Daikenchuto, a Kampo Medicine, Regulates Intestinal Fibrosis Associated with Decreasing Expression of Heat Shock Protein 47 and Collagen Content in a Rat Colitis Model

Ken Inoue, Yuji Naito, Tomohisa Takagi, Natsuko Hayashi, Yasuko Hirai, Katsura Mizushima, Ryusuke Horie, Kohei Fukumoto, Shinya Yamada, Akihito Harusato, Ikuhiro Hirata, Tatsushi Omatu, Naohisa Yoshida, Kazuhiro Uchiyama, Takeshi Ishikawa, Osamu Handa, Hideyuki Konishi, Naoki Wakabayashi, Nobuaki Yagi, Hiroshi Ichikawa, Satoshi Kokura, and Toshikazu Yoshikawa

A Department of Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science; Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602–8566, Japan; and b Department of Medical Life Systems, Doshisha University; Kyotanabe, Kyoto 610–0394, Japan.

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Heat shock protein (HSP) 47 may play an important role in the pathogenesis of intestinal fibrosis. Daikenchuto (DKT), a traditional Japanese herbal (Kampo) medicine, has been reported to ameliorate intestinal inflammation. The aims of this study were to determine time–course profiles of several parameters of fibrosis in a rat model, to confirm the HSP47-expressing cells in the colon, and finally to evaluate DKT’s effects on intestinal fibrosis. Colitis was induced in male Wistar rats weighing 200 g using an enema of trinitrobenzene sulfonic acid (TNBS). HSP47 localization was determined by immunohistochemistry. Colonic inflammation and fibrosis were assessed by macroscopic, histological, morphometric, and immunohistochemical analyses. Colonic mRNA expression of transforming growth factor β1 (TGF-β1), HSP47, and collagen type I were assessed by real time-polymerase chain reaction (PCR). DKT was administered orally once a day from 8 to 14 days after TNBS administration. The colon was removed on the 15th day. HSP47 immunoreactivity was coexpressed with α-smooth muscle actin-positive cells located in the subepithelial space. Intracolonic administration of TNBS resulted in grossly visible ulcers. Colonic inflammation persisted for 6 weeks, and fibrosis persisted for 4 weeks after cessation of TNBS treatment. The expression levels of mRNA and proteins for TGF-β1, HSP47, and collagen I were elevated in colonic mucosa treated with TNBS. These fibrosis markers indicated that DKT treatment significantly inhibited TNBS-induced fibrosis. These findings suggest that DKT reduces intestinal fibrosis associated with decreasing expression of HSP47 and collagen content in the intestine.

Key words Daikenchuto; heat shock protein 47; myofibroblast

In formulating therapeutic strategies for inflammatory bowel disease (IBD), especially Crohn’s disease (CD), it is important not only to inhibit inflammation but also to regulate intestinal fibrosis. Chronic transmural inflammation combined with dysregulated wound healing is thought to result in fibrostenotic stricture formation in more than one-third of patients with CD. Fibrotic stenosis often leads to surgery to relieve obstructions and reduces quality of life in IBD patients. Existing therapies, predominantly aminosalicylates, steroids, and immunomodulators, can relieve the inflammatory symptoms of intestinal fibrostenosis in CD, but they do not significantly improve the stricture lesions of the bowel. Recent data suggest that anti-tumor necrosis factor-α treatment might be effective in the early stages of CD, when fibrogenesis is still reversible. In other diseases, such as systemic sclerosis, pulmonary fibrosis, and rheumatoid arthritis, anti-fibrotic agents are already used or are in preclinical or clinical trials. Therefore, prevention and treatment of fibrosis in CD is a key point for further research in the field of IBD.

Although the exact molecular mechanisms of tissue fibrosis are not fully understood, increased synthesis and deposition of collagens are consistently observed in most fibrotic diseases, irrespective of their origin. Recent evidence has suggested that transforming growth factor (TGF) β1, heat shock protein (HSP) 47, fibroblasts, and subepithelial myofibroblasts (SEMFs) are involved in the processing and secretion of procollagen and play an important role in the molecular mechanism underlying fibrosis. HSP47 is a 47-kDa glycoprotein that is present mostly in the endoplasmic reticulum of collagen-producing cells, including SEMFs, and is involved in the molecular maturation of various types of collagens by assisting in the correct folding of procollagens. Increased HSP47 expression with excessive accumulation of collagens is consistently observed in various human and experimental fibrotic diseases. Recent in vivo studies have shown that blocking the bioactivities of HSP47 not only alters collagen production but also reduces the progression of fibrotic lesions, directly implicating HSP47 in fibrogenesis. Therefore, the profibrotic effects of HSP47 make it a potential target for developing anti-fibrotic therapy. Recently, Honzawa et al. demonstrated for the first time that the serum level of HSP47 is higher in patients with CD than in those with ulcerative colitis and control subjects. However, it is still unclear whether or not HSP47 is involved in intestinal fibrosis in CD or in which type of cells this chaperone protein is localized.

Recent studies frequently used an experimental colitis in rats induced by intracolonic administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) as a CD model. This model, maintained for 4–6 weeks, is used to investigate not only immune events underlying the acute inflammatory response...
in the colon but also intestinal fibrosis combined with abundant collagen deposition and fibrogenic extracellular matrix changes. The first aim of the present study was to determine time–course profiles of several parameters that are characteristic of fibrosis in this model of colitis in rats maintained for 6 weeks, and to confirm the HSP47-expressing cells in the colon.

Japanese herbal medicine, called Kampo, includes herbal therapies that have been used in Asia for thousands of years. Kampo medicines are now manufactured to qualitative and quantitative standards in Japan. DKT, a Kampo medicine consisting of extract powders from dried Japanese pepper, processed ginger, ginseng radix, and maltose powder, has been used to improve gastrointestinal motility, postoperative adhesion, and paralytic ileus after abdominal surgery; its clinical efficacy is well established. Moreover, DKT had a significant promotility effect in the small bowel and ascending colon transit in healthy subjects compared with placebo in a randomized, parallel-group, double-blind, placebo-controlled study. Recently, Kono et al. reported that DKT significantly attenuated mucosal damage and colonic inflammatory adhesions, and inhibited the elevation of proinflammatory cytokines tumor necrosis factor-α and interferon-γ in the colon in an acute inflammatory model of TNBS colitis. The second aim of this study was to investigate the effects of DKT on intestinal fibrosis and the expression of fibrosis-associated genes in TNBS-induced chronic colitis in rats.

MATERIALS AND METHODS

**Reagents** All chemicals were prepared immediately before use. DKT was obtained from Tsumura Co. (Tokyo, Japan). TNBS was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of reagent grade.

**Experimental Animals** Male Wistar rats weighing 180—200 g were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). The animals were housed at 22 °C in a controlled environment with 12 h of artificial light per day and allowed access to rat chow and water ad libitum. They were fasted for 24 h before the experiment but had free access to drinking water. The animals were maintained and the experimental procedures carried out in accordance with the NIH guidelines for the use of experimental animals. All experimental protocols were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (Kyoto, Japan).

**Induction of Colitis** TNBS-induced colitis was established in rats as previously described. In brief, rats were lightly anesthetized with pentobarbital following a 24-h fast, and then a rubber catheter (o.d. 2 mm) was inserted 8 cm into the anus. TNBS dissolved in 50% ethanol (120 mg/ml) was instilled into the lumen of the colon through the catheter (0.25 ml in volume). Following the instillation of 30 mg TNBS per rat, the anus was occluded with a clip for 60 min.

**Overview of Model** In the first experiment, in the control group, rats were killed 1 week after administration of the 50% ethanol vehicle, in the 1-, 2-, 4-, and 6-week groups, rats were killed 1, 2, 4, and 6 weeks after administration of TNBS dissolved in 50% ethanol.

In the second, all rats were divided into four groups at random on the day after the induction of colitis. The sham group was treated with vehicle (distilled water) after receiving an enema of 50% ethanol vehicle instead of TNBS. The TNBS group was treated with vehicle after TNBS administration. The sham plus DKT-treated group and the TNBS plus DKT-treated group was treated with DKT (900 mg/kg; Japanese pepper 20 mg/kg, processed ginger 50 mg/kg, ginseng radix 30 mg/kg, and maltose powder 800 mg/kg) after TNBS administration. The dose of DKT used in the present study was determined according to the previous study. DKT was dissolved in distilled water and administered via gastric intubation once a day from 1 week after TNBS administration (day 8) until day 14. The rats were killed on day 15.

**Assessment of Colonic Fibrosis** The distal colon was removed and opened by a longitudinal incision. The degree of colitis was evaluated by an independent observer who had no knowledge of the treatment. The wet weight of a 6-cm-long segment of the colon was measured. As indices of macroscopic injury, colonic damage was estimated macroscopically as the sum of the mucosal and serosal scores. The mucosal score was rated on a 6-point scale (0—5) according to the criteria established by Morris et al. The serosal score was rated on a 4-point scale (0—3) according to the criteria established by Yoshida et al. (Table 1). For microscopic study, specimens of the distal colon were fixed in 10% formalin and embedded in paraffin, then cut into serial thick sections. The sections were stained with hematoxylin–eosin (H&E) to assess the degree of inflammation and the colon thickness, and with Azan to detect connective tissue and fibrosis. The degree of colonic fibrosis was scored as absence (0), mild (1), moderate (2), or severe (3), depending on the density and extent of Azan-positive connective tissue staining and disruption of tissue architecture. The other section was used for the quantitative estimation of collagen. The Semi-Quantitative Collagen Assay Kit (Chondrex, Inc., Redmond, WA, U.S.A.) provides a simple quantitative microassay tool for determining the amounts of collagen and non-collagenous proteins in tissue sections by differential staining with two dyes, Sirius Red and Fast Green. Sirius Red binds specifically to collagen, whereas Fast Green stains non-collagenous proteins. This method has been applied to the measurement of collagen contents in various tissues. These dyes can be easily extracted from stained tissues, and the amounts (micrograms) of collagen and non-collagenous proteins in each section can be calculated based on OD 540 (Sirius Red) and OD 605 (Fast Green). The assay sensitivity is high enough to determine collagen and non-collagenous proteins in 10 μm×3 cm tissue sections prepared for general histological studies.

**Real-Time Polymerase Chain Reaction (PCR)** The expression levels of intestinal HSP47, collagenI, and transforming growth factor β1 (TGF-β1) mRNA were determined by real-time-PCR. Samples for mRNA isolation were removed from colonic tissue. Total RNA was isolated with the acid guanidinium phenol chloroform (AGPC) method using ISOGEN (Nippon Gene, Toyama, Japan). The RNA concentration was determined by absorbance at 260 nm in relation to that at 280 nm. The RNA was stored at −70 °C until used for reverse-transcription polymerase chain reaction (RT-PCR). One milliliter of RT product was added to a solution
containing 3 mM of each primer, HSP47, collagen I, TGF-β1, and β-actin (as an internal standard). The mixture underwent PCR amplification for 30 cycles (1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C). The primers had the following sequences: for HSP47, sense 5'-GACAACCGTGCTTCATGGT-3'; antisense 5'-TGTCGGTGACATCGTA-3'; for collagen I, sense 5'-GGAGAGTACTGGATCGACCCTACT-3' and antisense 5'-CTGACCTGTCCTCAGTTTGAC-3'; for TGF-β1, sense 5'-GCTGGTGAACCCACTGAT-3' and antisense 5'-GCCACTGCGGACAAGTCT-3'; for β-actin, sense 5'-GAGCAAACATCCCAAGTT-3' and antisense 5'-GCCGTGGATCTTGGAGT-3'.

Assessment of HSP47 Localization in Colonic Mucosa
To evaluate the co-localization of HSP47 and myofibroblasts, double labeling by immunofluorescence was performed using a confocal microscope (FV10i; Olympus, Tokyo, Japan). Serial 5-μm-thick cryostat sections were mounted on silanized slides and incubated overnight at 4 °C with a mouse monoclonal antibody to HSP47 (Stressgen, Brussels, Belgium) and a rabbit polyclonal antibody to α-smooth muscle actin (Abcam, Cambridge, MA, U.S.A.). Primary antibodies were then reacted with a goat anti-mouse immunoglobulin G (IgG) labeled with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, U.S.A.) or a chicken anti-rabbit IgG labeled with Alexa Fluor 594 (Invitrogen).

Statistics Colonic damage scores and Azan scores are presented as scatter plots and as medians. Differences between groups were compared by analysis of variance (Kruskal–Wallis test) followed by multiple-comparison Dunn’s test. Other data were presented as the mean±S.E.M. and were compared by one-way analysis of variance (ANOVA). If the ANOVA was significant, the differences between individual groups were analyzed by multiple-comparison Bonferroni’s test. Values of p<0.05 were considered statistically significant. All analyses were performed using GraphPad Prism5 (GraphPad Software, San Diego, CA, U.S.A.) on a Windows-based computer.

RESULTS

Localization of HSP47 in Colonic Mucosa As shown in Fig. 1, in colonic mucosa, HSP47 immunoreactivity was coexpressed with α-smooth muscle actin, indicating that these cells were myofibroblasts. Most of the HSP47-positive cells were located in the subepithelial space.

Effects of Intracolonic Administration of TNBS on Colon Weight, Wall Thickness, and Damage Score after Administration Intracolonic administration of TNBS resulted in grossly visible ulcers. Damage scores peaked one week after TNBS/ethanol administration, and remained at peak levels for the following 6 weeks. Colon weight and wall thickness also significantly increased and remained significantly above control levels for 2 weeks (Fig. 2). Histologically, ulceration and inflammation of the colon remained for 6 weeks (Fig. 3).

Effect of Intracolonic Administration of TNBS on Colonic Fibrosis After TNBS/ethanol administration, there were Azan-positive diffused collagen deposits in the mucosa and submucosa (Fig. 4). Azan-stained collagen deposits were still evident 2 weeks after TNBS/ethanol administration (Fig. 4). Quantitative estimation of collagen remained significantly (p<0.05) above control levels for 4 weeks (Fig. 4).

Expression of HSP47, Collagen1, and TGF-β1 mRNA in Colonic Tissue To define the mechanisms underlying the fibrogenic process, we used RT-PCR to elucidate changes in gene expression for a collagen-specific molecular chaper-
one (HSP47), collagen (collagen1), and inflammation-mediating growth factor (TGF-β1).

In the TNBS-treated colons, the mRNA expression levels of HSP47 and TGF-β1 remained significantly \((p<0.05)\) above control levels for 2 weeks, and that of collagen1 was significantly elevated for 1 week.

Effects of DKT on TNBS-Induced Colon Weight, Wall Thickness, and Damage Score

In the rats exposed to TNBS, macroscopic findings of the colon demonstrated severe colitis with hyperemia, edema, thickening, ulceration, and necrosis. In contrast, the rats treated with DKT at a dose of 900 mg/kg/d showed smaller erosions with mild edema in the colon (Fig. 6). As a result, the colonic damage score showed a significant increase by TNBS administration. The therapeutic effect of DKT was also confirmed histologically (Fig. 6). DKT treatment significantly inhibited the increase in the damage score (Fig. 7). Figure 8 shows typical histological features in TNBS- and DKT-treated groups.
Effect of DKT on TNBS-Induced Colonic Fibrosis

In the TNBS rats, the affected colonic wall area consisted of granulomatous tissue in which fibroblasts and fibrosis were evident in the submucosa along with regenerative changes in the overlying epithelium. Due to collagen deposition, this group of rats often exhibited severe disarrangement of colonic architecture. Compared to untreated TNBS rats, colonic fibrosis was significantly reduced in DKT-treated TNBS rats (Fig. 8).

Effects of DKT Administration on HSP47, Collagen1, and TGF-β1 mRNA in Colonic Tissue

The mucosal mRNA expression levels of HSP47 and collagen1 were increased significantly in untreated TNBS rats. These increases were significantly inhibited by treatment with DKT at a dose of 900 mg/kg. HSP47, n=6—11. Collagen type 1, n=6—7. TGF-β1, n=6—12. ∗p<0.05 compared to sham group rats. #p<0.05 compared to TNBS rats. #p<0.05 compared to TNBS rats.

DISCUSSION

In this study, we first assessed intestinal fibrosis in rat TNBS colitis by a variety of parameters, including histology,
quantitative estimation of collagen production, and measurement of mRNA expression for HSP47, collagen type I and TGF-β1. We then determined the localization of HSP47. Immunohistochemical study showed that HSP47 was localized predominantly in α-smooth muscle actin (SMA)-positive SEMFs. By each assessment, colonic transmural fibrosis and collagen accumulation persisted for 6 weeks after the cessation of TNBS treatment, accompanied by enhanced expression levels of HSP47, collagen type I, and TGF-β1 genes. In the next experiment, we found that DKT reduced the damage score, microscopic fibrosis, and the expression levels of HSP47 and collagen I mRNA in this experimental rat model of fibrosis. This is the first study to confirm that HSP47 expressed in SEMFs was upregulated in colons treated with TNBS, and that DKT reduces intestinal fibrosis associated with decreasing expression of HSP47 and collagen content in the intestine.

In this experiment, the rats developed mucosal injury and inflammation, as reflected by the increases in damage score, wall thickness, and wet weight. These scores peaked one week after TNBS administration, then decreased gradually for 6 weeks. Azan score, an index of fibrosis determined by the density and extent of Azan-positive connective tissue staining, and the levels of collagen accumulation peaked one week after TNBS administration. These findings, especially those of transmural inflammation and fibrosis, resemble human CD, which is similar to results in rats. Our findings indicate that the present experimental model is useful for assessing colonic fibrosis.

HSP47 is believed to be an important cause of various fibrotic diseases. Increased expression of HSP47 with excessive accumulation of collagens is consistently observed in various human and experimental fibrotic diseases such as idiopathic pulmonary fibrosis, 

fibrotic transplanted kidney, 

and peritoneal sclerosis. Previous studies have shown the essential roles of HSP47 not only in the maturation of type I collagen but also in the subsequent secretion, processing, and fibril formation of this collagen. Recent reports have demonstrated that HSP47 expression is highly tissue- and cell-specific, primarily restricted to phenotypically altered collagen-producing cells, and its expression correlates well with that of collagen. In the present study, HSP47-positive cells were predominantly localized in α-SMA-positive myofibroblasts located in the subepithelial space, which were confirmed by the merged immunofluorescence. The localization of HSP47 in myofibroblasts has been demonstrated also in lung interstitium, and the relative amounts of HSP47 mRNA in the lung correlate significantly with hydroxyproline content.

Intestinal myofibroblasts also were implicated in intestinal fibrosis in TNBS colitis as well as in a Lewis rat model injected with bacterial cell wall lipopolysaccharides. In the present study, HSP47 mRNA expression was significantly enhanced 1 week after TNBS administration, and this enhancement was correlated with changes in mRNA expression for TGF-β1 and collagen I. In human lung fibroblasts, TGF-β1 induced trimer formation of heat shock factor 1 (HSF1), which then binds to the heat shock promoter element (HSE) to induce the synthesis of HSP47. These data, including the present findings, suggest that the upregulated signaling pathway of TGF-β1—HSP47-collagen I genes may be related to the pathogenesis of intestinal fibrosis in rats treated with TNBS.

In the subsequent study, the oral administration of DKT prevented the development and progression of colonic fibrosis in TNBS-induced chronic colitis in rats. This prevention appeared to be related to the decreasing expression of HSP47 and collagen I in the colon. These findings were observed when DKT was administered for one week, beginning a week after the development of colitis, indicating its therapeutic potential against intestinal fibrosis after the resolution of acute inflammatory reaction. This is the first report to show that DKT ameliorated intestinal fibrosis associated with decreasing expression of HSP47 and collagen I induced by TNBS administration. Although HSP47-collagen pathway has been already reported in several papers, the mechanisms other than the HSP47-collagen pathway may present in the course of intestinal fibrosis. Further studies are necessary to elucidate the precise mechanism of intestinal fibrosis.

DKT, a traditional Japanese herbal (Kampo) medicine, is a mixture of extract powders from dried Japanese pepper, processed ginger, ginseng radix, and maltose powder. It has been used to improve gastrointestinal motility, postoperative adhesion, and paralytic ileus after abdominal surgery; its clinical efficacy is well established. These properties of DKT have been proven to be mediated by acetylcholine, nitric oxide, motilin, and calcitonin gene-related peptide and their receptor components. In the present study, DKT treatment did not affect TGF-β1 mRNA expression, whereas it significantly inhibited HSP47 expression in the colon. Because it has been demonstrated that ginsenoside Rb1 and hydroxy-a-sanshool are the main ingredients in DKT extract, further studies are needed to clarify the mechanism by which DKT exerts anti-fibrotic properties in chronic intestinal inflammation.

In summary, the present study establishes that the enhancement of HSP47 expression in myofibroblasts occurring in experimental colitis may play a crucial role in collagen accumulation and fibrosis during the chronic inflammation phase, and may indicate that DKT treatment can protect against TNBS-induced intestinal fibrosis, which is associated with a remarkable decrease in HSP47 overexpression. These results suggest that DKT administration may be therapeutically effective for chronic intestinal fibrosis. Further studies are necessary to elucidate the precise mechanism by which DKT acts on the expression of HSP47 and myofibroblasts, and to investigate DKT’s clinical efficacy in patients with CD.

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