Regular Article

Active Ingredients of Hange-shashin-to, Baicalein and 6-Gingerol, Inhibit 5-Fluorouracil-Induced Upregulation of CXCL1 in the Colon to Attenuate Diarrhea Development

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5-Fluorouracil (5-FU) is widely used as an anti cancer drug and is known to cause severe diarrhea. Recently we suggested that levels of chemokine (C-X-C motif) ligand 1 (CXCL1) and neutrophil recruitment in the colonic mucosa were drastically increased by the 5-FU administration in mice. Hange-shashin-to (HST) is prescribed in Japan for treat gastritis, stomatitis, and inflammatory diarrhea. We therefore examined the effects of HST and its active ingredients on 5-FU-induced CXCL1 upregulation in cultured colon tissue, and also examined the effects of HST on 5-FU-induced diarrhea development in the mouse. The distal colon isolated from the mouse was incubated with 5-FU and HST. Mice were given 5-FU (50 mg/kg, intraperitoneally (i.p.)) daily for four days. HST (300 mg/kg, *per os* (p.o.)) was administered 30 min before mice received 5-FU. mRNA levels of CXCL1 in the colon were examined using quantitative RT-PCR. 5-FU enhanced CXCL1 mRNA in the colon but the effect by 5-FU was markedly suppressed by application of HST and its active ingredients, baicalein and 6-gingerol. Nuclear factor kappa B (NF- κ B) was activated by 5-FU treatment in cultured colon tissue, which was also suppressed by HST and the combination of baicalein and 6-gingerol. Furthermore, HST reduced 5-FU-induced diarrhea development. Under such experimental condition, CXCL1 gene, protein levels of neutrophil elastase and myeloperoxidase upregulation induced by 5-FU in the colon was attenuated by HST. These findings suggest that HST, especially baicalein and 6-gingerol, prevent the development of neutrophil recruitment and diarrhea by the inhibition of NF- κ B activity.

Key words hange-shashin-to; 5-fluorouracil; diarrhea; chemokine (C-X-C motif) ligand 1; baicalein; 6-gingerol

Chemokine (C-X-C motif) ligand 1 (CXCL1) known as keratinocyte chemoattractant and growth-regulated oncogene α (GRO α), which is related to interleukin-8,¹⁾ has neutrophil-activating and neutrophil-hemoattractant properties. Neutrophils are most abundant type of granulocytes, that are activated in chronic inflammation, and that are attracted to the site with inflammation *via* chemokines, such as interleukin-8 in humans²⁾ and CXCL1 in mice.³⁾ The CXCL1 binds to the C-X-C chemokine receptor type 2 (CXCR2), which is predominantly expressed on neutrophils. Activated neutrophils release elastase, which cause tissue injury. CXCR2 is the receptor related ELR+ chemokines (glutamic acid-leucine-arginine containing), and is responsible for neutrophil chemotaxis.^{4,5)} Recently, we reported that 5-fluorouracil (5-FU) administration upregulated levels of CXCL1 and neutrophil recruitment in the mucosa of murine distal colon⁶⁾ and CXCL1 gene expressions upregulated by 5-FU were mediated by transcription factor nuclear factor-kappa B (NF- κ B) activation in murine distal colon.⁷⁾ Moreover, the SB225002, a CXCR2 antagonist attenuated the 5-FU administration-induced neutrophil recruitment and diarrhea in mouse.⁶⁾

Chemotherapy-induced diarrhea has been reported in 50–80% of treated patients,⁸⁾ with the diarrhea resulting from certain types of chemotherapy sometimes being severe. Although 5-FU is ordinarily used in the treatment of cancer

and is known to cause diarrhea, very little laboratory research has been carried out to elucidate the mechanisms underlying the physiopathology of this event. In chemotherapy-related diarrhea, loperamide is usually used. Although the loperamide slows the gastrointestinal motility and makes the stool less watery, it is not provided enough therapy of 5-FU-induced severe diarrhea by the use only for loperamide. Therefore, it is important to clarify mechanism of 5-FU-induced diarrhea, and to find the new specific treatment strategy.

Hange-shashin-to (HST), a Japanese herbal medicine, is composed of seven crude herbs: *Scutellariae radix*, *Pinelliae tuber*, *Zingiberis rhizoma*, *Ginseng radix*, *Glycyrrhizae radix*, *Zizyphi fructus*, and *Coptidis rhizoma*. The medicine has been prescribed in Japan for the empirical treatment of acute and chronic gastroenteric catarrh, fermentative diarrhea, and acute gastroenteritis.⁹⁾ HST has clinical evidence and its action mechanisms have been partially elucidated.^{10,11)} Although the active constituents and the pharmacological properties of their potency are not sufficiently clarified yet, there are reports that activation of NF- κ B pathway is inhibited by some ingredients of HST, such as baicalin, berberine and 6-gingerol.^{12–14)} Aim of this study was to examine the effect of HST and some of its ingredients on experimental model of 5-FU-induced diarrhea, which is mediated by NF- κ B activation.

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MATERIALS AND METHODS

Animals Male C57BL/6J mice (8–9 weeks of age, Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) were used. All procedures using animals were carried out according to protocols approved by the Animal Care Committee of the Hoshi University (Tokyo, Japan).

Chemicals and Reagents HST was obtained from Tsunuma and Co. (Tokyo, Japan). 5-FU, baicalin and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Baicalein, berberin chloride, wogonin hydrate, 6-gingerol and 6-shogaol were purchased from Sigma-Aldrich (MO, U.S.A.). RPMI 1640 medium and penicillin-streptomycin mixed solution were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Biosera Inc. (Ringmer, U.K.).

Tissue Culture Tissue culture samples were prepared from the murine distal colon (1.5 cm length), which was opened along its length and cleaned of fecal content in cold phosphate buffered saline (PBS) containing 100 U/mL penicillin and 100 μ g/mL streptomycin. The tissue itself was transferred into a culture tube (CELLreator Filter Top Tube, Greiner Bio-One, Kremsmuenster, Austria). To each tube, 3 mL of medium consisting of RPMI 1640, 5% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin were added. The tissue concentrations were 0.011–0.015 g/mL medium. The dried powdered extract form of HST was suspended in DMSO at 100 mg/mL, diluted with culture medium, and filtered through a 0.45- μ m membrane. HST was added to cultures at final concentrations of 300 μ g/mL. The tissue samples were incubated with 10 μ M 5-FU or its vehicle, PBS, in a humidified incubator at 37°C and 5% CO₂ for 24 h.

Quantitative RT-PCR (qRT-PCR) Expression levels of CXCL1 mRNA were determined by qRT-PCR as described previously.⁶⁾ Briefly, The PCR primer sets were used for CXCL1 (forward primer; GCTCCCTTGTTTCAGAAAATTG and reverse primer; TCACAGACAGGTGCCATCA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer; CCTCGTCCCGTAGACAAAATG and reverse primer; TCTCCACTTGCCACTGCAA). The thermal cycle profile used was 1) denaturing for 30 s at 95°C and 2) annealing for 30 s at 60°C. PCR amplification was performed for 40 cycles. Data are presented as the mRNA expression relative to that of the housekeeping gene GAPDH using the 2^{−ΔΔCt} method.¹⁵⁾

Enzyme-Linked Immunosorbent Assay (ELISA) The CXCL1 concentration in the medium was measured using the ELISA described by the manufacturer's instructions (PeproTech, NJ, U.S.A.). All samples were analyzed in duplicate.

Immunoblotting Preparation of protein samples and immunoblot analyses were performed as previously.¹⁵⁾ The primary antibodies used were rabbit anti-I κ B α antibody (1:1000 dilution; BioLegend Inc., CA, U.S.A.), mouse anti-neutrophil elastase (ELA2) (1:1000 dilution; R&D System, Inc., MN, U.S.A.), mouse anti-myeloperoxidase (MPO) (1:1000 dilution; R&D System, Inc.) and mouse anti-GAPDH antibody (1:5000 dilution; Sigma-Aldrich). The second antibodies used were horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; 1:5000 dilution; Cell Signaling Technology Japan, Tokyo, Japan) and anti-mouse (IgG; 1:5000 dilution; Cell

Signaling Technology Japan, Tokyo, Japan). The bands were detected using an enhanced chemiluminescent system (Wako Pure Chemical Industries, Ltd.).

Lactate Dehydrogenase (LDH) Assay Quantification of cell toxicity was performed by measuring LDH release into the medium. LDH activity was measured using a Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. One percent Triton X-100 was used as a positive control.

Treatment Protocol Mice were given a single intraperitoneal (i.p.) injection of 5-FU (50 mg/kg) daily for four days (Days 0–3), with its vehicle, saline used as a control. Twenty-four hours after the final injection of 5-FU, mice were euthanized under deep anesthesia using isoflurane and the distal colon was removed, washed with cold PBS, and stored in TRI Reagent™ at −80°C. In HST administration, mice were treated with HST (500 mg/kg, *per os* (*p.o.*)) or with olive oil (vehicle) during Days 0–3.

Diarrhea Assessment Diarrhea assessment was carried out blind by four investigators, as described previously.^{6,15)}

Statistical Analysis All the data are expressed as the mean $\pm$ standard error of the mean (S.E.M.). Statistical significance of difference was assessed by one-way ANOVA followed by the Bonferroni/Dunn *post-hoc* test. A value of $p < 0.05$ was considered significant.

RESULTS

We examined 5-FU-induced changes in CXCL1 gene expression in cultured distal colon tissue. As shown in Fig. 1A, the gene expression of CXCL1 was significantly upregulated by 10 μ M 5-FU treatment for 24 h. Hence, we examined the effects of HST on 5-FU-associated upregulation of CXCL1 gene expression *in vitro*. The 5-FU-induced augmentation of CXCL1 gene expression was significantly inhibited by 300 μ g/mL HST.

Recently, Kono *et al.* showed that HST contains at least 10 active ingredients; baicalin, berberine, baicalein, wogonin, 6-gingerol, 6-shogaol, 8-gingerol, 10-gingerol, 8-shogaol, and 10-shogaol with using LC tandem mass spectrometry.¹⁶⁾ As the concentration of baicalin, berberine, baicalein, wogonin, 6-gingerol and 6-shogaol exceeded 0.1 μ M in 300 μ g/mL HST.¹⁶⁾ We therefore used these six ingredients and examined their effects at concentrations equal (20 μ M baicalin, 3.4 μ M berberin, 1 μ M baicalein, 0.6 μ M wogonin, 0.4 μ M 6-gingerol and 0.18 μ M 6-shogaol) to 300 μ g/mL HST¹⁶⁾ on 5-FU-induced upregulation of CXCL1 gene expression. This was significantly attenuated by 1 μ M baicalein or 0.4 μ M 6-gingerol (Fig. 1A). We also investigated the effects of HST and its active ingredients on the upregulation of CXCL1 secretion induced by 5-FU in the medium. This increase in CXCL1 concentration was decreased by 300 μ g/mL HST, 1 μ M baicalein or 0.4 μ M 6-gingerol, as well as their effects on CXCL1 gene expression (Fig. 1B).

CXCL1 gene expression has been previously demonstrated to be mediated by transcription factor NF- κ B activation.¹⁷⁾ Activation of the I κ B kinase (IKK) complex leads to phosphorylation by IKK β of two specific serines near the N terminus of NF- κ B inhibitor alpha (I κ B α), thereby targeting I κ B α for ubiquitination and degradation by the 26S proteasome after NF- κ B activation.¹⁸⁾ We therefore examined I κ B α expression levels

Fig. 1. Effects of Hange-shashin-to (HST) and Its Active Ingredients Baicalin, Berberin, Baicalein, Wogonin, 6-Gingerol and 6-Shogaol, on 5-Fluorouracil (5-FU)-Induced Upregulation of the CXCL1 mRNA in Distal Colon Isolated from Mouse (A)

The effects of HST and its active ingredients on 5-FU-induced secretion of CXCL1 from the distal colon (B). Ten micromolar 5-FU-induced upregulation was inhibited by 300 μg/mL HST, 1 μM baicalein and 0.4 μM 6-gingerol. Each column represents the mean ± standard error of the mean (S.E.M.) of 4–8 independent experiments. Values represent the means ± S.E.M. of 6–8 independent experiments. ****p* < 0.001 vs. PBS + DMSO. ***p* < 0.01 and ****p* < 0.001 vs. 5-FU + DMSO.

and NF-κB was significantly activated by 5-FU. This activation was also inhibited by both 300 μg/mL HST and 1 μM baicalein. NF-κB activity was significantly inhibited by the combination of 1 μM baicalein with 0.4 μM 6-gingerol, while IκBα expression was not significantly increased by 0.4 μM 6-gingerol (Figs. 2A, B).

To examine cell toxicity of 5-FU and HST, we measured LDH release from cultured tissues. LDH release was increased by 10 μM 5-FU treatment for 24 h. The LDH release induced by 5-FU was significantly attenuated by 300 μg/mL HST co-treatment (Fig. 2C).

We then investigated the effects of HST on 5-FU-induced development of diarrhea. HST dramatically inhibited 5-FU-

Fig. 2. Effects of Hange-shashin-to (HST) and Its Active Ingredients Baicalin, Berberin, Baicalein, Wogonin, 6-Gingerol and 6-Shogaol, on 5-FU-Induced Nuclear Factor-kappa B (NF-κB) Activity (Degradation of IκBα) in the Distal Colon

Typical photographs of bands for IκBα and GAPDH (A). NF-κB activation was inhibited by 300 μg/mL HST and 1 μM baicalein, and the combination of 1 μM baicalein with 0.4 μM 6-gingerol (B). Values represent the means ± S.E.M. of three independent experiments. ***p* < 0.01 and ****p* < 0.001 vs. PBS + DMSO. #*p* < 0.05 and ****p* < 0.001 vs. 5-FU + DMSO. Effects of 10 μM 5-FU and 300 μg/mL HST on lactate dehydrogenase (LDH) release. Data are expressed as percent of the LDH release induced by 1% Triton X-100. Values are the means ± S.E.M. of four independent experiments. ***p* < 0.01 vs. PBS + DMSO. #*p* < 0.05 vs. 5-FU + DMSO.

induced diarrhea (Fig. 3A). Under these conditions, 5-FU-induced upregulation of CXCL1 gene expression in the distal colon was significantly reversed by administration of HST (Fig. 3B). To investigate neutrophil recruitment in distal colon, we examined expression levels of ELA2 and MPO. Both ELA2 and MPO, those are stored in azurophilic granules of neutrophil, were increased by 5-FU in distal colon. Furthermore, the increased MPO and ELA2 expression were inhibited by HST administration (Figs. 3C–E). These findings suggest neutrophil recruitment induced by 5-FU was attenuated by HST administration.

DISCUSSION

We show here that upregulation of CXCL1 by 5-FU was significantly inhibited by HST administration and its active ingredients baicalein and 6-gingerol. NF-κB was activated by 5-FU treatment in cultured colon tissue, which was inhibited by HST and the combination of baicalein and 6-gingerol. *in*

Fig. 3. Effects of Administration of Hange-shashin-to (HST) on 5-Fluorouracil (5-FU)-Induced Changes in the Diarrhea Score

HST (500mg/kg, *p.o.*) or its vehicle (olive oil, *p.o.*) was administered for four days, and 30min before treatment with 5-FU (50mg/kg, *i.p.*) or its vehicle (saline, *i.p.*). Development of diarrhea by 5-FU was inhibited by administration of HST (A). Each point represents the mean $\pm$ S.E.M. of four independent experiments. ** $p < 0.01$ vs. Vehi+vehi. ## $p < 0.01$ vs. Vehi+5-FU. Effect of HST on 5-FU-induced upregulation of mRNA of CXCL1 in the murine distal colon (B). Each column represents the mean $\pm$ S.E.M. of four independent experiments. *** $p < 0.001$ vs. Vehi+vehi. ## $p < 0.01$ vs. Vehi+5-FU. Effect of 5-FU and HST administration on expression of neutrophil elastase (ELA2) and myeloperoxidase (MPO) in distal colon of mouse. Representative photos showing bands for ELA2, MPO and internal control, GAPDH (C). Levels of ELA2 and MPO expressed as the ratios of the intensities of ELA2 and MPO to GAPDH protein bands (D, E, respectively). Results are expressed the mean $\pm$ S.E.M. from four experiments. *** $p < 0.001$ vs. Vehi+vehi. # $p < 0.05$ and ## $p < 0.01$ vs. Vehi+5-FU.

in vivo, administration of HST reduced 5-FU-induced upregulation of ELA2 and MPO expression in the distal colon and diarrhea development in the mouse.

Recently, we found that the CXCL1 mRNA in the colon was drastically increased by 5-FU administration,⁶⁾ and that 5-FU also considerably increased the number of Ly-6G-positive neutrophils in the distal colonic mucosa of mouse.⁶⁾ Moreover, neutrophil recruitment was dramatically attenuated by treatment of the CXCR2 antagonist, SB225002.⁶⁾ These suggest that neutrophil recruitment could be related to the pathophysiology of 5-FU-induced diarrhea in the mouse.⁶⁾ In the current study, we showed that the mRNA of CXCL1 was enhanced by 5-FU *in vitro* and *in vivo*. Moreover, ELA2 and

MPO were increased by 5-FU administration. This finding is consistent with a recent study.⁷⁾ We also indicated that 5-FU induced activation of NF- κ B, and that this activation was attenuated by HST. Therefore, although the detailed mechanism underlying NF- κ B activation induced by 5-FU is unclear from this study, it is likely that IKK is activated by 5-FU.

The CXCL1 has been reported to be expressed in various cells, epithelial, endothelial and inflammatory cells.^{19–21)} Although we showed that the CXCL1 expression in distal colon was enhanced by 5-FU, the cell which CXCL1 upregulated by 5-FU was not identified in the current study. Therefore, a further study is necessary.

HST has been reported to downregulate expression of the pro-inflammatory prostaglandins, such as prostaglandin E₂, in a colitis animal model.^{10,22)} However, it is not clear that the downregulation of prostaglandin E₂, and its associated antibacterial activity was involved in the attenuation of 5-FU-induced diarrhea resulting from HST administration observed in this study. Moreover, one of the main ingredients of HST berberine, which has broad-spectrum antibacterial activity, has been shown to inhibit butyrate-induced colonic epithelial cell death.^{23,24)} Furthermore, Kase *et al.* demonstrated that HST is an effective agent in the prevention and/or treatment of irinotecan-induced chronic diarrheal symptoms in rat.²⁵⁾

In the present study, NF- κ B activation induced by 5-FU *in vitro* was inhibited by HST, baicalein and the combination of baicalein with 6-gingerol. It has been reported that baicalein inhibits NF- κ B activation in various cell types such as RAW 264.7 cells,²⁶⁾ human mast cells²⁷⁾ and a retinal pigment epithelial cell line.²⁸⁾ There are reports that 6-gingerol also inhibits NF- κ B activation in mouse skin,²⁹⁾ macrophages¹⁴⁾ and HuH-7 hepatoma cells.³⁰⁾ Furthermore, it has been recently reported that 6-gingerol has protective effects against ischemia/reperfusion-induced intestinal mucosa injury mediated by the NF- κ B pathway.³¹⁾ Taken together, it is possible that attenuation of 5-FU-induced upregulation of CXCL1 by HST involves inhibition of NF- κ B activation.

Although the pathogenesis of 5-FU-induced diarrhea is not fully understood, its cytotoxicity, including apoptosis and abnormal inflammation, is considered to be one of the pathogenesis.^{32,33)} In this study, HST inhibited completely 5-FU-induced diarrhea, although HST inhibited partially gene expression of CXCL1, protein levels of ELA2 and MPO *in vivo*. While further studies are necessary to clarify the difference, these findings may indicate that HST also inhibits 5-FU-induced apoptosis, resulting in attenuation of diarrhea. Indeed, we suggested that HST inhibited the increases in LDH release induced by 5-FU *in vitro*.

5-FU and its prodrugs are routinely used in various cancer chemotherapy.³⁴⁾ However, their usefulness is limited by a number of gastrointestinal toxicity, including diarrhea, which could result in severe mortality and morbidity. The current therapy for 5-FU-induced diarrhea is not specific, and its objectives are to attenuate the dysphoria and inconvenience of frequent and watery bowel movements, and to prevent the need for enteral and/or parenteral replenishment of fluids and electrolytes during recovery from the mucositis by antineoplastic drugs. Diarrhea has been occurred up to half of cancer patients receiving weekly 5-FU therapy. In addition, the severe diarrhea can increase when 5-FU is administered by bolus injection rather than intravenous infusion.^{8,35)} In conclu-

sion, HST attenuated the CXCL1 mRNA expression increased by 5-FU treatment *in vitro*, which was mediated by activation of NF- κ B. HST attenuated the development of neutrophil recruitment and diarrhea, caused by 5-FU in mice model.

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Conflict of Interest The authors declare no conflict of interest.

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