The Kampo Medicine Daikenchuto Inhibits Peritoneal Fibrosis in Mice

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Long-term peritoneal dialysis therapy causes inflammation and histological changes in the peritoneal membrane. Inflammation generally activates fibroblasts and results in fibroblast–myofibroblast differentiation. Heat-shock protein 47 (HSP 47), a collagen-specific molecular chaperone, is localized in myofibroblasts and is involved in the progression of peritoneal fibrosis. Daikenchuto (DKT), a Kampo medicine, is used to prevent postoperative colon adhesion. It inhibits inflammation and HSP 47 expression in the gastrointestinal tract. We examined the effect of DKT on chlorhexidine gluconate (CG)-induced peritoneal fibrosis in mice injected with 0.1% CG dissolved in 15% ethanol. DKT was dissolved in the drinking water. Histological changes were assessed using Masson trichrome staining. Cells expressing α-smooth muscle actin (α-SMA), HSP 47, phospho-Smad 2/3, F4/80, and monocyte chemotactic protein-1 were examined immunohistochemically. Compared with the control group, the peritoneal tissues of the CG group were markedly thickened, and the number of cells expressing α-SMA, HSP 47, phospho-Smad 2/3, F4/80, and monocyte chemotactic protein-1 was significantly increased. However, these changes were inhibited in the DKT-treated group. These results indicate that DKT can prevent peritoneal fibrosis by inhibiting inflammation and HSP 47 expression.

Key words Daikenchuto; heat-shock protein 47; inflammation; peritoneal dialysis; peritoneal fibrosis

Peritoneal dialysis (PD) is a beneficial therapy for end-stage renal disease. However, long-term PD therapy leads to peritoneal fibrosis. Recent studies have reported that some patients with peritoneal fibrosis develop encapsulating peritoneal sclerosis in which the gastrointestinal tract is covered with a thickened fibrous tissue, which leads to serious ileus. Encapsulating peritoneal sclerosis is associated with high mortality, which is one of the most serious complications of PD therapy. The precise pathogenesis of peritoneal fibrosis remains unknown; however, marked peritoneal fibrosis and a massive accumulation of collagen are typical in patients under long-term PD therapy.

There have been many therapeutic candidates for peritoneal fibrosis such as vitamin D, tamoxifen, and corticosteroids. However, evidence-based effective therapy is unavailable. Japanese herbal medicine, also called Kampo medicine, includes herbal therapies that have been used in Asia for thousands of years. In Japan, Kampo medicines are manufactured to qualitative and quantitative standards. Daikenchuto (DKT), a Kampo medicine, consists of extract powders from dried Japanese pepper, processed ginger, ginseng radix, and maltose powder. It has been used to improve gastrointestinal motility and prevent postoperative adhesion and paralytic ileus after abdominal surgery effectively. Cumulative evidence reveals that DKT attenuates inflammation in rats treated with monocrotaline and in a rat model of Crohn’s disease. Furthermore, Inoue et al. reported in a rat model that DKT significantly attenuated colitis with decreased expression of the collagen-specific molecular chaperone heat shock protein 47 (HSP 47), which is essential for the biosynthesis and secretion of collagen molecules. These results strongly indicate that HSP 47 has crucial roles in collagen accumulation in various disorders such as human peritoneal fibrosis.

DKT suppresses intestinal inflammation, but its effect on peritoneal fibrosis remains to be clarified. In this study, we examined the effect of DKT on the development of experimental peritoneal fibrosis that is induced by intraperitoneal injections of chlorhexidine gluconate (CG) in mice.

MATERIALS AND METHODS

Animals The animals used in this study were 8-week-old male ICR mice (Japan SLC, Inc., Shizuoka, Japan). They weighed approximately 35 g. The mice had free access to laboratory chow and were housed in a light- and temperature-controlled room in the Laboratory Animal Center of the Nagasaki University School (Nagasaki, Japan). The experimental protocol was inspected by the Animal Care and Use Committee of the Nagasaki University School and approved by the President of the Nagasaki University School (approval number: 1004050846-5).

Reagents DKT was prepared from the Daikenchuto dried extracts, which were purchased from Tsumura and Company (Tokyo, Japan; lot E20542). The DKT extracts (0.125%, 0.625%, 1.25%) were dissolved in drinking water and heated at 50°C by a hot stirrer. They were then filtered with a 0.2-µm pore size filter. The DKT contains processed ginger (Zingiber officinale Roscoe, rhizome), ginseng (Araliaceae, Panax ginseng C.A. Meyer, radix), and zanthoxylum fruit (Rutaceae, Zanthoxylum piperitum De Candolle). Detailed information for preparing DKT is described in a previous report.

According to preliminary examination, the average intake of 1.25% DKT-containing water was nearly 7 mL/d. Therefore, the concentration would be nearly equal to a dose
Administration of CG and DKT To induce peritoneal fibrosis, the animals were injected intraperitoneally with 1.25% CG in 15% ethanol in saline (i.e., the vehicle). Three times weekly for 3 weeks, 2 groups of mice received either the CG-containing solution or an equal volume of vehicle. Both groups were further subdivided into 2 groups: mice that received 1.25% DKT in their drinking water for 21 d or mice that received drinking water alone for 21 d. Therefore, there were 4 groups: vehicle+drinking water (defined as the control group); CG+drinking water (defined as the CG group); CG+DKT (defined as the DKT group), and the vehicle+DKT (vehicle+DKT) group. Each group consisted of 5 mice. The bottles of DKT-containing drinking water were replaced every 3 d and the DKT intake of each mouse was measured. The body weights of all mice were measured once weekly, and the amount of DKT ingestion was measured every 3 d.

According to a previous report, DKT has a dose-dependent effect. To confirm the dose-dependent effect of DKT in this model, we administered 0.125% and 0.625% of DKT (the daily concentrations of which would be 250 mg/kg/d and 1250 mg/kg/d, respectively). In addition, to examine the therapeutic effect of DKT on peritoneal fibrosis, we also administered 1.25% DKT 2 weeks after CG administration. Each group consisted of 5 mice.

The mice were killed on day 22. Their peritoneal tissues were dissected, removed, and fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). For Western blotting, the parietal peritoneal tissues were snap frozen and maintained at −80°C.

Histological Analysis and Immunohistochemistry For histological examination, 4-μm thick paraffin-embedded tissues were stained with Masson trichrome. An indirect immunohistochemical technique was used for HSP 47, α-SMA, and F4/80. Indirect staining was performed as described previously. In brief, deparaffinized tissue sections were incubated for 30 min with a blocking buffer containing 5% normal goat serum, 5% fetal calf serum, 5% bovine serum albumin, and 20% normal swine serum in phosphate-buffered saline. The sections were then reacted with the primary antibodies, and diluted in the same blocking buffer. After reacting with F4/80, the sections were reacted with HRP-conjugated rabbit anti-IgG antibody and HRP-conjugated swine anti-rabbit IgG antibody for 1 h at room temperature (RT). For HSP 47 and α-SMA, the sections were reacted with primary antibodies for 16 h at RT, and HRP-conjugated goat anti-mouse IgG antibody, diluted to 1/100, for 1 h at RT.

For pSmad 2/3 and MCP-1, the tissues were stained by an avidin–biotin complex technique using the Vectastain Elite ABC kit (Vector Laboratories, Inc.), after reacting with the primary antibody, as described previously. In brief, pSmad 2/3 was reacted for 1 h at RT. For MCP-1, the primary antibody was reacted for 16 h at RT. The reaction products were visualized by treating the sections with 70 μL of 30% hydrogen peroxide and 50 μg of DAB in 100 μL of Tris-buffered saline (TBS; 50 mM Tris buffer, pH 7.6, and 150 mM NaCl). The sections were finally counterstained with methyl green and mounted.

Morphometric Analysis To assess the thickness of submesothelial compact zone, we used image analysis software (Lumina Vision Ver. 2.04; Mitani Corp., Fukui, Japan), after Masson trichrome staining. We measured the thickness at 5 positions that were randomly selected in each tissue at ×100 magnification. The results were averaged.

In each sample, we counted the number of HSP 47-expressing cells, α-SMA-expressing cells, pSmad 2/3-expressing cells, F4/80-positive macrophages, and MCP-1-expressing cells in 5 fields at ×100 magnification. The results for each tissue were averaged.

Western Blotting Seventy-five microliters of the tissue lysates (with a protein concentration of approximately 5 mg/mL) were mixed with 25 μL of sample buffer and boiled for 5 min at 100°C. Each sample (10 μL) was mounted on sodium dodecyl sulfate polyacrylamide gel (γ-Page gradient gel 10–20%, 2331840, ATTO, Tokyo, Japan) and separated. They were then electrophoretically transferred to a polyvinylidene fluoride membrane (0.2-μm pore size). The membranes were
blocked with 5% nonfat milk in 20mM TBS (pH 7.6) for 1 h at RT, and then incubated for 1 h with mouse anti-HSP 47. After a brief wash with TBS containing 0.1% Tween 20 (TBS-T), the second antibody, HRP-goat anti-mouse, was diluted in TBS-T and incubated on membranes for 1 h at RT. The membranes were washed 6 times with TBS-T. The blots were developed with the Amersham ECL plus Western blotting Detection System (GE Healthcare), and chemiluminescence was captured with the Alpha Imager 3400/2200 (Alpha Innotech, San Leandro, CA, U.S.A.). Densitometric analysis was also performed with the Alpha Imager.

β-Actin was the internal control for peritoneal fibrosis.\textsuperscript{22) For detecting the internal control after washing with Restore Western blot Stripping Buffer (Thermo Fisher Scientific, Inc.), rabbit anti-β-actin antibody was reacted on the same membrane and the signal was detected, as described previously.

\textbf{Statistical Analysis} Data are expressed as the mean±the standard error of the mean (S.E.M.). Differences between groups were examined for statistical significance by repeated measures ANOVA (Bonferroni/Dunn test). A $p<0.05$ denoted a statistically significant difference. All statistical analyses were performed with Stat View version 5.0 software (SAS

**Fig. 1. Masson Trichrome Staining of Peritoneal Tissues**

A, In normal mice, the monolayer of mesothelial cells was covered by the surface of the peritoneum. B, In the control mouse group, which was injected with the vehicle, the peritoneum was only slightly thickened. C, Injections of CG induced significant thickening of the peritoneum. D, The administration of DKT in the CG-treated mice significantly suppressed the progression of peritoneal thickening. E, The daily administration of DKT had little effect on peritoneal thickening, compared to the control group. For A–E, the magnification is $\times$200 and the bars indicate the thickness of the submesothelial compact zone. F, The bar graph represents the thickness of the submesothelial compact zone. The data were expressed as the mean±the standard error of the mean (S.E.M.). **$p<0.01$. CG, chlorhexidine gluconate; DKT, Daikenchuto.
RESULTS

Body Weight and DKT Ingestion Throughout the experimental period, the body weights of the control group and the vehicle+DKT group were not significantly different. At 1 week and 2 weeks, the body weights in the groups administered CG (i.e., the CG group and the DKT group) were significantly lower than the body weight of the control group. However, at 3 weeks, the body weight of the groups administered CG and the control group was the same. Supplemental Table 1 shows these results. The average daily amount of DKT ingested during the observation period was 3091 mg/kg in the DKT group and 4307 mg/kg in the vehicle+DKT group (Supplemental Fig. 1). The amount of DKT ingested by the vehicle+DKT group was beyond the amount ingested by the DKT group; however, the vehicle+DKT group showed no adverse effects.

Histological Examination Following Masson Trichrome Staining In the normal and control mice, the peritoneal tissue consisted of a peritoneal mesothelial monolayer and sparse connective tissues just below this layer (Figs. 1A, B). Com-

Institute Inc., Cary, NC, U.S.A.).
pared to the control group, the submesothelial compact zone in the peritoneum of the CG group was significantly thicker and enriched with numerous cells (Figs. 1C, F). The thickness of the submesothelial compact zone in the DKT group was significantly less than that of the CG group (Figs. 1D, F). The submesothelial compact zone in the half-dose DKT group was thicker than that of the DKT group. The CG group showed gastrointestinal adhesion and some mice developed a cocoon; these changes, all of which reflect the features of human peritoneal fibrosis, were significantly suppressed in the DKT group (data not shown). To assess the toxicity of DKT, we further analyzed the vehicle+DKT group. Drinking water containing DKT had no significant effect on the peritoneum and liver (Figs. 1E, F). The peritoneal thickness was greater in mice administered 0.125% DKT or 0.625% DKT than in mice administered 1.25% DKT (defined as the DKT group) (Supplemental Figs. 2A, B, D). In addition, the late administration of DKT had little anti-fibrotic effect (Supplemental Figs. 2C, D).

Expression of HSP 47 As shown in Fig. 2A, in the control group, HSP 47 was weakly and primarily expressed in the mesothelial cells. In the CG group, the number of HSP 47-positive cells was increased in the thickened submesothelial compact zone (Fig. 2B), whereas in the DKT group, the number of HSP 47-positive cells was decreased (Figs. 2C, D). Most HSP 47-positive cells were morphologically identified as mesothelial cells on the surface of the peritoneum and spindle-shaped fibroblasts in the submesothelial compact zone.

The expression of HSP 47 in the parietal peritoneal tissue was also analyzed by Western blotting. A single band corresponding to 47 kDa was detected in the peritoneal tissue (Fig. 2E). Densitometric analysis showed a significant increase in the CG group, compared to the control group. On the other hand, the expression of HSP 47 in the DKT group was significantly suppressed, compared to the expression in the CG group (Figs. 2E, F).

Expression of α-SMA, Transforming Growth Factor-β and pSmad 2/3 We investigated the number of α-SMA-expressing cells as a marker of myofibroblasts, which are collagen-producing cells. In the control group, we observed the expression of α-SMA only in vascular smooth muscle cells in the peritoneum (data not shown). In the CG group, we observed numerous α-SMA-expressing myofibroblasts in the submesothelial compact zone (Fig. 3A, Table 1). In contrast, the number of α-SMA-positive myofibroblasts in the DKT group was markedly decreased (Fig. 3B, Table 1). We next evaluated the activation of TGF-β-signaling by the phosphorylation of Smad 2/3. As can be seen in Fig. 3C, the number of pSmad 2/3-positive cells was higher in the CG group than in the control group (Fig. 3C, Table 1). However, in the DKT group pSmad 2/3-expressing cells were decreased in the submesothelial compact zone (Fig. 3D, Table 1).

Expression of F4/80 and MCP-1 To investigate the in-

![Fig. 3. Immunohistochemistry for α-SMA and pSmad 2/3](image-url)

A, Numerous α-SMA-expressing cells were present in the thickened submesothelial compact zone of the CG group. B, In the DKT group, the number of α-SMA-expressing cells was reduced. C, pSmad 2/3 was strongly expressed in the thickened submesothelial compact zone in the CG group. D, In the DKT group, the expression of pSmad 2/3 was decreased. For A–D, the magnification was ×200 and the bars indicate the thickness of the submesothelial compact zone. CG, chlorhexidine gluconate; pSmad 2/3, phospho-Smad 2/3; α-SMA, α-smooth muscle actin.
Involvement of inflammation in the development of peritoneal fibrosis, we performed immunohistochemistry for F4/80 as a marker of macrophages. In the CG group, we observed numerous macrophages in the thickened peritoneal tissues (Fig. 4A, Table 1). However, the DKT group contained significantly fewer macrophages, compared to the CG group (Fig. 4B, Table 1).

We also investigated the number of MCP-1-expressing cells, which exert an important effect on macrophage infiltration. In the CG group, the number of MCP-1-positive cells was significantly higher than the number in the control group (Fig. 4C, Table 1). Compared to the CG group, the DKT group contained significantly fewer MCP-1-expressing cells (Fig. 4D, Table 1).

DISCUSSION

In the present study, we demonstrated that DKT in a dose-dependent manner prevented CG-induced peritoneal fibrosis in a mouse model of peritoneal fibrosis. DKT diminished collagen accumulation in the thickened submesothelial area via suppressing HSP 47-positive cells and myofibroblasts. Furthermore, treatment with DKT reduced the number of MCP-1-positive cells and F4/80-positive macrophages. In this report, DKT had an anti-fibrotic effect in a dose-dependent manner, as reported previously.16,23) These findings indicate that DKT may be useful in preventing the progression of peritoneal fibrosis.

Peritoneal fibrosis is a serious complication of continuous
ambulatory PD therapy and is characterized by collagen accumulation.\(^1\) The precise mechanisms of peritoneal fibrosis remain unclear, although HSP 47 is known to be involved in fibrosis as a collagen-specific molecular chaperone that is closely associated with collagen synthesis.\(^{1,9}\) We have previously demonstrated that HSP 47 expression was significantly correlated with the accumulation of collagen type I and type III in an animal model of peritoneal fibrosis.\(^{17,18}\) Furthermore, we demonstrated that the number of infiltrating macrophages in the peritoneum was positively correlated with peritoneal fibrosis,\(^ {17}\) which suggests that inflammation has a key role in the development of peritoneal fibrosis.

Daikenchuto is a complex mixture that consists of ginseng, processed ginger, Japanese pepper, and maltose.\(^ {23}\) Daikenchuto is commonly prescribed to prevent postoperative colon adhesion in Japanese patients and this effect has been proven in clinical trials.\(^ {24,25}\) Animal models also demonstrate various beneficial effects such as improving bowel movement,\(^ {26}\) preventing tala-induced colon adhesion,\(^ {27}\) and attenuating inflammation in an animal model of Crohn’s disease.\(^ {10,11}\) Inoue et al. have reported that DKT prevented intestinal fibrosis in trinitrobenzene sulfonic acid-induced rat colitis through inhibiting HSP 47 expression.\(^ {27}\) In addition, shogaol and san-shoo, which are the active ingredients of DKT, have a strong anti-inflammatory effect.\(^ {2,28}\) Shogaol inhibits the production of interleukin (IL)-1\(\beta\) and tumor necrosis factor (TNF)-\(\alpha\),\(^ {29,30}\) which are induced by CG injection and involved in macrophage infiltration. In accordance with the previous results, we could verify that HSP 47 expression and inflammation in our model were inhibited by DKT administration.

Since the effect of DKT was dose-dependent, we set the amount of DKT as nearly equal to the maximum dose of previous studies.\(^ {16,23,28}\) This dose is greater than the dose for clinical use in humans. However, the mice administered DKT had no adverse effect on renal and liver function and DKT administration improved liver function, although the precise mechanism remains unclear (data not shown).

In the present study, we prepared the DKT extract as 1.25% by weight, which is equal to the maximum dose of preliminary examination. It is unclear, although HSP 47 expression and inflammation in our model were inhibited by DKT administration. It is unclear, although HSP 47 expression and inflammation in our model were inhibited by DKT administration.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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