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Rebamipide inhibits indomethacin-induced small intestinal injury: Possible involvement of intestinal microbiota modulation by upregulation of α-defensin 5

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ABSTRACT

Enterobacteria play important roles in the pathophysiology of small intestinal injuries induced by nonsteroidal anti-inflammatory drugs (NSAIDs). We investigated the effects of rebamipide, a gastrointestinal mucoprotective drug, on indomethacin-induced small intestinal injuries, intestinal microbiota, and expression levels of α-defensin 5, which is a Paneth cell-specific antimicrobial peptide and is important for the regulation of intestinal microbiota. Indomethacin (10 mg/kg) was orally administered to mice after oral administration of rebamipide (100 or 300 mg/kg) or vehicle for 1 week, and the small intestinal injuries were assessed. After oral administration of rebamipide, the small intestinal contents were subjected to terminal restriction fragment length polymorphism (T-RFLP) analysis to assess the intestinal microbiota composition. Further, the expression levels of mRNA and protein for α-defensin 5 in the ileal tissue were determined by real-time reverse transcription–polymerase chain reaction and western blotting analysis, respectively. Rebamipide inhibited indomethacin-induced small intestinal injuries and T-RFLP analysis showed that rebamipide increased the percentage of *Lactobacillales* and decreased the percentage of *Bacteroides* and *Clostridium* than that in vehicle-treated controls. The mice that were treated with rebamipide showed an increase in α-defensin 5 mRNA expression and protein levels in the ileal tissue compared to vehicle-treated control mice. Indomethacin reduced expression of α-defensin 5 mRNA in ileal tissue, while rebamipide reversed expression of α-defensin 5 mRNA. In conclusion, our study results suggest that rebamipide inhibits indomethacin-induced small intestinal injuries, possibly by modulating microbiota in the small intestine by upregulation of α-defensin 5.

1. Introduction

Recent clinical studies using video capsule endoscopy and balloon-assisted endoscopy have revealed that gastrointestinal toxicities, including ulcerations and bleeding, are a common adverse effect in the small intestine after the use of nonsteroidal anti-inflammatory drugs (NSAIDs) (Goldstein et al., 2005; Maiden et al., 2005). Proton pump inhibitors have no inhibitory effects on NSAID-induced small intestinal injuries because this injury is independent of gastric acid. Moreover, the results of a recent animal experimental study suggest that proton pump inhibitors may exacerbate NSAID-induced intestinal injuries through the induction of dysbiosis, which is an imbalance of small intestinal commensal flora (Wallace et al., 2011).

Enterobacteria are regarded as an important factor in the pathophysiology of NSAID-induced small intestinal injuries. In a previous study, we demonstrated that ampicillin, which is an antibiotic, inhibit indomethacin-induced small intestinal injuries, and this is accompanied by a decrease in the number of enterobacteria those are invading the intestinal mucosa (Konaka et al., 1999). Toll-like receptor (TLR) 4, which is a receptor for lipopolysaccharide (LPS) originating from gram-negative bacteria, was shown to play a key role in NSAIDs-induced small intestinal injuries (Watanabe et al., 2008). In addition, we previously demonstrated that the probiotic *Lactobacillus casei* strain Shirota prevents indomethacin-induced small intestinal injuries (Watanabe et al., 2009). These results suggest that the condition of small intestinal microbiota greatly influence NSAID-induced small intestinal injuries.

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Rebamipide (2-(4-chlorobenzoylamino-3-[2(1*H*)-quinolinon-4-yl] propionic acid), which is a mucosal protective agent, has been used clinically for treating gastritis and peptic ulcers (Arakawa et al., 2005, 1998). The results of recent clinical studies indicate that rebamipide has preventive effects on NSAID-induced small intestinal injuries (Fujimori et al., 2011; Mizoguchi et al., 2001; Niwa et al., 2008). However, the precise mechanisms by which rebamipide suppresses NSAID-induced small intestinal injuries remain unknown.

In the present study, we investigated the effects of rebamipide on indomethacin-induced small intestinal injuries in mice and we demonstrated that rebamipide modulates small intestinal microbiota accompanied by upregulation of mouse α-defensin 5, also known as cryptdin 5, which has an important role in the regulation of the small intestinal microbiota.

2. Materials and methods

2.1. Animals and induction of small intestinal injury

Seven-week-old specific pathogen-free C57BL/6 mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). All animals were housed in polycarbonate cages with paper chip bedding in an air-conditioned biohazard room with a 12-h light-dark cycle. All animals had free access to food and water. All experimental procedures were approved by the Animal Care Committee of the Osaka City University Graduate School of Medicine. To induce small intestinal injury, 10 mg/kg indomethacin (Sigma Chemical Company, St. Louis, MO) with 0.5% carboxymethylcellulose was orally administered to nonfasted animals that were sacrificed 24 h later. For evaluation of macroscopic injury, 1% Evans blue was injected intravenously 30 min before sacrifice in order to delineate the injury, and the small intestine was collected and opened along the anti-mesenteric attachment. The areas (mm^2) of the macroscopically visible lesions were measured, summed per small intestine, and used as the lesion index. For histological evaluation, 10 mg/kg indomethacin was administered to animals that were sacrificed 24 h later and small intestinal tissue samples which possessed typical mucosal injury was fixed with 10% buffered formalin and 4-μm-thick tissue sections mounted on glass slide was subjected to hematoxylin and eosin (H&E) staining.

2.2. Histological evaluation of small intestinal damage

Tissue sections stained with H&E were viewed under a white-light microscope. For each mouse, at least 10 random villi at injured areas were scored in a masked fashion by two investigators independently (Y.N and T.W). For evaluation, we used a modified histological scoring system (Nadatani et al., 2012). The histology score ranged from 0 to 13 and was subdivided into the following six categories: epithelium (0=normal, 1=flattened, 2=loss of epithelial continuity, 3=severe denudation), villus shape (0=normal, 1=short and rounded, 2=extremely short and thick), villus tip (0=normal, 1=damaged, 2=severely damaged), stroma (0=normal, 1=slightly retracted, 2=severely retracted), inflammation (0=no infiltration, 1=mild infiltration, 2=severe infiltration), and crypt status (0=normal, 1=mild crypt loss, 2=severe crypt loss).

2.3. Experimental groups

In order to investigate the effects of rebamipide on small intestinal microbiota and indomethacin-induced small intestinal injury, mice received oral administration of vehicle (0.5% carboxymethylcellulose) or rebamipide (100 or 300 mg/kg body weight), which was supplied by Otsuka Pharmaceutical Co., Tokyo, Japan, once daily for 1 week before the administration of indomethacin. To clarify the effects of rebamipide on expression of mRNA for α-defensin 5, mice received oral administration of vehicle or rebamipide (100 or 300 mg/kg body weight) once daily for 1 day and 1 week. Studies were carried out using 6 to 8 animals.

2.4. DNA extraction and the molecular analysis of small intestinal microbiota using a 16S ribosomal DNA library and terminal restriction fragment length polymorphisms

DNA extraction from the contents of the lumen of the small intestine and the analysis of the small intestinal microbiota using a 16S ribosomal (*r*) DNA library and terminal restriction fragment length polymorphisms (T-RFLP) were outsourced to TechnoSuruga Laboratory Co, Ltd (Shizuoka, Japan). After ligation of the ileocecal junction with surgical thread in order to avoid contamination of the intestinal contents with colonic contents, the ileum was longitudinally incised, and the contents of the lumen of the ileum were collected. The contents were suspended in a buffer containing 4 M guanidium thiocyanate, 100 mM Tris–HCl (pH 9.0), and 40 mM Tris-EDTA and agitated in the presence of zirconia beads using a FastPrep FP100A Instrument (MP Biomedicals, Irvine, CA) to homogenize the samples. DNA was extracted from the bead-treated suspension using an automatic nucleic acid extractor (Precision System Science, Chiba, Japan). The reagent used for the automatic nucleic acid extraction was GC series Genomic DNA whole blood (Precision System Science). The 16S rRNA gene was amplified from the DNA with the fluorescent-labeled 516F primer (5′-TGCCAGCAGCCGCGGTA-3′; *Escherichia coli* positions 516 to 532) and the 1510R primer (5′-GGTTACCTTGTTACGACTT-3′; *E. coli* positions 1510 to 1492). The 5′-ends of the forward primers were labeled with 6′-carboxyfluorescein, which was synthesized by Applied Biosystems Japan, Ltd. (Tokyo, Japan). The polymerase chain reaction (PCR) amplifications of the DNA samples (10 ng of each DNA) were performed according to a protocol previously described (Nagashima et al., 2003). The PCR products were purified using MultiScreen PCR micro96 Plates (Millipore, Billerica, MA, USA).

The restriction enzymes were selected according to a protocol previously described (Nagashima et al., 2003). The purified PCR products were digested with 20 U of the restriction enzyme (*Bsl*I) at 55 °C for 1 h. The fragment analysis was conducted with the ABI PRISM 3130×1 genetic analyzer (Applied Biosystems) using analysis software (GeneMapper, Applied Biosystems). As a size standard marker, the MapMarker X-Rhodamine Labeled 50–1000 bp (BioVentures, TN, USA) was used. An operational taxonomic unit (OTU) was used to describe the clusters of clone sequences that differed from known species by about 2% and that were at least 98% similar to members of their cluster (Suau et al., 1999). Because the apparent size of identical terminal restriction fragments (T-RFs) can vary over a range of 1–3 bp among different gels and/or lanes of the same gel, major T-RFs that were similar in size within 1–3 bp were summarized to OTUs (Nagashima et al., 2003). The major T-RFs were identified by computer simulation, which was performed using a T-RFLP analysis program and a phylogenetic assignment database for the T-RFLP analysis of human colonic microbiota (Matsumoto et al., 2005).

Hierarchical cluster analysis was performed using Gene Maths (Applied Maths, Kortijk, Belgium) in order to measure the similarities in the flora patterns between the samples, and the results were represented graphically with dendrograms. A Pearson's similarity coefficient analysis and the unweighted pair-group