Exacerbation of indomethacin-induced small intestinal injuries in Reg I-knockout mice

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NONSTEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs) are known to damage the upper gastrointestinal (GI) tract as a serious adverse event (1). Recent advances in diagnostic methods, including double-balloon endoscopy (DBE) and capsule endoscopy (CE), enable examination of the entire small intestine (28, 44). Studies using these techniques have shown that the prevalence of small bowel mucosal breaks in patients receiving NSAIDs is higher than that reported previously (13, 26, 27). Although the clinical importance of NSAID-induced small intestinal injuries has been acknowledged, suitable therapeutic strategies have not been sufficiently established. Recently, animal models with NSAID-induced small intestinal injuries have been successfully employed to reveal the mechanisms of NSAID-mediated tissue damage, as well as to delineate the effectiveness of several essential endogenous and exogenous factors for therapy (32, 49). However, owing to the complex mechanism of small intestinal damage induced by NSAIDs, a successful therapeutic strategy has not been clearly established.

The regenerating gene (Reg) was originally isolated from rat regenerating pancreatic islets in 1988. Reg I (regenerating product I), one of four Reg subfamilies (14, 29, 30), is expressed in the GI tract of various species and plays important roles not only in regeneration but also in proliferation, inflammation, and carcinogenesis (36, 48). We previously found that Reg I is a potent regulator of cell growth that is required to generate and maintain the villous structure of the small intestine (31). These findings suggest that Reg I may be important for establishment of new therapeutic strategies for NSAID-induced small intestinal injuries on the basis of its abilities to control cell proliferation, and maintain intercellular integrity and gut homeostasis. In the present study, we used Reg I-knockout (KO) mice to evaluate the role of Reg I and its therapeutic potential in NSAID-induced small intestinal injuries. Our results indicate that Reg I plays a key role in the pathogenesis of NSAID-induced small intestinal injuries and may be useful as a therapeutic agent.

MATERIALS AND METHODS

Animals. All of the following experimental procedures were approved by the animal care committee of Shimane University Graduate School of Medicine. ICR mice (Charles River Japan, Kanagawa, Japan) were used for the experiments to evaluate Reg I expression in the small intestine and the efficacy of recombinant Reg I (rReg I) in mice with indomethacin administration. Reg I-KO (Reg I−/−) mice and wild-type (Reg I+/+) littermates were used in the experiment to evaluate the role of Reg I in indomethacin-induced small intestinal injuries. The Reg I−/− mice were generated on an ICR background as previously described (46). All mice were housed in cages with chopped paper bedding in an air-conditioned biohazard room with a 12-h light-dark cycle and allowed access to food and water.

Reg I mRNA expression in small intestine of wild-type mice with indomethacin administration. Initially, we determined the time course changes of Reg I mRNA expression in the small intestine of wild-type mice after administration of indomethacin. Ten wk-old male ICR mice were injected subcutaneously twice, with a 24-h interval, with indomethacin at a dose of 80 mg/kg body wt dissolved in 5% nonsteroidal anti-inflammatory drug; regenerating gene product I; intestinal permeability

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using an optical microscope. For this evaluation, we used a previously blinded to the treatment groups independently assessed each section for histopathological evaluation by hematoxylin-eosin staining. Two observers were trained by using the image analysis software Photo

Table 1. Criteria for histological damage score

<table>
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<tr>
<th>Score</th>
<th>Width of ulceration</th>
<th>Depth of lesion</th>
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<tr>
<td>0</td>
<td>No ulcer</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Small ulcer (&lt;3 mm)</td>
<td>Not visible</td>
</tr>
<tr>
<td>2</td>
<td>Large ulcer (&gt;3 mm)</td>
<td>Visible</td>
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Microscopic evaluation of small intestinal injuries. For histological examinations, injured segments of the small intestine were trimmed, fixed in 10% formalin, and processed as a tissue block for histopathological evaluation by hematoxylin-eosin staining. Two observers blinded to the treatment groups independently assessed each section using an optical microscope. For this evaluation, we used a previously proposed histological damage scoring system. As shown in Table 1, the histological damage score included the following four factors: width of the ulceration, depth of the lesion, degree of inflammatory infiltrate, and presence of thrombi.

Inflammatory parameters of small intestinal injuries. For assessment of proinflammatory agents, production of IL-1β and MPO in the experimental tissues was measured by enzyme immunoassay (EIA). First, the small intestinal mucosal layer was scraped off, weighed, and ground with liquid nitrogen-chilled mortar and pestles. The tissue was then completely lysed with 200 μl of lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycine, 1 mM PMSF, 1 μg/ml leupeptidase, and 28 μg/ml aprotinin) for every 10 mg of tissue. Tissue lysates were centrifuged at 13,000 rpm at 4°C for 20 min and the supernatants were stored at −70°C until being assayed. The amounts of IL-1β and MPO were assessed by using EIA kits for IL-1β (R&D Systems, Minneapolis, MN) and MPO (Hycult Biotechnology, Uden, Netherlands), respectively.

Therapeutic efficacy of recombinant Reg I protein in indomethacin-induced small intestinal injuries. The therapeutic potential of Reg I was assessed by administration of recombinant Reg I protein (rReg I). Briefly, rReg I was prepared using a mammalian expression system in which the recombinant Reg I gene was inserted into the expression vector pET28a (Novagen). The recombinant protein was purified by affinity chromatography and characterized by mass spectrometry. The protein was then administered intraperitoneally to the mice at a dose of 1 mg/kg body weight. The mice were euthanized at 3, 6, 12, or 24 h after the last injection of indomethacin and the small intestines were dissected. The intestines were then disaggregated into single-cell suspensions and the expression of Reg I mRNA was measured by real-time PCR. Error bars indicate the standard error of mean values.

Fig. 1. Regenerating gene (Reg) I mRNA expression and histological findings in wild-type mice with indomethacin-induced small intestinal injuries. Ten-week-old male ICR mice were injected subcutaneously twice with indomethacin, with a 24-h interval, then 24 h after the last injection. A: time course changes of Reg I mRNA expression in the small intestines of wild-type mice after second injection of indomethacin. Total RNA was extracted from each small intestine sample and Reg I mRNA expression was evaluated by real-time PCR. Error bars indicate the standard error of mean values. B: representative histological images of small intestine samples from the experimental mice (hematoxylin-eosin, original magnification ×40).
our laboratory as described previously (16) and was diluted in distilled water. The rReg I solution or vehicle (distilled water) was injected into 10-wk-old male ICR mice at a dose of 500 ng/body into the lateral tail vein. Six hours after rReg I administration, indomethacin or the vehicle (5% NaHCO₃) was injected subcutaneously twice, with a 24-h interval, at a dose of 80 mg/kg of body wt dissolved in 5% NaHCO₃. Twenty-four hours after the last indomethacin injection, the animals were euthanized and small intestinal injuries were assessed with respect to the area of macroscopically visible ulcers or hemorrhagic lesions, the length of the small intestine, histopathology, and production of IL-1β and MPO in the experimental tissues.

Effect of rReg I protein on IL-1β production by macrophages. To evaluate the effect of rReg I protein on IL-1β production, an in vitro system was established. The mouse macrophage cell line RAW264.7 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco’s modified Eagle medium (Invitrogen, CA), supplemented with 10% fetal bovine serum (ICN Biomedicals, Aurora, OH) and penicillin-streptomycin-amphotericin B (Invitrogen). Cells were cultured in 24-well plates (5 x 10⁴ cells/well) pretreated with Reg I protein (1 ng/ml) for 6 h, then stimulated with lipopolysaccharide (LPS; 100 ng/ml) (Invivogen) for 24 h. IL-1β contents in the cell culture supernatants were determined by using an IL-1β EIA kit (R&D Systems).

Statistical analysis. All values represent means ± SE. Since all data had statistically nonnormal distributions, nonparametric statistical methods were used to analyze the data. Comparisons between two groups were made by a Mann-Whitney U-test. A P value of <0.05 was considered to be statistically significant and all P values are two sided. Data were analyzed via statistical software IBM SPSS Statistics for Macintosh version 18.0 (SPSS, Chicago, IL).

RESULTS

Increased Reg I expression in the small intestine of wild-type mice after indomethacin administration. First, we examined the time course of changes in Reg I mRNA expression in the small intestine of wild-type and Reg I–/– mice after indomethacin administration. Ten-week-old male Reg I+/+ mice and Reg I–/– littermates were injected subcutaneously twice with indomethacin, with a 24-h interval, then 24 h after the last indomethacin injection the small intestines were dissected. A: gross findings of small intestines. B: macroscopically visible injured lesions. C: histological findings (hematoxylin-eosin, original magnification ×40).

Fig. 2. Representative images of small intestines dissected from Reg I+/+ and Reg I–/– mice at 24 h after the last injection of indomethacin or the vehicle. Ten-week-old male Reg I+/+ mice and Reg I–/– littermates were injected subcutaneously twice with indomethacin, with a 24-h interval, then 24 h after the last indomethacin injection the small intestines were dissected. A: gross findings of small intestines. B: macroscopically visible injured lesions. C: histological findings (hematoxylin-eosin, original magnification ×40).
small intestines of wild-type mice and compared these with microscopic findings to clarify the pattern of Reg I expression in indomethacin-induced small intestinal injuries. A single dose of indomethacin treatment was not adequate to create significant injuries in the intestinal mucosa (data not shown); thus we gave two doses of indomethacin with a 24-h interval and noted that Reg I mRNA expression rapidly increased and peaked at 6 h after the second indomethacin injection, followed by a gradual decline toward a normal level (Fig. 1A).

Similar to the findings noted above, histological assessment did not reveal any significant damage in the intestinal tissues after the first dose of indomethacin (data not shown), whereas those were clearly distinguished as early as 6 h after the second injection, and the small intestinal mucosa showed an altered villous morphology with disarrangement. Mucosal damage was maximal at 12 h and histopathological examination showed development of small intestinal ulcers (Fig. 1B). These results indicate that after indomethacin administration, the expression of Reg I mRNA is upregulated in the early phase of small intestinal injuries, followed by the development of small intestinal ulcers.

**Exacerbation of indomethacin-induced small intestinal injuries in Reg I-KO mice.** On the basis of speculation that upregulated Reg I plays some role in the pathogenesis of indomethacin-induced small intestinal injuries, we administered indomethacin to both Reg I−/− and Reg I+/+ mice. Figure 2, A–C, shows representative pictures of small intestines dissected from the experimental mice at 24 h after the last injection of indomethacin or the vehicle. The small intestines of the indomethacin-treated mice were filled with melena, and the length of the small intestine in the indomethacin-treated Reg I−/− mice was significantly shorter than that in the indomethacin-treated Reg I+/+ mice (Fig. 3A; P < 0.01). Administration of indomethacin provoked lesions in the small intestine, whereas both vehicle-treated Reg I−/− and Reg I+/+ mice had no small intestinal lesions (Fig. 2B).

The area of macroscopically visible lesions in the Reg I−/− mice was significantly greater than that in the Reg I+/+ mice at 24 h after the last injection of indomethacin (Fig. 3B; P < 0.01). Histological examinations revealed deep ulcers with prominent inflammatory cell infiltration and vascular thrombosis present in both Reg I−/− and Reg I+/+ mice (Fig. 2C). However, this trend was more conspicuous in Reg I−/− mice, and the histological damage scores of indomethacin-treated Reg I−/− mice were significantly higher than those of Reg I+/+ mice (Fig. 3C; P < 0.05). In contrast, histological examinations showed no active inflammation in either vehicle-treated Reg I−/− or Reg I+/+ mice.

Since the scores assessed by histopathological examinations were significantly different between Reg I−/− and Reg I+/+ mice, we further evaluated the expression of proinflammatory cytokines and MPO in the small intestine samples obtained from indomethacin- or vehicle-treated mice. Twenty-four hours after the second indomethacin injection, both IL-1β and MPO contents in the mucosa were significantly higher in Reg I−/− mice than in Reg I+/+ mice (Fig. 3, D and E; P < 0.05), whereas, as expected, those were unchanged in vehicle-treated mice. These findings suggest that Reg I may play a protective role in indomethacin-induced small intestinal injuries.

**Inhibitory effect of rReg I on indomethacin-induced small intestinal injuries.** On the basis of the above experiments using Reg I-KO mice, we speculated that rReg I administration might prevent indomethacin-induced small intestinal injuries. To test our speculation, we injected rReg I into the mouse model of indomethacin-induced small intestinal injuries. Figure 4, A–C, shows the gross and microscopic findings of the small intestines from both rReg I-treated mice and vehicle-treated mice at 24 h after the last indomethacin injection. Although the small intestines of the experimental animals were shortened, this shortening in rReg I-treated mice was attenuated compared with vehicle-treated mice (Fig. 5A; P < 0.01). The area of small intestinal injuries in the rReg I-treated mice was significantly smaller than that in the vehicle-treated mice (Fig. 5B; P < 0.01). Microscopically, more shallow ulcers with less inflammatory cell infiltration were observed in the rReg I-treated mice. Consequently, the histological damage scores for the rReg I-treated mice were lower than those of the vehicle-treated mice (Fig. 5C; P < 0.05).
The mucosal content of IL-1β after indomethacin administration in the rReg I-treated mice was also significantly lower than that in the vehicle-treated mice (Fig. 5D; \( P < 0.05 \)). Although the mucosal content of MPO also tended to be lower in the rReg I-treated mice than the vehicle-treated mice, this difference was not statistically significant (data not shown). In this model, small intestinal injuries were inhibited in rReg I-treated mice, suggesting that rReg I treatment has some preventive effect against small intestinal injuries induced by indomethacin administration.

**Inhibitory effect of rReg I protein on LPS-induced IL-1β production by macrophages.** Indomethacin treatment causes intestinal tissue damage in vivo, which permits interactions between luminal bacterial components and mucosal immune cells including macrophages to induce innate immune responses. Since IL-1β is mainly produced by infiltrating macrophages in injured intestinal mucosa, we used a mouse macrophage cell line, RAW264.7, in an in vitro experiment to determine whether rReg I protein directly inhibits LPS-induced IL-1β production by macrophages. As shown in Fig. 6, the macrophages were responsive to LPS stimulation and produced abundant IL-1β, whereas LPS-induced IL-1β production by cells pretreated with rReg I was significantly lower than that by nontreated macrophages.

**DISCUSSION**

In the present study, we analyzed Reg I expression and demonstrated its role in the pathogenesis of NSAID-induced small intestinal injuries. Reg I mRNA expression rapidly increased up to 6 h after indomethacin administration and reached a peak prior to development of small intestinal ulcers. *Reg I<sup>−/−</sup>* mice developed more severe indomethacin-induced small intestinal injuries than *Reg I<sup>+/+</sup>* mice. Furthermore, rReg I administration inhibited the development of indomethacin-induced small intestinal injuries. This therapeutic...
effect of rReg I was confirmed morphologically and biochemically. These results imply that Reg I may be useful as a therapeutic agent for indomethacin-induced small intestinal injuries.

NSAIDs are the most widely used drugs for various acute or chronic conditions, such as relief of pain and inflammation, prevention of colorectal cancer (3, 15, 34, 43), and treatment of cancer (20) and cardiovascular disease (4, 45). On the other hand, their use is strongly associated with a broad spectrum of unexpected adverse effects in various organs (24). Particularly, GI injuries are the most common adverse events of these agents (10, 23, 37). In addition to gastric injuries, which are known as a classical adverse effect of NSAIDs, it is becoming widely accepted that NSAIDs are also responsible for small intestinal injuries. NSAID-induced small intestinal injuries result in various manifestations, such as ulcers, bleeding, and protein-losing enteropathy (6), which are sometimes critical, especially in elderly NSAID users. Recent epidemiological reports have suggested that NSAID-induced small intestinal injuries are more frequent than previously thought. Graham et al. (13) reported that examinations using CE revealed small intestinal injuries in 71% of chronic NSAID users. Maiden et al. (26) also used CE and determined that a 2-wk administration of an NSAID caused small intestinal injuries in 68% of healthy volunteers. Matsumoto et al. (27) reported that examinations using DBE revealed NSAID-induced small intestinal injuries in 51% of NSAID users. Although the clinical importance of NSAID-induced small intestinal injuries is emerging, a suitable therapeutic strategy has not been fully established. This difficulty is mainly due to the complicated mechanism of NSAID-induced small intestinal injuries.

The main mechanism of NSAID-induced gastric injuries is inhibition of prostaglandin (PG) synthesis transmitted by both cyclooxygenase (COX)-1 and COX-2 (25). In the small intestine, NSAIDs inhibit COX-1 and COX-2 (39); however, various other specific factors are also associated with the development of mucosal injuries. Numerous studies have demonstrated that enterohepatic recirculation of NSAIDs is more important than suppression of PG synthesis in the pathogenesis of small intestinal injuries (32). NSAIDs enter epithelial cells via damage to the brush border and cause increased intestinal perme-

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**Fig. 5.** rReg I treatment attenuated indomethacin-induced small intestinal injuries. Shown are the length of the small intestine (A), the area of macroscopically visible injured lesions (B), histological damage scores (C), and tissue content of IL-1β in the experimental mice (D). For measurement of IL-1β, total protein was extracted from each small intestine tissue sample. IL-1β content was evaluated using EIA. Error bars indicate the standard error of mean values obtained independently from 5 mice. #P < 0.05, *P < 0.01 vs. vehicle-treated mice.

**Fig. 6.** Inhibitory effect of rReg I protein on IL-1β production by macrophages. RAW264.7 cells were cultured in 24-well plates (5 × 10⁴ cells/well) with or without rReg I protein for 6 h, then stimulated with LPS (100 ng/ml) for 24 h. IL-1β contents in the cell culture supernatants were measured by EIA. Error bars indicate the standard error of mean values obtained from 3 independent experiments. #P < 0.05 vs. nontreated cells.
ability (41). Elevated intestinal permeability facilitates the entrance of luminal agents including bacteria (5, 11, 19), bile, pancreatic juice, and various toxins, which promote the release of proinflammatory mediators from the epithelial cells with subsequent production of reactive oxygen species, nitrogen intermediates, and proteolytic enzymes by infiltrating neutrophils (42). Once the mucosal barrier is disrupted by the effects of the NSAID, mucosal damage is accelerated via the above-mentioned steps. Thus intestinal permeability is now regarded as a key element in the development of NSAID-induced small intestinal injuries (40).

Reg I was initially isolated as a growth factor from a cDNA library of rat regenerating pancreatic islets. Reg I expression has also been identified outside of the pancreas, particularly in gastric mucosa. We previously reported that Reg I protein is mainly expressed in gastric fundic enterochromaffin-like cells in the stomach and that its production is upregulated by a variety of stimuli, including gastrin, proinflammatory cytokines, water immersion restraint stress, and indomethacin administration (2, 17, 18, 21). These studies with experimental animal models clarified that Reg I protein is a potent growth factor of gastric epithelial cells and plays an important role in gastric mucosal regeneration. More recently, we evaluated the intestinal tracts of Reg I−/− mice and found that Reg I is an essential regulator of cell growth that is required to generate and maintain the villous structure of the small intestine. In that study, immunohistochemistry detected epithelial cells in the lower half of the intestinal villi expressing Reg I, and pathological analysis using electron microscopy revealed that attachment of villous epithelial cells to the basement membrane in Reg I−/− mice appeared weaker compared with that of Reg I+/+ (31). These findings suggest that Reg I plays essential roles in the maintenance of intercellular integrity and that its deficiency may lead to increased permeability of the small intestine. Because NSAID administration causes increased intestinal permeability, we speculated that Reg I may exert certain protective effects in indomethacin-induced small intestinal injuries.

On the basis of the above speculation, we initially evaluated Reg I expression in the small intestine of indomethacin-treated wild-type mice. In this model, Reg I expression was increased in the early phase of small intestinal injury after indomethacin administration, which is similar to our previous result obtained from an experimental model with indomethacin-induced gastric injury. This finding suggests that Reg I may also function as a protective factor in the small intestine, as well as in the stomach, against indomethacin-induced mucosal injuries (17). To understand the precise role of Reg I in indomethacin-induced small intestinal injuries, we designed additional experiments using Reg I−/− mice. Reg I deficiency significantly exacerbated shortening of the small intestines of the mice and increased the area of macroscopically visible injured lesions induced by indomethacin administration. Histological scores were also markedly increased in indomethacin-treated Reg I−/− mice, which coincided well with our findings regarding the small intestine tissue contents of IL-1β and MPO. Furthermore, our in vitro results clearly indicated that LPS-induced IL-1β production by macrophages pretreated with rReg I protein was significantly lower than that by nontreated macrophages. Together, these findings support our view that Reg I plays a protective role in indomethacin-induced small intestinal injuries.

Various strategies for the prevention and treatment of NSAID-induced small intestinal injuries have been studied for a long time. Bjarnason et al. (8, 9) showed that PGE1 attenuates indomethacin-induced increased intestinal permeability in humans. The effects of antibiotics have also been well examined, since Robert et al. (33) reported germ-free rats exhibiting resistance to indomethacin-induced small intestinal injuries. They demonstrated that treatment with antibiotics such as kanamycin sulfate (22) and metronidazol (7) maintains intestinal permeability and inhibits indomethacin enteropathy. Saud et al. (35) recently reported that administration of anti-TNF-α monoclonal antibody reduces inducible nitric oxide synthase expression and improves indomethacin-induced enteropathy in rats. Although such previous studies may provide better therapeutic options for indomethacin-induced injuries of the small intestine, they are not as entirely effective as expected. In the present study, we observed increased Reg I expression in the small intestine of wild-type mice after indomethacin administration; however, it did not completely inhibit the development of mucosal injury. On the basis of this protective effect of Reg I against small intestinal injuries induced by indomethacin, we evaluated the therapeutic potential of rReg I in wild-type mice with indomethacin administration. Treatment with rReg I significantly decreased the pathological damage scores and production of inflammatory mediators in the small intestines of indomethacin-treated mice. These results suggest that rReg I may become an effective tool for preventing NSAID-induced small intestinal injuries. Although we did not find mechanistic evidence in the present study showing Reg I–mediated therapeutic potential for indomethacin-induced small intestinal injury, it is possible that the extraordinary role of Reg I in cell proliferation and maintenance of intracellular integrity may be involved in this process (2, 17, 18, 21, 31). Furthermore, Reg I may also be effective against other small intestinal injuries such as inflammatory bowel disease, since it has been demonstrated that Reg I is upregulated and promotes cell proliferation under other stress conditions (12). Nevertheless, additional investigations addressing the in vivo efficacy and safety should be carefully performed before rReg I can be considered for use in a clinical therapeutic strategy.

In summary, we investigated the role of Reg I and its therapeutic potential in indomethacin-induced small intestinal injuries in mice. The present results show for the first time that Reg I has a protective role against NSAID-induced small intestinal injuries and that rReg I effectively attenuates such injuries. Additional analysis of Reg I function in the small intestine may contribute to the development of a new therapeutic strategy for NSAID-induced small intestinal injuries.

DISCLOSURES
The authors have no conflicts of interest to disclose.

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