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Mucus reduction promotes acetyl salicylic acid-induced small intestinal mucosal injury in rats

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ABSTRACT

Background: Acetyl salicylic acid (ASA) is a useful drug for the secondary prevention of cerebrocardiovascular diseases, but it has adverse effects on the small intestinal mucosa. The pathogenesis and prophylaxis of ASA-induced small intestinal injury remain unclear. In this study, we focused on the intestinal mucus, as the gastrointestinal tract is covered by mucus, which exhibits protective effects against various gastrointestinal diseases.

Materials and Methods: ASA was injected into the duodenum of rats, and small intestinal mucosal injury was evaluated using Evans blue dye. To investigate the importance of mucus, Polysorbate 80 (P80), an emulsifier, was used before ASA injection. In addition, rebamipide, a mucus secretion inducer in the small intestine, was used to suppress mucus reduction in the small intestine of P80-administered rats.

Results: The addition of P80 reduced the mucus and exacerbated the ASA-induced small intestinal mucosal injury. Rebamipide significantly suppressed P80-reduced small intestinal mucus and P80-increased intestinal mucosal lesions in ASA-injected rats, demonstrating that mucus is important for the protection against ASA-induced small intestinal mucosal injury. These results provide new insight into the mechanism of ASA-induced small intestinal mucosal injury.

Conclusion: Mucus secretion-increasing therapy might be useful in preventing ASA-induced small intestinal mucosal injury.

1. Introduction

The antithrombotic effects of acetyl salicylic acid (ASA) are used in the secondary prevention of cardiovascular diseases [1–3]. The most common side effect of ASA is gastrointestinal mucosal injury. Video capsule endoscopy and balloon-assisted endoscopy allow us to visualize the small intestine in greater detail; hence, the incidence of ASA-induced small intestinal mucosal injury has been reported more often [4–6].

We have reported that nonsteroidal anti-inflammatory drugs (NSAIDs), including ASA, induce damage to small intestinal epithelial cells in an in vitro model [7–10]. In the initial stage of ASA-induced small intestinal mucosal injury, malfunctioning of the tight junction causes mucosal barrier dysfunction [7]. However, not all ASA users develop ASA-induced mucosal injury. No experimental evidence has indicated the difference among individual ASA users.

In this study, we focused on the role of intestinal mucus, because a considerable amount of evidence indicated its importance in maintaining mucosal homeostasis. Mucus covers and protects the intestinal epithelium from bacteria by promoting their clearance and separating them from the epithelial cells, thereby inhibiting inflammation and infection as a “first line of defense” [11–13]. Gastrointestinal diseases, such as peptic gastric ulcer [14], infectious enteritis [12], and ulcerative colitis [15,16], were reported to be associated with mucus reduction. Therefore, we hypothesized

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that reduction of intestinal mucus is one of the major reasons for ASA-induced small intestinal mucosal injury. In this study, we aimed to elucidate the importance of mucus in the pathogenesis of ASA-induced small intestinal mucosal injury.

2. Materials and Methods

2.1. Experimental animals

Nine-week-old male Sprague-Dawley (SD) rats weighing 340–380 g were obtained from Shimizu Laboratories (Kyoto, Japan) and were housed at 22 °C in a controlled environment, with 12 h of artificial light per day. They were fed and had free access to drinking water before the experiments. All experiments were carried out in accordance with the National Institutes of Health (NIH) guidelines for the use of experimental animals, and the protocols were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan).

2.2. Experimental procedure

The protocol for ASA-induced small intestinal mucosal injury in rats was reported and described by Nonoyama et al. [17,18]. We used a modified model to study the protective effect of mucus in ASA-induced small intestinal injury. Briefly, under isoflurane, the abdomen of each rat was incised, and the duodenum was exposed. Different concentrations of ASA (0, 50, 100, and 200 mg/kg; Sigma-Aldrich, St. Louis, MO) suspended in 0.5% carboxymethyl cellulose (CMC; Wako Pure Chemical Industries Ltd, Kyoto, Japan) were injected into the proximal duodenum with an 18-gauge needle. Sixty minutes after ASA injection, 1% Evans blue was injected into a penile vein to easily detect mucosal damage; this method was modified from Nonoyama et al. [17]. Thirty minutes after Evans blue injection, the entire small intestine was removed, cut open, and divided into eight equal parts.

In some experiments, Polysorbate 80 (P80; Sigma-Aldrich, St. Louis, MO), a representative emulsifier, was given to rats via drinking water (1%) for 2 weeks, according to previous studies [19–21].

2.3. Assessment of ASA-induced small intestinal mucosal injury

To assess ASA-induced small intestinal mucosal injury, we used the following methods:

1. Measurement of Evans blue–stained area: All rats received intra-duodenal ASA treatment, followed by Evans blue. ASA induces vascular hyperpermeability in damaged mucosal areas, resulting in leakage of Evans blue from the vascular lumen into the mucosa [22]. We quantified the damaged mucosal areas by measuring the amount of Evans blue leakage. The removed small intestine was cut open and divided into eight equal parts. The samples were photographed, and the area stained with Evans blue was measured as the damaged area using Image J 1.4 software (NIH, Bethesda, MD). Each group was compared using the Evans blue lesion index (%) (Evans blue–stained area/total small intestinal area).
2. Quantification of Evans blue leakage: Evans blue leakage was measured using a modified protocol described by Lu et al. [23]. Briefly, 1 h after the ASA treatment, Evans blue was injected into a penile vein. In our model, 30 min after Evans blue injection, the chest was accessed through the diaphragm; a catheter was placed into the inferior vena cava, and the left ventricle was opened. Phosphate-buffered saline (PBS) was perfused into the inferior vena cava until it came clear out of the left ventricle. Subsequently, a 5-cm-long mucosa of the first part of the small intestine, 10 cm to the pylorus, was removed from all rats. The stripped mucosa from the removed tissue was incubated in 1 ml of formaldehyde and then homogenized with an ultrasonic cell disruption device (digital homogenizer; Iuchi Corporation, Osaka, Japan). Another 3 ml of formaldehyde was added to the homogenized tissue for preservation. After being maintained at room temperature for 48 h, the samples were centrifuged (1000 rpm, 5 min), and the absorbance of the supernatants was measured at the wavelength of 650 nm using a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA). After the Evans blue standard curve was plotted, the Evans blue content was calculated based on its concentration in the small intestinal tissue protein and was presented as μg/μg protein.
3. Histological scoring: We evaluated ASA-induced small intestinal mucosal injury using the histological scoring method of Nonoyama et al. [17]. Briefly, after ASA treatment, the entire small intestine was removed, and the proximal 10-cm section was discarded because it was damaged by the needle prick and the subsequent ASA injection. The histological samples (each 10 cm) were then fixed in formalin solution. After the slides were decoded, representative sections for treatment were photographed under low-power microscopy. The damaged areas were classified as follows: 0, normal; 1, weak damage; 2, severe damage. The sum score of these 10-cm sections at × 100 magnification was the designated histological score for each individual rat. The total score was calculated by an average of the histological score per rat in a designated concentration category (0, 50, 100, and 200 mg/kg).

2.4. Qualification of intestinal mucus (Alcian blue staining and MUC2 staining)

Some emulsifiers reduce mucus thickness [19–21] by decreasing the viscosity and promoting liquefaction of the mucus [24]. Therefore, in this study, rats were administered with P80, one of the representative emulsifiers, via drinking water (1%) for 2 weeks to reduce mucus in the small intestine. The samples (5-cm long) that were 10 cm proximal to the pylorus were removed, and the samples from the rats administered with P80 were fixed in Carnoy (Wako). The samples were stained with Alcian blue (Wako) and MUC2 (rabbit polyclonal antibody; Santa Cruz Biotechnology, CA, USA) to visualize the intestinal mucus, and the amount of mucus between sham and P80-administered rats was compared.

2.5. Quantification of intestinal mucus (periodic acid-Schiff–positive substance)

The 5-cm-long samples 10 cm proximal to the pylorus were removed, and the amount of mucus (periodic acid-Schiff [PAS]–positive substance) was measured; this method was reported by Garcia et al. [25,26]. In brief, the small intestines were removed, homogenized, and suspended in 1 ml of PBS. At 4 °C, the homogenized samples were centrifuged at 15,000 rpm for 15 min. Periodic acid (0.1%, 100 μl) was pipetted into each sample and incubated at 37 °C for 2 h. Subsequently, 100 μl of Schiff reagent (Sigma-Aldrich) was added and incubated at room temperature for 30 min. The optical density of the resulting solution at the 555-nm wavelength was taken as the amount of PAS-positive product. The amount of mucus in each sample was calculated based on the calibration curve of known concentrations of pig gastric mucin (Sigma-Aldrich).

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