely et al., 1985; DeBell et al., 1987), DAO has been

Fig. 5. Effects of rebamipide on proinflammatory cytokines. Tissues from the small intestines of C57BL/6 mice exposed abdominally to 13 Gy of IR and treated with saline or rebamipide (Rb) treatment were collected on day 6 post-IR (5 mice per condition). Total RNA was isolated and quantitative real-time PCR was performed to measure expression of IL-1β (A), IL-6 (B), TNF-α (C), TGF-β1 (D), IL-4 (E), and MMP9 (F). The bars represent mean values, and the error bars show standard deviations. *p < 0.05 versus control group; †p < 0.05 versus IR group.
suggested as a candidate marker to measure the integrity of the intestinal epithelium (Fukudome et al., 2014). The normal concentration of plasma DAO is 62.57 ± 9.27 unit/L, and the plasma DAO concentration in the IR group was reduced to 15.05 ± 6.79 unit/L (p < 0.05). Upon rebamipide treatment, the plasma DAO concentrations increased to 29.83 ± 5.06 unit/L in the IR + Rb200 group and 32.15 ± 5.90 unit/L in the IR + Rb400 group on day 6 post-IR (p < 0.05, Fig. 6C). These data indicate that rebamipide administration after radiation-induced intestinal injury promotes proliferation of intestinal epithelial cells.

3.6. Rebamipide upregulated Wnt/β-catenin signaling in the irradiated small intestine

To investigate whether rebamipide treatment affects the signaling pathway for intestinal regeneration, mRNA levels of Wnt3A and β-catenin in small intestines were measured and found to be reduced in response to IR, whereas upon rebamipide treatment, Wnt3A and β-catenin mRNA levels were significantly upregulated when compared with the IR group on day 6 post-IR (Fig. 7A, B). In addition, β-catenin protein expression in the small intestine was evaluated by immunohistochemistry and was clearly observed throughout the membranes of intestinal epithelial cells (Fig. 7E). In the IR group, expression of β-catenin in the villi and crypts of intestinal epithelial cells were reduced, and this IR-induced reduction of β-catenin expression was attenuated upon rebamipide administration.

It has recently been demonstrated that LIG4 and c-myc are upregulated by Wnt/β-catenin signaling in irradiated intestinal epithelial cells (Betess et al., 2005; Jun et al., 2016), thus we also analyzed expression of LIG4 and c-myc in the small intestines of our experimental groups (Fig. 7C, D). The mRNA levels of LIG4 and c-myc in the IR group were lower than in the control group (p < 0.05, Fig. 7A–C). In the rebamipide-treated groups, the LIG4 and c-myc mRNA levels were significantly higher than in the IR group. These data suggest that rebamipide activates the Wnt/β-catenin signaling pathway, which functions in intestinal regeneration.

4. Discussion

Enteritis is the most common side effect of radiation therapy for the treatment of abdominal cancer, and the susceptibility of the small intestine to radiation-induced damage is the limiting factor when determining the prescription dose of radiation therapy. However, to our knowledge, there are no widely used methods to reduce the severity of radiation-induced enteritis. Herein, we provide evidence that treatment with rebamipide after IR protects against radiation-induced intestinal injury.

Administration of rebamipide showed structural recovery of the small intestine in irradiated mice. In addition, treatment with rebamipide reduced bacterial translocation across the intestinal epithelium and restored expression of junctional complexes in the intestine indicating that rebamipide exerts beneficial effects on intestinal barrier function in mice. These beneficial effects can be explained by two mechanisms: (1) suppression of inflammatory cytokine production and MMP9 expression in the small intestine and (2) an increase in intestinal epithelial cell proliferation.

In this study, the maximum impairment of the intestinal barrier was reached on day 6 post-IR. In the rebamipide treatment groups, the structural damage induced by irradiation, such as reduced heights of small intestinal villi and the number of intestinal crypts, were reversed. These results show that rebamipide attenuates radiation-induced bowel damage.

Impairment of the intestinal barrier leads to activation of the immune response and a loss of solutes, which leads to leak-flux diarrhea (Sandle et al., 1990; Podolsky, 2002), thus we investigated intestinal barrier function in mice treated with rebamipide. Irradiation significantly increased bacterial translocation in MLNs and increased FITC-dextran concentrations in sera. In contrast, rebamipide administration after irradiation significantly reduced bacterial translocation and the serum concentration of FITC-dextran. Moreover, expression of intestinal junctional complex molecules, such as occludin and claudin-3, significantly increased after rebamipide treatment, which demonstrates that rebamipide restores the damaged structures in intestinal barriers that were induced by irradiation. These results indicate that rebamipide improves barrier function by upregulating synthesis of intracellular junctional molecules.

Because inflammation that occurs after radiation may result in various symptoms, including impairment of the intestinal barrier, reducing inflammation is generally beneficial. We observed that inflammation in the small intestines of mice in the rebamipide treatment group was milder than in the small intestines of mice in the IR group. We also showed that mRNA levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6, as well as MMP-9, were higher in the irradiated small intestine. Further, MMP-9 is the most abundantly expressed protease in injured intestinal tissues (Tarlton et al., 2000; Castaneda et al., 2005). MMP-9 is released from intestinal epithelial cells in response to proinflammatory cytokines and is responsible for the loss of mucosal integrity (Naito and Yoshikawa, 2005). Among proinflammatory cytokines, TNF-α and IL-1β are the primary inducers of MMP protein production.
mRNA levels of TNF-α, IL-1β, and MMP9 were significantly reduced in the rebamipide treatment group. These results suggest that rebamipide administration after radiation-induced enteritis alleviates inflammation and improves mucosal integrity in the small intestine.

Because recovery from mucosal injury relies on cellular proliferation, we evaluated proliferation of intestinal epithelial cells in mice treated with rebamipide. Cellular proliferation was measured by Ki-67 labeling. Ki-67 labeling analysis showed a reduction in the proliferation of small intestinal cells after irradiation, and proliferation was upregulated after rebamipide administration. Although the amount of proliferation did not vary significantly between the two groups treated with different rebamipide dosages, both groups showed significantly increased cell proliferation compared with the IR group. The plasma DAO level is a candidate marker for measuring radiation-induced epithelial cell damage (Ely et al., 1985; DeBell et al., 1987). The DAO plasma level in the mouse intestine decreased after IR and recovered to a normal level after rebamipide administration indicating that intestinal injury was reduced and the integrity of the intestinal epithelium improved in mice treated with rebamipide.

Wnt/β-catenin signaling regulates proliferation of small intestinal stem cells. Loss of β-catenin leads to a rapid loss of intestinal epithelial cells beginning with crypt loss, inhibition of cellular proliferation, and an increase in enterocytic differentiation. In this study, Wnt3A and β-catenin mRNA levels and intestinal cell proliferation decreased after IR, and rebamipide administration reversed these reductions in gene expression and cell proliferation. We also investigated expression of LIG4 and c-myc, which are signaling targets of Wnt/β-catenin. Recent studies have demonstrated that Wnt/β-catenin signaling upregulates LIG4, a DNA repair gene, in irradiated intestinal epithelial cells (Jun et al., 2016) and that c-myc is associated with proliferation and formation of intestinal crypts (Pinto et al., 2003; Bettes et al., 2005). Expression of LIG4 and c-myc were reduced after IR, and this reduction was reversed upon treatment with rebamipide. These results suggest that rebamipide activates β-catenin signaling to promote intestinal cell proliferation and regeneration.

In conclusion, rebamipide may ameliorate inflammation in the small intestine and may improve the structure of junctional complexes between small intestinal epithelial cells during radiation-induced intestinal injury. Rebamipide administration may also increase cell proliferation and regeneration of the intestinal epithelium by activating Wnt/β-catenin signaling. Although a higher dose of rebamipide did not show an equivalent increase in benefits, rebamipide has been shown to be safer than conventional drugs, including NSAIDs, immunosuppressants, and TNF-α blockers (Zhang et al., 2013). Furthermore, rebamipide has recently been shown to suppress invasion of cancer cells (Kang et al., 2013). Therefore, rebamipide is a promising therapeutic for patients with radiation-induced enteritis.

Disclosure statement

The authors have no competing interests.
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