Short Communication

Scutellariae Radix augments ulceration but attenuates proinflammatory cytokine and chemokine gene induction in the small intestine during indomethacin-induced enteropathy in mice

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Our previous report described the enhancement of intestinal bleeding and anemia associated with indomethacin (INDO)-induced enteropathy in mice by oral administration of Scutellariae Radix (SR) extract. We here demonstrate that SR extract enhanced ulceration but attenuated the elevation of expression levels of interleukin (IL)-1β, IL-6 and CCL2 gene transcripts in the small intestine during INDO-induced enteropathy. Our observations further support that INDO-induced enteropathy is aggravated by the oral administration of SR extract. This effect of SR extract may be due to the modulation of immune-inflammatory processes possibly through the suppression of proinflammatory cytokine and chemokine induction.

Key words non-steroidal anti-inflammatory drug, cytokine, chemokine.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are extensively used as anti-pyretics and anti-inflammatory analgesics, although long-term ingestion of NSAIDs induces enteropathy characterized by ulceration in the small intestine and blood loss due to intestinal bleeding. The experimental studies have also confirmed that the administration of several types of NSAIDs can induce enteropathy with predominant damage in the small intestine accompanied by intestinal bleeding and blood loss. On the other hand, early inflammatory responses such as the increased expression of proinflammatory cytokines in the small intestine are reported to be associated with NSAID-induced enteropathy, suggesting a role of immuno-inflammatory processes in this disease.

Our previous study demonstrated that the oral administration of SR extract enhanced intestinal bleeding and anemia in indomethacin (INDO)-treated mice. These results suggest that SR extract aggravated INDO-induced enteropathy, although further detailed analyses such as histological evaluation have not been carried out. We also speculate that the alterations of inflammatory responses in the small intestine are associated with the enhancement of intestinal bleeding by SR extract. Therefore, we examined the effects of SR extract on ulceration and the induction of proinflammatory cytokine and chemokine genes in the small intestine during INDO-induced enteropathy in mice.

Materials and Methods

Drugs. Lyophilized powders of water extract of SR were kindly provided from Tsumura & Co. (Tokyo, Japan). High-performance liquid chromatographic analysis for the SR extract was carried out in our previous report and the content of baicalin, a major flavonoid in SR, was determined to be 26.1 % (w/w). INDO was purchased from Wako Pure Chem (Osaka, Japan).

Animals and drug treatment. Male ddY mice at 5 weeks of age (SLC Japan, Shizuoka, Japan) were housed in plastic cages placed in an air-conditioned room (temperature at 23 ± 2 °C and humidity at 55 ± 5 %) under a 12hr:12hr light-dark cycle; the light was turned on at 0800 h. The animals were maintained under the above conditions at least for 1 wk prior to the experiments below. Experimental schedule for the present study was illustrated in Fig. 1.

![Fig. 1. Schedule for drug administration and animal collection.](image-url)
INDO was dissolved in dimethylsulfoxide (DMSO) and was subcutaneously injected at 5 mg/kg in a volume of 1 ml/kg at 1300 h on days 0 and 1. The same volume of DMSO without INDO was subcutaneously injected in the control groups. SR extract was dissolved in distilled water and was orally administered two times daily (0900 h and 1700 h) at 500 mg/kg on days -1, 0 and 1. This dose was reported to enhance intestinal bleeding in INDO-treated mice.10 Mice were sacrificed by cervical dislocation 24 hr after the second INDO injection and the small intestine was harvested for histological evaluation and gene transcript analysis.

**Histological evaluation of ulceration in the small intestine.** Evans blue dye dissolved in saline at 0.25 % (w/v) was intravenously injected (10mL/kg) 30 min before the mice were sacrificed. Small intestine including duodenum, jejunum and ileum was harvested and opened along an antimesenteric margin on dry filter paper. The specimens were immersed into 10 % (v/v) formalin over night. Fecal contents adhered on the mucosal surface of small intestine were washed with saline and then observed under stereoscope. All ulcers were photographed using a digital camera (MDCE-5B, Shodensha, Inc., Osaka, Japan) connected to a stereoscope. The number of ulcers was counted and the total area of ulcers was determined using Motic Plus 2.0S software (Causeway Bay, Hong Kong). Measurement of number and area of ulcer was performed by an observer who could not identify the treatments for tissue specimens.

**Determination of gene transcript levels in the small intestine.** Total RNA was extracted from the frozen tissues using RNAiso (Takara Bio, Tokyo, Japan) and dissolved in nuclease-free water. RNA concentration was determined by measuring ultraviolet absorption and a constant amount (1 µg) of total RNA was treated with a RNase-free DNase (Nippon Gene, Toyama, Japan) and was reverse-transcribed using Superscript III (Invitrogen, Carlsbad, California) in the presence of random hexamer primers (Invitrogen). The mixture containing first-strand cDNA was diluted with a constant volume of nuclease-free water and its aliquot was introduced into a real-time polymerase-chain reaction (PCR) system in the presence of SYBR Green using SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan). A sequence of the gene transcript of β-actin, IL-1β, IL-6, or TNFα was amplified using LineGene real-time PCR system (Toyobo, Tokyo, Japan). The primers used to amplify these gene transcripts are listed in Table 1. The PCR conditions were 95 °C for 10 minutes, followed by 35 (β-actin), 40 (IL-1β, TNFα and CCL2) or 45 (IL-6) cycles of 94 °C for 15 seconds, 58 °C (IL-1β), 60 °C (IL-6, TNFα and CCL2) or 61 °C (β-actin) for 15 seconds and 72 °C for 30 seconds. A standard curve using serial 1:10 dilutions of pooled cDNA for each target transcript was generated in every PCR experiment to determine the relative amounts of the sequences amplified with the respective primers. The amplified products were verified by checking melting curves after the final cycle of each PCR. The amounts of gene transcripts were expressed as the relative value standardized to that of β-actin in each sample.

**Statistical analysis.** All data were expressed as the mean ± standard error. Statistical analysis for the comparison of ulceration was carried out by Student’s t-test and that of gene transcript levels was done by analysis of variance (ANOVA) followed by Bonferroni’s post hoc test.

**Results and Discussion**

A typical representative of ulcerated mucosa of small intestine 24 hr after the second INDO administration was shown in Fig. 2. Ulcerated lesions stained with Evans blue dye was easily discriminated from non-ulcerated area. It was found that the number of ulcers in the small intestine from the mice in the SR/INDO group was significantly greater than in INDO group (p<0.01) (Fig. 3, left). The total area of ulcers tended to be greater in the SR/INDO group than in INDO group (0.05<p<0.1) (Fig. 3, right). No ulceration was induced in the small intestine from mice treated with Water/DMSO.

We found that the expression levels of gene transcripts of IL-1β, IL-6 and CCL2 in the small intestine were significantly elevated in INDO-treated mice (water/INDO) compared with the vehicle-treated mice (water/DMSO) (p < 0.05) (Fig. 4). The expression level of TNFα gene transcript

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**Fig. 2. Small intestinal mucosa from INDO-treated mice**

Arrows indicate the positions of ulcers which were stained with Evans blue dye.
**Fig. 3.** Effects of SR extract on the number of ulcers and the total ulcerated area in the small intestine from INDO-treated mice.
Mice (10 mice/group) orally administered with the SR extracts or vehicle (Water) and treated with INDO (5 mg/kg). Small intestine was harvested one day after the second INDO injection. The number of ulcers and the total ulcerated area were determined.

**Fig. 4.** Effects of SR extract and INDO on the expression levels of IL-1β, IL-6, Tnfα and CCL2.
Mice (10 mice/group) orally administered with SR extracts or vehicle (Water) every day from day -1 and treated with INDO on days 0 and 1 (5 mg/kg). Small intestines were harvested and total RNA was extracted. RT-PCR. The values represent the mean ± SEM and an asterisk (*) represents significant difference with the Water/DMSO group at p<0.05 (ANOVA followed by Bonferroni’ post hoc test).

In the water/INDO group was slightly higher than in the water/DMSO group whereas this difference was not statistically significant. However, the expression levels of gene transcripts of IL-1β, IL-6 and CCL2 in the SR/INDO group were not significantly lower (p >0.05) than those in the DMSO/INDO groups, although these levels were not significantly higher (p >0.05) than those in the water/DMSO group.

Although the number of ulcers in the SR/INDO group was significantly higher than in the DMSO/INDO group, the total area of ulcers was not significantly different between these two groups (Fig. 3). In addition, marginal effects of SR extract in lowering the expression levels of gene transcripts of proinflammatory cytokines in the small intestine of INDO-treated mice were shown (Fig. 4). Thus, SR extract might not strongly modulate the pathological and
immunological responses associated with INDO-induced enteropathy in mice. These effects of SR extract should be compared with those of a reference drug. On the other hand, a difference in the animal species or ages would affect the effects of SR extract on IDNO-induced enteropathy. It is also necessary to examine whether the administration of SR extract enhances differentially INDO-induced enteropathy in different species and ages of experimental animals.

There are reports indicating that the administration of SR or SR-containing Kampo formulations are effective in attenuating pathological responses during experimental in flammatory bowel diseases.\textsuperscript{11-14} In addition, some flavonoids contained in SR such as baicalein and wogonin have been reported to attenuate the induction of proinflammatory cytokines.\textsuperscript{15-18} It is possible that the beneficial effects of SR on some experimental models of inflammatory bowel diseases are implicated to the modulation of inflammatory responses through the suppression of proinflammatory cytokine and chemokine induction. Although the suppression by SR extract of the elevation of expression levels of IL-1\(\beta\) and IL-6 gene transcripts during INDO-induced enteropathy was also observed in the present study, these changes were associated with the enhancement of ulceration in INDO-treated mice (Figs. 3 and 4). There are several reports indicating that the induction of proinflammatory cytokines in the small intestine associated with INDO-induced enteropathy,\textsuperscript{19-20} although a direct role of these molecules in the development of this pathology has not been established. Instead, our observations allow us to speculate that these proinflammatory cytokines may play a protective role in mucosal injury during INDO-induced enteropathy. However, it remains to be examined whether other herbs which suppress the induction of proinflammatory cytokines enhance INDO-induced enteropathy.

It is known that INDO treatment impairs microcirculation and induces ischemia in the mucosal tissues, which can be implicated to mucosal injury during INDO-induced enteropathy.\textsuperscript{19,20} Apoptosis is a major cellular response leading to epithelial damage during INDO-induced enteropathy\textsuperscript{21,22} and is known to be induced under the above hypoxic conditions. It is reported that hypoxia-induced apoptosis is suppressed by IL-6.\textsuperscript{23} Recently, IL-15 was demonstrated to protect intestinal epithelial cells possible through the inhibition of apoptosis.\textsuperscript{24} The protective effects of IL-15 are suggested to be mediated through the generation of several proinflammatory cytokines such as TNF\(\alpha\) and IL-6.\textsuperscript{20} These observations may support protective roles of proinflammatory cytokines in INDO-induced enteropathy. On the other hand, the induction of several chemokines is reported under the inflammatory conditions of bowel diseases in human.\textsuperscript{19,20} Since leukocyte infiltration into mucosal tissues occurs during NSAID-induced enteropathy,\textsuperscript{9} chemokines may play a role in this response. However, we showed that CCL2 gene transcript in the small intestine was elevated during INDO-induced enteropathy and was reduced by SR extract similarly to those of IL-1 and IL-6 (Fig. 5). Further detailed analyses for the role of CCL2 as well as other chemokines in INDO-induced enteropathy remain to be investigated.

SR is a major herbal constituent in many kinds of Kampo formulations. Therefore, there may be cases in which SR-containing Kampo formulations are co-administered in the course of the therapy of inflammatory diseases with NSAIDs. However, no attention has been paid to the effects of Kampo formulations on the therapeutic efficacies or side-effects of NSAIDs. Our previous study demonstrated that, in contrast to SR extract, SR-containing Orengedokuto and San’oshashintosho suppressed INDO-induced intestinal bleeding and anemia in INDO-treated mice.\textsuperscript{10} These results suggest that the combination with other herbal medicines alters the effects of SR on NSAID-induced enteropathy. Further studies are necessary to examine the influences of the combined use of SR-containing Kampo formulations and NSAIDs and to explore the role of proinflammatory cytokines and chemokines in the modulation of NSAID-induced enteropathy by Kampo formulations.

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**References**


