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Rebamipide ameliorates radiation-induced intestinal injury in a mouse model

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ABSTRACT

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.

1. Introduction

The treatment of malignant tumors with radiation therapy also affects surrounding healthy tissues (Haydont 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 metallopeptidase 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

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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; Ulluwishewa 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®; Careside, 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 dosemeter (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 mg 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 1000*g* 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 I Master Mix (Roche Diagnostics, Mannheim, Germany) was used for real-time RT-PCR reactions. The primers sequences were as follows: IL-1β sense 5′-GCAACTGTTCCTGAACTCA-3′ and antisense 5′-CTCGGAGCCTGTAGTGCAG-3′, IL-6 sense 5′-CTTGGGACTGATGCTGGTGA-3′ and antisense 5′-TGCAAGTGCATCATCGTTGT-3′, IL-4 sense 5′-CGGCATTTTGAACGAGGTC-3′ and antisense 5′-GAAAAGCCCGAAAGAGTCTC-3′, TNF-α sense 5′-GCCTCTTCTCATTCCTGCTT-3′ and antisense 5′-CACTTGGTGGTTTGCTACGA-3′, TGF-β1 sense 5′-CCCTATATTTGGAGCCTGGA-3′ and antisense 5′-CTTGCGACCCACGTAGTAGA-3′, MMP9 sense 5′-GCCCTGGAACTCACACGACA-3′ and antisense 5′-TTGGAAACTCACACGCCAGAAG-3′, Wnt3A sense 5′-TTCTTACTTGAGGGCGGAGA-3′ and antisense 5′-ACCCGTATCCCAGACAGGA-3′, β-catenin sense 5′-ACTGCTGGGACTCTG-3′ and antisense 5′-TGATGGCGTAGAACAG-3′, LIG4 sense 5′-TTAGTTGTATCCGCACGCT-3′ and antisense 5′-TGTGAGGAAGCCATAGAAGC-3′, Claudin-3 sense 5′-AAGCCGAATGGACAAAGAA-3′ and antisense 5′-CTGGCAAGTAGCTGCAGTG-3′, Claudin-4 sense 5′-CGCTACTCTTGCCATTACG-3′ and antisense 5′-ACTCAGCACACCATGACTTG-3′, ZO-1 sense 5′-AGGACACCAAAGCATGTGAG-3′ and antisense 5′-GGCATTCCTGCTGGTTACA-3′, Occludin sense 5′-GCTGTGATGTGTGTGAGCTG-3′ and antisense 5′-GACGGTCTACCTGGAGGAAC-3′, c-myc sense 5′-ACACGGAGGAAAACGACAAG-3′ and antisense 5′-AGAGGTGAGCTTGTGCTCGT-3′, and β-actin sense 5′-TCCCTGGAGAAGAGCTATGA-3′ and antisense 5′-CGATAAAGGAAGGCTGGAA-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 $2^{-\Delta\Delta Ct}$ 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 2000*g* for