



Toki-shakuyaku-san, a Japanese kampo medicine, reduces colon inflammation in a mouse model of acute colitis



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ABSTRACT

Toki-shakuyaku-san (TOKI) is a Japanese kampo medicine, which consists of a mixture of herbal medicines and considered to be a promising remedial agent due to its immunomodulatory and anti-inflammatory effects. We examined the beneficial effects of TOKI in inflammatory bowel disease associated with the inflammation of the intestinal barrier. A study was designed, using C57BL/6 female mice and were administered with 3% DSS in drinking water for 8 days with or without 1 g/kg/day TOKI orally for the last 3 days and a normal group supplied with plain drinking water for 8 days. TOKI treatment attenuated the clinical symptoms of acute murine colitis and also alleviated the inflammatory mechanism by reducing the inflammatory mediators, such as IL-1 β , IL-2, TGF- β , RAGE and TLR2. It has also decreased the levels of CHOP, caspase12, cleaved caspase3 and cleaved caspase7 and thereby down-regulated the endoplasmic reticulum stress and apoptotic signaling induced by DSS. Moreover, the expression levels of cyclin D1 and c-kit have also confirmed the beneficial role of TOKI in colitis. All these data suggested that TOKI can be a promising agent for the treatment of colitis since it alleviates the disease progression and severity.

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1. Introduction

Inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis (UC) are characterized by chronic and relapsed inflammation in the intestine, but the etiology and pathogenesis have not been completely understood [1–3]. UC is an intractable IBD that causes inflammation and sores on the inner lining of the digestive tract mainly large intestine [3]. Key features of UC include diffuse mucosal inflammation that reverts proximally from the rectum to a varying degree in conjunction with severe inflammation and the coincident production of a complex mixture of inflammatory mediators, extensive superficial mucosal ulceration and is characterized by rectal bleeding, diarrhea, and abdominal pain [1]. Researchers presumed a number of factors associated with the pathogenesis of this disease probably environmental and genetic factors, which interact with the intestinal mucosal barrier and trigger an event that leads ultimately to a chronic activation of immune and nonimmune cells in the gut [4,5]. Oral administration of

dextran sulfate sodium (DSS) instigates UC in hamster and mice [5,6]. DSS consumption provokes immense macrophage and granulocyte infiltration, distortion of the anatomy of glands, and ulceration in the colon, which replicates the pathological features in the acute phase of colitis patients and therefore used as a conventional animal model of UC. DSS can damage enterocytes directly and disturb the integrity of the gut barrier and eventually promotes the invasion of intraluminal commensal bacteria [6–8]. Therapy for IBD mainly focuses on the regulation of inflammatory cells and their secretion of various inflammatory mediators like proinflammatory cytokines, chemokines, etc. [9]. Recently, endoplasmic reticulum (ER) stress has been recognized for the induction of inflammatory cytokines [10,11]. Inflammatory cytokines released after the induction of ER stress functions as an alarming or danger signal to communicate with other cells or to recruit immune cells [12].

The incorporation of natural products into the therapeutic regimens is an attractive approach for improving disease treatment due to their generally low toxicity profiles and high patient compliance [13]. Japanese herbal medicines of traditional Chinese origin, also called kampo medicines, are highly standardized for their quality and widely

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used for the treatment of various diseases [14]. Recently several kampo medicines have been investigated using animal studies and clinical trials to evaluate their potential beneficial effects [15]. Almost 150 kampo formulations have been approved as prescription drugs by the Ministry of Health, Labour and Welfare, Japan, and the treatment with kampo medicine is a symptom- and patient-based, therefore, a form of tailor-made drug therapy [16]. Toki-shakuyaku-san (TOKI; “Dang gui shao yao san” in Chinese) is a traditional kampo medicine, which is widely used in Japan, Korea and China to improve blood circulation and to treat various gynecological disorders such as irregular menstruation, dysmenorrhea, endometriosis, menopausal syndromes and sterility [17–22]. The TOKI kampo formulation is made from six different herbs such as *Hoelen* (Fu ling), *Cnidium rhizome* (Chuan xiong), *Angelica sinensis* (Dang gui), *Peony root* (Shao yao), *Atractylodes rhizome* (Bai zhu), and *Alisma rhizome* (Ze xie) [23]. In this study, we used TOKI in an animal model of UC to find out the cardinal effects of this drug in IBD and to find out the role of this drug in the anti-inflammatory cascade.

2. Materials and methods

2.1. Drugs and chemicals

DSS (MW 36,000–50,000) was purchased from Wako, Japan. TOKI was obtained from Tsumura & Co., Tokyo, Japan (Prepared under GMP; Standard Commodity Classification No. of Japan–875200). All other chemicals used in this study were purchased from Sigma, Tokyo, Japan until mentioned otherwise.

2.2. Animals

C57BL/6J female mice were maintained and housed in the animal facility of the Faculty of Pharmacy, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan. The mice were fed with normal chow diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. Animals were kept under temperature and humidity controlled conditions and a light-dark cycle of 12:12 h. All the experiments were approved by the regulations of the Committee on Bioethics for Animal Experiments of our institute (approval number–H2705) and were performed under the relevant regulations and guidelines during the light phase of the cycle [24].

2.3. Experimental design

A protocol was designated for 8 days and the mice were allocated into three groups of matching age, sex and number ($n = 6-8$). In the first group, designated as the normal control, the mice received normal diet and plain drinking water (DW) ad libitum. The second group, designated as the DSS control, received 3% DSS in DW throughout the protocol. The third group, designated as the TOKI group, received 3% DSS in DW and once daily treatment with TOKI suspension in water (1 g/kg/day) orally during the last three days.

2.4. Change in body weight and disease activity index (DAI)

Body weight was measured on a daily basis from the prior day of colitis induction and throughout the study protocol. The stool consistency and the presence of bloody stools, as well as rectal bleeding, were observed and scored as described previously [25]. Weight loss was scored as 0, none; 1, 1–5%; 2, 5–10%; 3, 10–20%; 4, over 20. Stool consistency was scored as 0, well-formed pellets; 2, loose stools; and 4, diarrhea. Fecal blood was scored as 0, negative hemocult test; 2, positive hemocult test; and 4, gross bleeding. The clinical disease activity index (DAI) was the sum of the above parameters.

2.5. Colon length analysis and histological scoring

The mice were sacrificed at the end of the protocol and their colons were excised from cecum to one cm above to anus. The colon length was measured, which indirectly stipulate the inflammatory index of the colon. For the histological analysis, the distal colon samples were fixed immediately in 10% formaldehyde solution, embedded in paraffin, cut into 5 μ m thick transversal sections, mounted on glass slides, deparaffinized and stained with hematoxylin and eosin stain (H&E). The slides were monitored under light microscope to check the colonic damage [26].

2.6. Western immunoblotting analysis

Western immunoblotting analysis was carried out as per the earlier method [27]. The tissue samples obtained from the colons were homogenized in lysis buffer. Protein concentration of the homogenates was then measured by the bicinchoninic acid method. For Western blots, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and identified with the following antibodies to quantify their levels: rabbit anti-growth arrest damage-inducible protein (GADD)-153 (also called as C/EBP homologous protein (CHOP)), rabbit anti-caspase12, rabbit anti cleaved caspase3, rabbit anti-cleaved caspase7, rabbit anti-interleukin (IL)-1 β , goat anti-receptor for advanced glycation end products (RAGE), goat anti-toll like receptor (TLR)-2, mouse anti-cyclin D1, and rabbit anti-c-kit and rabbit anti-glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibodies (Santa Cruz Biotechnology, TX, USA or Cell Signaling Technology, Tokyo, Japan). We have used 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA), and the proteins separated were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk (Sigma, St. Louis, MO, USA) in TBS-T (20 mM/L Tris, pH 7.6, 137 mM/L NaCl and 0.05% Tween) and incubated with primary antibody diluted in the blocking solution (1:1000) at 4 °C overnight. The bound antibody was visualized with respective horseradish peroxidase coupled secondary antibody (Santa Cruz Biotechnology, TX, USA) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK). The level of GAPDH was estimated in every sample. Films were scanned and band densities were measured by densitometric analysis using the Scion Image program (Epson GT-X700, Tokyo, Japan). Finally, Western blot data were normalized with GAPDH.

2.7. Evaluation of colonic gene expression by realtime reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was carried out by the already prescribed method from our lab [27]. Colon tissues were preserved in RNAlater (Ambion Inc., Austin, TX, USA) immediately after sacrifice. RNA extraction was performed by using MagNA Pure Compact RNA Isolation Kit (Roche Diagnostics K. K., Tokyo, Japan) according to the manufacturer's protocol. Synthesis of cDNA was performed by reverse transcription using total RNA (2 μ g) as a template (Super Sript II; Invitrogen Corp. Carlsbad, CA, USA). Analysis of gene expression was carried out by RT-PCR (Smart cycler; Cepheid, Sunnyvale, CA, USA) using cDNA synthesized from colon tissues. RT-PCR was performed by monitoring with the following TaqMan probes; IL-1 β (Mm00434228_ml), IL-2 (Mm00434256_ml), transforming growth factor (TGF)- β (Mm03024053_m1) and GAPDH (Mm99999915_g1) (Applied Biosystems, Foster City, CA, USA), according to the following protocol: 600 s at 95 °C, followed by thermal cycles of 15 s at 95 °C, and 60 s at 60 °C for extension. Relative standard curves representing several 10 fold dilutions (1:10:100:1000:10,000:100,000) of cDNA from colon tissue samples. Results were used for linear regression analysis of other samples. Results were normalized to GAPDH mRNA as an internal control and thus shown as relative mRNA levels.

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