BPB Reports

Report

Effects of Indomethacin Administration on the Small Intestines of 40-Week-Old Mice

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Received June 15, 2023; Accepted July 19, 2023

It is well known that administration of non-selective cyclooxygenase (COX) non-steroidal anti-inflammatory drugs (NSAIDs) increases the risk of developing gastric and duodenal ulcers. Previously, we reported that administration of indomethacin, one of these COX non-selective drugs, results in acute inflammation of the small intestine and that the subsequent increased release of cytokines, such as interleukin-6 and tumor necrosis factor-α, and histamines can lead to the development of dry skin. However, the effect of indomethacin administration in aging mice remains unknown. We aimed to investigate the effect of indomethacin administration on the small intestine of 40-week-old adult mice. As a result, both macroscopic and histologic abnormalities were observed in their small intestines. Expression of diamine oxidase, a histamine-degrading enzyme in the jejunum, was also decreased. In addition, expressions of mucin-2 and zonula occludens-1, both of which regulate the intestinal barrier function, were also decreased. Therefore, it was suggested that administration of indomethacin to aging mice may cause the release of histamines, which consequently increase in concentration in the small intestine, infiltrate into the blood, and circulate throughout the body as the intestinal barrier function declines.

Key words indomethacin, diamine oxidase, mucin-2, zonula occludens-1

INTRODUCTION

In super-aging societies like Japan, non-steroidal anti-inflammatory drugs (NSAIDs) are widely administered to elderly patients not only for immune diseases such as rheumatism, but also for lower back and joint pain caused by sarcopenia.[1)] Since January, 2020, the incidence of sarcopenia in the elderly has progressed rapidly due to self-restraint to prevent the spread of the new coronavirus infection, leading to an increase in the frequencies of falls and compression fractures, as well as consequent administration of NSAIDs.[2)] NSAIDs exert their therapeutic effect by inhibiting cyclooxygenase (COX) and suppressing prostaglandin (PG) production.[3)] COX has two isoforms, COX-1, COX-2.[4)] Selective COX-1 inhibitors include ketoprofen, which is only available for external administration, and indomethacin, which has both internal and external routes of administration. Celecoxib and etodolac are two examples of selective COX-2 inhibitors.

Furthermore, it is well known that non-selective COX NSAIDs increase a patient's risk of developing gastric and duodenal ulcers as a result of their suppression of PG production.[5,6)] While there are few subjective symptoms, inflammation is known to develop in the mucosa of the small intestine prior to the formation of gastric or duodenal ulcers.[7,8)] As previously described, administration of indomethacin caused inflammation of both the jejunum and ileum as well as an increase in mast cells, followed by an increase in the expression of inflammatory cytokines and histamines, and their mediators, which entered the bloodstream and were involved in the development of dry skin.[9,10)] It has also been elucidated.

Histamines are known mediators of the symptoms associated with hay fever and atopic dermatitis.[11)] Since the 1980s, the enzyme diamine oxidase (DAO) has attracted attention to its therapeutic potential. Specifically, DAO catalyzes oxidative deamination[12)] and is also a major enzyme involved in the degradation of histamines.[12)] Additionally, its primary site of action is within the intestines, which also function as a DAO storage organ. Indeed, it is thought that intestinal DAO plays a role in regulating the effects of histamines on the whole body.[13,14)] Moreover, DAO is suggested to play an important role in histamine intolerance (HIT), specifically through its reduced activity.[13,14)] Despite the potential associations between the administration of non-selective COX NSAIDs, changes in DAO activity, effects of histamines, and aging, little relevant research has been published.

In this study, we administered indomethacin, a non-selective COX NSAID, to adult mice to induce acute inflammation in the small intestine, investigating its effect on DAO activity and the barrier function of the small intestine.

MATERIALS AND METHODS

Animals and Experimental Design First, 9- and 40-week-old specific-pathogen-free (SPF) hairless male mice (HOS:HR-1) were purchased from SLC (Hamamatsu, Shizuoka, Japan). The mouse rearing environment was maintained at a temperature of 23 ± 2°C and 55 ± 10% humidity with a 12-h light-dark cycle. The mice were fed laborato-

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ry chow (CE-2; Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*. The mice were randomly assigned to one of two groups (n=4 per group): control or oral administration of 40 mg/kg indomethacin (Wako, Osaka, Japan) once daily for four days. This dosage of indomethacin (40 mg/kg) was selected based on our preliminary examination of the method of Maruyama *et al.* [9] and is the maximum concentration at which no adverse health changes (weight loss, behavioral abnormalities) were observed upon long-term administration to mice. Delivery vehicle was administered to the control mice according to the same schedule. This study strictly followed the recommendations and guidelines for the care and use of laboratory animals at the Suzuka University of Medical Science (Approval No. 34). In this study, further efforts were made to minimize animal suffering in all surgical procedures.

Tissue Staining On the final day of administration, the small intestine (jejunal part) was collected from each mouse 6 h after the final administration. Jejunal samples were fixed with 4% formaldehyde (Wako) in phosphate-buffered saline and were cut into 6-μm-thick sections. The sections were stained with hematoxylin-eosin (H&E) in accordance with established procedures, enabling histological analysis. Additionally, the jejunal sections were stained using antibodies for immunofluorescence analysis, according to a previously published method.[10] Briefly, the specimens were incubated with the following primary antibodies: rabbit polyclonal anti-DAO (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-mucin-2 (MUC2) (1:100; Bioss, Woburn, MA, USA), and rabbit polyclonal anti-zonula occludens-1 (ZO-1) (1:100; Cell Signaling Technology Inc., Danvers, MA, USA). Next, the sections were incubated with the Alexa Fluor™ goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) secondary antibody. Through immunohistochemical examination, the expression levels of DAO were assessed using confocal microscopy (FV1000, Olympus, Tokyo, Japan), while ZO-1 and MUC-2 expression levels were evaluated using fluorescence microscopy (BZ-X800, Keyence, Tokyo, Japan). DAO expression was measured as the DAO-positive area from five random visual fields using Image J software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis All data are presented as their mean ± standard deviation. Using Microsoft Excel 365, one-way ANOVA with a Tukey's *post-hoc* test was used to compare more than two groups. Differences were considered statistically significant at $*p < 0.05$ or $**p < 0.01$.

RESULTS

Effects of Indomethacin Treatment on the Small Intestine Macroscopically, the entire small intestine was hyperhydrated and distended in indomethacin-treated 40-week-old mice compared to the other groups. Furthermore, exudation of yellow liquid (intestinal contents mixed with intestinal bacteria) outside the small intestine was also observed in the indomethacin-treated 40-week-old mice. (Fig. 1a). In addition, the H&E-stained jejunal tissues were further evaluated microscopically. Compared to other groups, the indomethacin-treated 40-week-old mice showed increased intestinal villi indistinguishability and leukocyte infiltration (Fig. 1b).

Effects of Indomethacin Treatment on the Expression of DAO in the Jejunum DAO expression was examined immunohistologically (Fig. 2). Indomethacin-treated 9-week-old mice showed a decreased expression of DAO compared to that of the control mice of the same age. Additionally, within the control group, the DAO expression of the 40-week-old mice decreased more than that in the 9-week-old mice. Finally, the indomethacin-treated 40-week-old mice showed the greatest decrease in DAO expression among all test groups.

Effects of Indomethacin Treatment on the Expressions of MUC2 and ZO-1 in Jejunum The expressions of MUC2 and ZO-1, both of which play an important role in intestinal barrier function, were examined (Fig. 3). Neither MUC2 nor ZO-1 showed any differences in expression between 9-week-old and 40-week-old control mice. In contrast, their expressions decreased in the indomethacin-administered group compared with that of the control group, with the 40-week-old mice showing the greatest decrease.

DISCUSSION

When administered indomethacin, the small intestines of 40-week-old mice filled with water and showed other abnormal symptoms. In contrast, no abnormalities were observed in the small intestines of mice in the control group, nor the 9-week-old mice administered indomethacin. Indomethacin-treated 40-week-old mice also showed the lowest expression of DAO, an enzyme involved in the degradation of histamines, of any of the treatment groups. In addition, the expressions of MAC2 and ZO-1, both of which control the intestinal barrier function, were lowest in the indomethacin-treated 40-week-old mice compared to the other treatment groups.

In this study, administration of indomethacin to 40-week-old mice decreased the expression of DAO, a histamine-degrading enzyme. The small intestine is a known DAO storage organ.[12] Indomethacin is known to induce inflammation in the small intestine of aging mice. In addition, it was shown that the number of mast cells increased in the intestinal tract of 40-week-old mice upon treatment with indomethacin (data not shown). This suggests that histamines, metalloproteases, and other related substances are secreted into the intestinal tract and thereby exist there in large amounts.[10] While these histamines are typically degraded by DAO, the expression of DAO was remarkably decreased in 40-week-old indomethacin-treated mice, suggesting a large amount of histamines remained in the small intestine in these mice.

Furthermore, 40-week-old indomethacin-treated mice show decreased expression of ZO-1 and MUC2. As ZO-1 mediates the polymerization of claudins that are involved in tight junction formation, ZO-1 loss is associated with intestinal barrier dysfunction.[15,16] MUC2 is a major component of the small intestinal gel layer produced by embryonic cells and protects epithelial cells from microorganisms in the gastrointestinal tract as well as the host's own degradative enzymes.[17,18] Therefore, it was suggested that decreases in the expressions of ZO-1 and MUC2 may cause further deterioration of the intestinal barrier function. Based on the above, 40-week-old indomethacin-treated mice develop small intestinal abnormalities and show a reduction in DAO expression. The results also demonstrated the possibility that histamines, inflammation-related substances produced in the small intestine, are maintained in the intestinal tract in large quantities without being degraded and are consequently released into the blood upon deterioration of the intestinal barrier function.

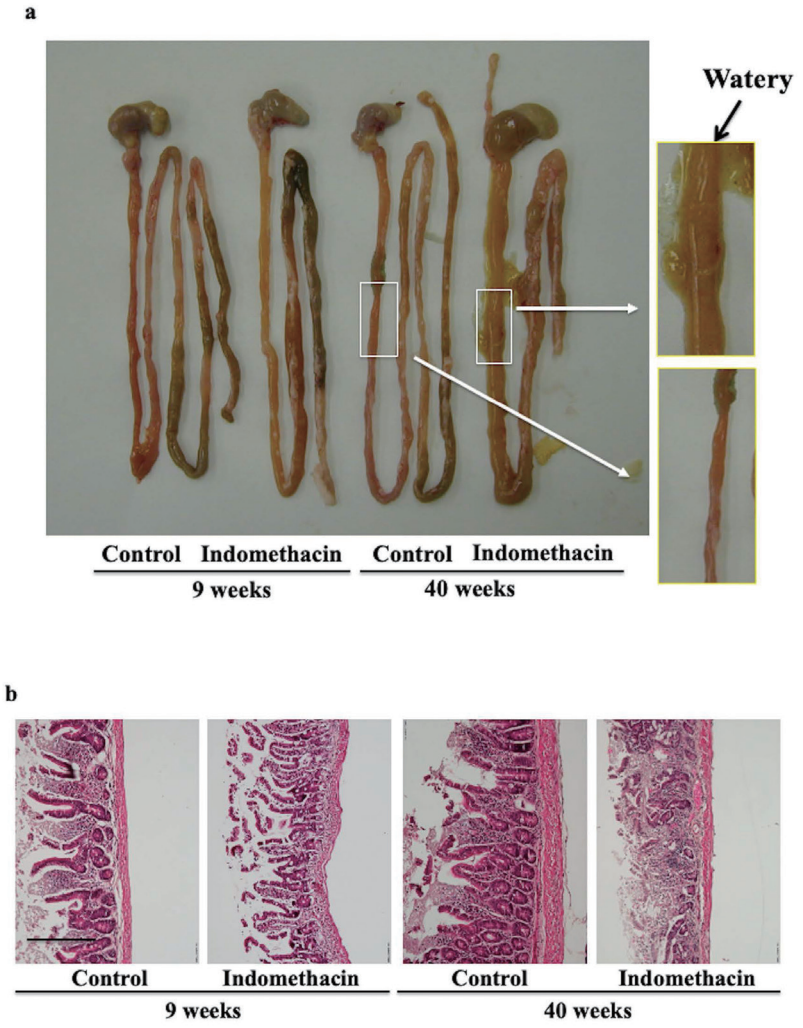

Fig. 1. Effect of Indomethacin Treatment on the Small Intestine.

Macroscopic changes (a) and HE-stained histological changes (b) to the jejunum. Watery: exudation of yellow liquid outside the small intestine. Scale bar = 100 μm.

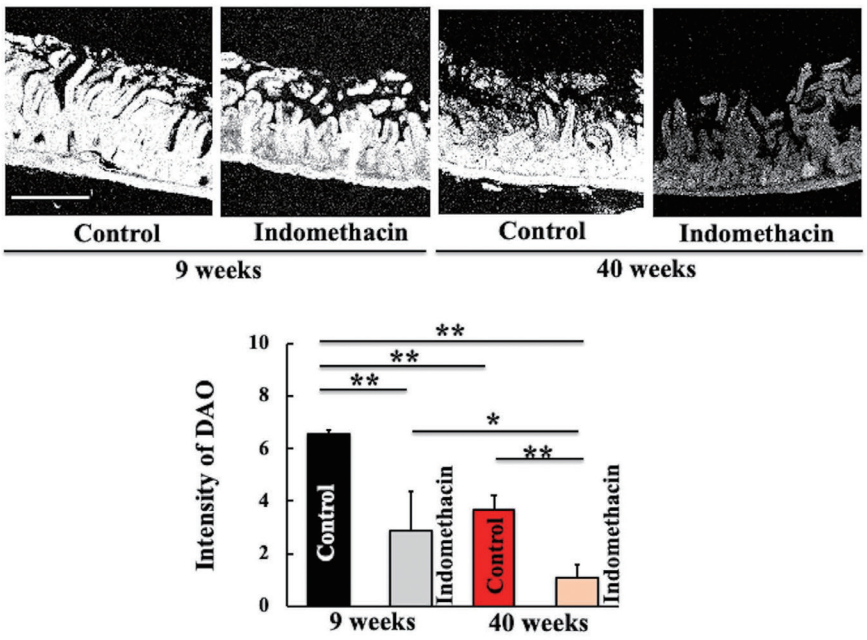

Fig. 2. Effect of Indomethacin Treatment on the Expression of Diamine Oxidase (DAO) in the Jejunum.

Values represent mean ± SD (Tukey's test, *$p < 0.05$ and **$p < 0.01$ indicate statistically significant differences). Scale bar = 100 μm.

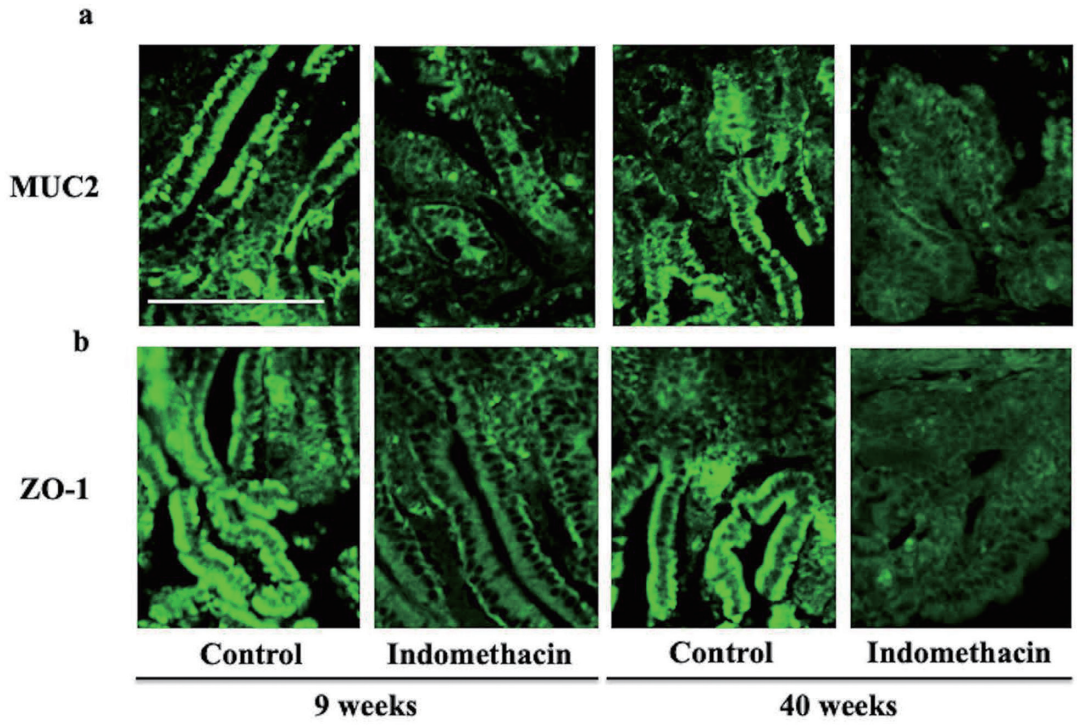

Fig. 3. Effect of Indomethacin Treatment on the Expressions of Mucin-2 (MUC2) (a) and Zonula Occludens-1 (ZO-1) (b) in the Jejunum. Scale bar = 100 μm.

As we previously reported, administration of indomethacin to 9-week-old mice induced skin dryness.[10] As a mechanism of this phenomenon, indomethacin induces inflammation of the small intestine and releases TNF-α and histamine from the small intestine into the blood. It was shown that these inflammatory substances migrate to the skin, affect mast cells in the skin, increase the secretion of MMP-1 and MMP-9, and cause skin dryness. In this study, administration of indomethacin to aged mice exacerbated small bowel damage and caused a decrease in DAO, MUC2 and ZO-1 levels. These findings suggested that a further increase in TNF-α and histamine in plasma was induced to exacerbate dry skin. While we did not examine the amount of histamines in the blood or dryness of the skin in this study, we plan to conduct a detailed examination to include the skin in the future.

In today's aging society, the use of NSAIDs is increasing due to various diseases that occur in the elderly. It is expected that NSAID side effects will increase and worsen with increasing use. In this study, administration of indomethacin, one of the NSAIDs, to aged mice exacerbated small intestinal disorders. This suggests that small intestinal disorders have a great impact on the skin, induce skin disorders, and increase the risk of infections and allergies. The results of this study clarified the mechanism of side effects on the small intestine and skin caused by administration of indomethacin, and it is thought that a safer method of use can be established.

Acknowledgments This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI program (Grant No. 23K06074).

Conflict of interest The authors declare no conflict of interest.

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