The Chinese Prescription Wen-Pi-Tang Extract Delays Disease Onset in Amyotrophic Lateral Sclerosis Model Mice While Attenuating the Activation of Glial Cells in the Spinal Cord

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Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by the selective loss of motor neurons. There is no effective treatment or drug against ALS, and the precise mechanisms leading to the selective loss of motor neurons are still unknown. We investigated the effect of a Chinese prescription, Wen-Pi-Tang, on the ALS model mouse SOD1G93A. Although the oral administration of Wen-Pi-Tang extract to SOD1G93A mice had no significant effect on body weight loss and survival time, Wen-Pi-Tang delayed disease onset. Therefore, we evaluated immunohistological changes in the spinal cord of SOD1G93A mice during the early disease period, and found that Wen-Pi-Tang extract inhibited neuronal loss in the lumbar segment of the spinal cord of mice. Furthermore, increased astrocytes and microglial cells, which increase prior to neuronal loss, in spinal cords were significantly reduced in the Wen-Pi-Tang treated group. Since oxidative markers, heme oxygenase-1 and inducible nitric oxide synthase, in the spinal cord were also reduced as well as the change in microglia, the administration of Wen-Pi-Tang was thought to delay disease onset by inhibiting glial cell activation.

Key words Wen-Pi-Tang; amyotrophic lateral sclerosis; astrocyte; microglial cell; heme oxygenase-1; inducible nitric oxide synthase

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by selective loss of motor neurons. Degeneration of the upper (motor cortex) and lower (brain stem and spinal cord) motor neurons causes muscle atrophy, paralysis, and bulbar symptoms. Its progression is rapid, and average survival times range from 3 to 5 years after the onset of symptoms. The annual incidence of ALS is 1—2 per 100000, and the prevalence is 6 per 100000 in most populations. There is no effective treatment or drug against ALS, and the precise mechanisms leading to the selective loss of motor neurons are unknown.

Almost all ALS cases are sporadic (SALS: sporadic ALS), while approximately 10% of cases are inherited (FALS: familial ALS). In FALS, more than 100 gene mutations have been identified, and it is known that about 20% of FALS cases are due to mutations of the gene coding for the enzyme copper-zinc superoxide dismutase (SOD1). Expression of mutated SOD1, and so on.

Wen-Pi-Tang is a traditional Chinese prescription composed of 5 crude drugs: Rhei Rhzoma (Rheum officinale BAILLON), 3 g of Ginseng Radix (Panax ginseng C. A. MEYER), 9 g of Aconiti Tuber (Aconitum japonicum THUNBERG), 3 g of Zingiberis Rhizoma (Zingiber officinalis ROSCOE), and 5 g of Glycyrrhizae Radix (Glycyrrhiza glabra LINN. var. glandulifera REGEL et HERDER). Ginseng Radix was produced in Korea, Aconiti Tuber was from Japan, and all the other ingredients were from China. All crude drugs were kindly supplied by Uchida Wakan-Yaku Co., Ltd. (Tokyo, Japan). The above crude drugs were chopped finely and extracted with distilled water at 100 °C for 65 min, as described previously. After the removal of insoluble matter by filtration, the filtrate was evaporated under reduced pressure and lyophilized (yield: 23.9%). For analysis of the Wen-Pi-Tang component, the aqueous extract has a strong anti-oxidative activity, and its anti-oxidative effects contribute to the improvement of renal dysfunction. In our previous study, Wen-Pi-Tang and its constitutive extracts showed radical scavenging effects against superoxide, nitric oxide, and peroxynitrite in vitro. In addition, the administration of Wen-Pi-Tang extract to influenza virus-infected mice, showing increasing oxidative stress after infection, improved impaired body weight gain and lung consolidation. This study demonstrates that Wen-Pi-Tang extract has the potential to prevent damage especially from oxidative stress in vivo. Since, generally, herbal medicines have a broad spectrum of effects, they are sometimes more effective against diseases than pure compounds.

In this study, we investigated the effect of Wen-Pi-Tang extract on ALS model mice carrying a mutated human SOD1 gene.

MATERIALS AND METHODS

Preparation of Wen-Pi-Tang Extract The Chinese prescription, Wen-Pi-Tang, consists of five crude drugs: 15 g of Rhei Rhzoma (Rheum officinale BAILLON), 3 g of Ginseng Radix (Panax ginseng C. A. MEYER), 9 g of Aconiti Tuber (Aconitum japonicum THUNBERG), 3 g of Zingiberis Rhizoma (Zingiber officinalis ROSCOE), and 5 g of Glycyrrhizae Radix (Glycyrrhiza glabra LINN. var. glandulifera REGEL et HERDER). Ginseng Radix was produced in Korea, Aconiti Tuber was from Japan, and all the other ingredients were from China. All crude drugs were kindly supplied by Uchida Wakan-Yaku Co., Ltd. (Tokyo, Japan). The above crude drugs were chopped finely and extracted with distilled water at 100 °C for 65 min, as described previously. After the removal of insoluble matter by filtration, the filtrate was evaporated under reduced pressure and lyophilized (yield: 23.9%). For analysis of the Wen-Pi-Tang component, the aqueous extract has a strong anti-oxidative activity, and its anti-oxidative effects contribute to the improvement of renal dysfunction. In our previous study, Wen-Pi-Tang and its constitutive extracts showed radical scavenging effects against superoxide, nitric oxide, and peroxynitrite in vitro. In addition, the administration of Wen-Pi-Tang extract to influenza virus-infected mice, showing increasing oxidative stress after infection, improved impaired body weight gain and lung consolidation. This study demonstrates that Wen-Pi-Tang extract has the potential to prevent damage especially from oxidative stress in vivo. Since, generally, herbal medicines have a broad spectrum of effects, they are sometimes more effective against diseases than pure compounds.

In this study, we investigated the effect of Wen-Pi-Tang extract on ALS model mice carrying a mutated human SOD1 gene.
was filtered and subjected to treatment with an Alumina cartridge (Bond Elute Co., Ltd.). HPLC equipped with a LC-10AD pump (Shimadzu, Tokyo, Japan) and an SPD-M10A VP absorbance detector was performed using a TSK GEL ODS-80Ts column (250×4.6 mm). The solvents were (A) 0.05 M AcOH–AcONH₄ (pH 3.6) and (B) 100% CH₃CN. A linear gradient of 90% A and 10% B changing over 60 min to 100% B was used. The flow rate was 1.0 ml/min. The eluent from the column was monitored with a UV detector. (+)-Catechin, epicatechin 3-O-gallate, liquiritin apioside, aloe-emodin 8-O-glucoside, and chrysophanol 1-O-glucoside, and rhein were observed as the major compounds of Wen-PiTang; isolindleyin, glycyrrhizin, and chrysophanol were also observed.

**Animals** Mice carrying a mutated human SOD1 gene (SOD1G93A; B6SJL-Tg (SOD1-G93A)1Gur/J) were obtained from Jackson Laboratory (Bar Harbor, ME, U.S.A.), and then the line was maintained as hemizygote by mating SOD1G93A transgenic males with wild-type B6SJL females. The offspring were genotyped by PCR using genomic DNA from ear tissue. Mice were housed at an ambient temperature of 23±1 °C and under a 12 h light/dark cycle. Water and food were available ad libitum. All experiments were carried out in accordance with the Animal Experimental Guideline of Niigata University of Pharmacy and Applied Life Science.

**Experimental Protocols** SOD1G93A transgenic mice and wild-type littermates at 6 weeks after birth were used in this experiment. Wen-PiTang extract was dissolved in water, and administered orally by a stomach tube at a dose of 100 or 200 mg/kg body weight/d from 7 weeks of age (W100 and W200, respectively). Riluzole was administered only water. The body weight of each mouse was measured twice a week, and the motor function was determined once a week. The disease onset was determined by counting neurons exhibiting a diameter of at least 20 µm under the light microscope (BH-2, Olympus, Tokyo, Japan). Seven slices from each mouse were used for this determination.

**Grip Strength Test** The mouse was lifted by its tail until it released the wire mesh, and the maximum grip force assessed by a spring scale attached to the wire mesh was measured. The test was performed once a week from the age of 6 weeks, and each mouse underwent three trials in a test.

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**Tissue Preparation** Animals (n=4—5) undergoing the same treatments as described above (experimental protocols) were sacrificed at the age of 16 weeks (9 weeks after the initiation of administration), and the spinal cord was removed after saline perfusion. The removed spinal cord was used for immunohistochemistry and immunoblotting. For immunohistochemistry, the removed spinal cord at lumbar area was fixed with 4% paraformaldehyde in PBS at 4 °C, and then lumbar segments (L2—4) were embedded in paraffin. Tissue blocks were sectioned coronally using a microtome, and 3—4 µm-thick slices were obtained. For immunoblotting, removed spinal cords were stored at −80 °C until analysis.

**Motor Neuron Number** For histological analysis, sectioned tissues were deparaffinized and we performed Nissl staining. Motor neuron numbers were determined by counting neurons exhibiting a diameter of at least 20 µm under the light microscope (BH-2, Olympus, Tokyo, Japan). Seven slices from each mouse were used for this determination.

**Immunohistochemistry** Deparaffinized spinal cord sections were autoclaved in 10 mM sodium citrate buffer, pH 6.0, and then stained with an antibody against glial fibrillary acidic protein (GFAP; SHIMA Laboratories Co., Ltd., Japan, 1:300; astrocyte marker), CD11b (Clone OX-42, Chemicon International, Temecula, CA, U.S.A., 1:50; microglia marker), heme oxygenase-1 (HO-1; Calbiochem, 1:300; oxidative stress marker), and inducible nitric oxide synthase (iNOS; Lab Vision, 1:500). For the secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) and Alexa Fluor 546-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, Oreg., U.S.A., 1:300) were used. The fluorescent images in the 7 spinal cord slices from each mouse (ventral and dorsal horns) were captured using fluorescent microscopy (BH-2, Olympus). To evaluate the expression levels of GFAP, HO-1, and iNOS in each region, the fluorescence intensities of immunopositive areas in those captured images were quantified using ATTO densitography (ATTO). CD11b-active microglia were determined by counting from the captured image (356×472 mm²).

**Western Blotting** Spinal cord samples were homogenized with ice-cold lysis buffer (pH 8.0, 50 mM Tris–HCl, 150 mM NaCl, 1% Triton-X-100, 0.1% sodium dodecyl sulfate (SDS), 10 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride (PMSF)) including a protease inhibitor cocktail tablet (complete, Roche Diagnostics, Mannheim, Germany). The homogenates were then centrifuged at 3000×g for 10 min at 4 °C, and the supernatants were used. Protein concentrations of the samples were determined by a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin (BSA) as the standard. Samples were subjected to 12.5% SDS-polyacrylamide gel electrophoresis (PAGE), and separated proteins were transferred electrothermally to a polyvinylidene difluoride (PVDF) membrane (ATTO, Tokyo, Japan). The membrane was blocked with 3% nonfat dry milk for 1 h at room temperature, and then incubated with primary antibody (iNOS: Cayman Chemical, 1:1000; neuronal NOS: nNOS: Zymed Laboratories, 1:500, Actin, Sigma, 1:1000) overnight at 4 °C. After
washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Chemicon, 1:10000). Immunoreactive proteins were visualized with chemiluminescent HRP substrate (Immobilon, Millipore, Billerica, MA., U.S.A.), and each band density was quantified with an image analyzer (Quantity One, Bio-Rad).

**Statistical Analyses** Data are given as the mean±S.E. Statistical analysis of differences between Wild and Tg groups was performed using Student’s t-test, and other differences were analyzed with one-way analysis of variance (ANOVA) followed by post-hoc Dunnett’s test. Body weight changes and rota-rod test results were analyzed by repeated-measures two-way ANOVA followed by pairwise multigroup comparisons by Holm–Sidak method. Survival and disease onset data were compared using Kaplan–Meier survival analysis with the log-rank test. A p-value <0.05 was considered significant.

**RESULTS**

**Body Weight and Motor Function** The administration of Wen-Pi-Tang extract (100 or 200 mg/kg B.W./d; W100 and W200, respectively) to ALS model mice was started at the age of 7 weeks, and the body weight and motor function were monitored by the Rota-rod test, wire hang test, and grip strength test until the age of 15 weeks. Figure 1 shows the body weights of mice. During the total experimental period, wild-type mice gained body weight steadily, while vehicle-treated Tg mice showed body weight loss starting at the age of 13 weeks, because ALS development and progression were reflected in body weight loss by muscle atrophy.29) Wen-Pi-Tang-treated Tg mice, however, didn’t show any marked differences in body weight compared to vehicle-treated Tg mice. Regarding Rota-rod test results (Fig. 2), wild-type mice maintained a normal motor function over the experimental period. In contrast, vehicle-treated Tg mice showed the rapid deterioration of motor function after 10 weeks of age. However, W100- and W200-treated Tg mice maintained their motor function until 12 and 13 weeks of age, respectively, and the latency periods of Wen-Pi-Tang-treated Tg mice at 11—13 weeks of age were significantly longer than those of vehicle-treated Tg mice at the same age. In the wire hang test, Wen-Pi-Tang-treated mice tended to maintain the same latency time at the age of 15 weeks, whereas wild-type mice showed a significant deterioration (Fig. 3A). Wen-Pi-Tang-treated mice also showed the same effects in the grip strength test (Fig. 3B). In the Rota-rod test
and wire hang tests, the administration of riluzole, a glutamate release inhibitor, led to significant changes in the decline of the latency time at the age of 15 weeks.

**Disease Onset and Survival Time** The disease onsets were determined based on the day when the mouse reached peak body weight. The results are shown using a Kaplan–Meier curve (Fig. 4). There was no change between vehicle-treated and W100-treated Tg mice, but the onset in W200-treated Tg mice was significantly delayed compared to the vehicle-treated Tg group. The average disease onsets of vehicle-, W100-, W200-, and riluzole-treated groups were 95.8±1.9, 97.0±1.8, 103.6±2.2, and 98.3±2.1 d, respectively. On the other hand, the average survival times were 130.2±3.2 for vehicle, 128.8±2.0 for W100, and 130.3±1.4 for W200-treated groups, and there were no differences.

**Number of Motor Neurons in Spinal Cord** Next, we examined the histological changes in the spinal cord of ALS model mice treated with Wen-Pi-Tang or vehicle for 9 weeks from 7 weeks of age (in the early disease phase). To determine the loss of motor neurons, we counted the neurons exhibiting a diameter of at least 20 μm in laminae VIII and IX of lumbar spinal cords (Fig. 5). Neuron numbers in the vehicle-treated Tg group were markedly decreased, and the percentage of remaining neurons was 31% compared with the Wild group. In contrast, Wen-Pi-Tang treatment inhibited neuronal loss from spinal cords in a dose-dependent manner (38% for W100 and 48% for W200 compared with the Wild group), and W200 treatment significantly inhibited neuronal loss in ALS model mice.

**Astrocytic and Microglial Activation in Spinal Cord** In ALS model mice, it has been reported that activated astrocytes and microglial cells that increase before the onset and/or early disease phase, are related to motor neuronal death. Therefore, we examined the effects of Wen-Pi-Tang on glial cell activation in lumbar spinal cords by imaging.
expression tended to be increased in vehicle-treated Tg mice. In contrast, a significant increase in astrocytes was observed in vehicle-treated Tg mice. Wen-Pi-Tang treatment inhibited the astrocyte increase dose-dependently. Significant differences in GFAP immunoreactivity between the vehicle-treated and W200-treated Tg groups in both the ventral and dorsal horns of the spinal cord were observed (Fig. 6B). Meanwhile, CD11b-positive microglial cells in the spinal cord were also significantly increased in vehicle-treated Tg mice along with the change of GFAP-positive astrocytes (Fig. 7A). Wen-Pi-Tang treatment, especially in W200, inhibited the microglial increase (Fig. 7B). In SOD1G93A mice, the immunoreactivities of both glial cells markedly increased, especially in the ventral horn where motor neurons localize. The inhibitory effects of Wen-Pi-Tang on gliosis were similarly seen both in the ventral and dorsal horns.

**HO-1 and NOS Proteins** We examined the expression of HO-1 and NOS in the spinal cord as a marker of oxidative stress and a marker of NO production, respectively. HO-1 expression in vehicle-treated Tg mice was significantly increased in both the ventral and dorsal horns when compared with the wild-type mice (Figs. 8A, B). The HO-1 level in the W200-treated Tg group was significantly decreased in both ventral and dorsal horns when compared with the vehicle-treated Tg mice. In Western blotting using the spinal cord, nNOS expression was significantly increased in both the ventral and dorsal horns.

**DISCUSSION**

ALS is an adult-onset neurodegenerative disease characterized by a selective loss of motor neurons, and there is almost no effective treatment or drug at present. Numerous compounds were investigated based on many hypotheses, but effective compounds for clinical use have not been developed. In this study, we investigated the effect of Wen-Pi-Tang, a Chinese prescription consisting of 5 crude drugs, on ALS model mice carrying a mutated human SOD1 gene.

Wen-Pi-Tang administration to ALS model SOD1G93A mice had almost no effect on the disease progression and survival time, but it significantly delayed the disease onset, determined as the time when the mice reached the peak body weight in the W200 group. Also in the Rota-rod test results,
Wen-Pi-Tang extract treatment significantly prolonged the start of rapid deterioration for 2—3 weeks compared with the vehicle-treated Tg group. In addition, Wen-Pi-Tang-treated mice tended to maintain their motor functions evaluated by the wire hang and grip strength tests at the age of 15 weeks. These results indicate that Wen-Pi-Tang treatment delayed the disease onset as the body weight peaked. On the other hand, riluzole treatment had no effect on the disease onset determined by the body weight peak, but it inhibited the deterioration of latency in the Rota-rod and wire hang tests at the age of 15 weeks. It has been reported that riluzole prolongs survival and does not delay disease onset in ALS model mice. Therefore, this result was thought to indicate that riluzole inhibited disease progression and that Wen-Pi-Tang had distinctly different effects on ALS model mice compared to riluzole.

Furthermore, we investigated the histological changes in the lumbar spinal cord of SOD1<sup>G93A</sup> mice treated with Wen-Pi-Tang or vehicle for 9 weeks from 7 weeks of age, since Wen-Pi-Tang delayed disease onset. As a result, Wen-Pi-Tang treatment improved motor neuronal loss and inhibited astrocytic and microglial activations in the lumbar spinal cord of SOD1<sup>G93A</sup> mice. In recent research on ALS, it has been reported that non-neuronal cells, especially astrocytes and microglia, play an important role in neurodegeneration in the mutant SOD1 ALS mouse model. It is known that reactive microglia and macrophages accumulate in the spinal cord of ALS patients. In addition, it has been reported that expressions of chemokines and cytokines related to these cells are also increased in ALS patients. Microglia activated before motor neuron degeneration produce an abundance of proinflammatory cytokines, induce COX-2 and iNOS expression, release a large amount of reactive oxygen species (ROS), and thus contribute to motor neuron degeneration. Microglia are thought to play an important role in neurodegenerative diseases, such as Alzheimer’s and Parkinson’s disease, and are also thought to be involved in ALS. On the other hand, Nagai et al. reported that rodent astrocytes expressing mutated SOD1 kill primary motor neurons by releasing a substance, and a non-cell autonomous effect of astrocytes on neuronal cells has also attracted attention in ALS research. The activation and accumulation of astrocytes and microglia in the spinal cord before ALS onset are involved in motor neuron degeneration, and, therefore, it is thought that the inhibition of astrocytic and microglial activation contributes to protection from spinal motor neuron loss.

Yamanaka et al. demonstrated that reducing mutant SOD1 toxicity within microglia or astrocytes significantly slowed disease progression of ALS, and indicated the important role of glial cells in ALS disease. In this study, Wen-Pi-Tang extract inhibited motor neuronal loss from the spinal cord accompanying the attenuation of the proliferation of glial cells. Wen-Pi-Tang extract has the potential to prevent damage, especially from oxidative stress, as described above, so we determined the oxidative stress condition based on the HO-1 level. Heme oxygenase is an enzyme catalyzing the degradation of heme to biliverdin (biliverdin is converted to bilirubin), and HO-1 is the inducible isof orm of heme oxygenase. Although the function of HO-1 has not been fully elucidated, it is known that this enzyme is induced by many kinds of factors, especially by the oxidative stress condition. Therefore, HO-1 is used as a marker of oxidative stress, and we determined the expression of this enzyme in the SOD1<sup>G93A</sup> mice spinal cord. HO-1 expression in vehicle-treated Tg group spinal cords was significantly increased when compared with the Wild group, and this finding suggested that oxidative stress was increased in ALS pathogenesis. In contrast, significantly decreased HO-1 expression on Wen-Pi-Tang treatment when compared with the vehicle-treated Tg group indicates that oxidative stress in the spinal cord was ameliorated by Wen-Pi-Tang administration. In addition, HO-1 expression was localized in mainly microglial cells, and so microglial cells subjected to high oxidative stress levels must induce the further activation, proliferation, and release of pro-inflammatory factors. Proliferated and activated microglial cells can be a source of ROS that injure motor neurons; thus, we determined NOS expression as one of the markers of ROS production. As a result, iNOS expression tended to increase in the SOD1<sup>G93A</sup> mice spinal cord, and it was derived from microglial cells. Although Wen-Pi-Tang treatment lowered the increase of iNOS expression (W200 group), it changed in parallel to microglial proliferation. Based on this result, it is thought that Wen-Pi-Tang may not inhibit iNOS expression, but inhibit microglial activation and proliferation. Additionally, the significant decrease in nNOS expression may be due to the loss of motor neurons that nNOS is derived from in the SOD1<sup>G93A</sup> mice spinal cord, because Wen-Pi-Tang treatment improved motor neuronal loss in the spinal cord of these mice.

At present, the mechanism of glial cell activation and the neurotoxic factor of activated glial cells acting on motor neurons in ALS are still unclear. In this study, we demonstrated that Wen-Pi-Tang extract administration improved motor neuronal loss from the SOD1<sup>G93A</sup> mice spinal cord by ameliorating astrocytic and microglial activation occurring from before disease onset. However, we also could not clarify the detailed mechanism behind the effect of Wen-Pi-Tang on glial cell activation. The Chinese prescription Wen-Pi-Tang and its constituents, 5 crude drugs, exhibit a variety of biological effects. Rhei Rhizoma, the main constituent of Wen-Pi-Tang, has many biological effects such as diarrheal, antidiarrheal, antibacterial, antimicrobial, anti-inflammatory, psychotropic, and other effects. In ALS research, it has been reported that minocycline, a second-generation antibiotic with anti-inflammatory effects, inhibited microglial activation in ALS model mice and cyclooxygenase-2 inhibitor ameliorated disease onset in ALS model mice. Wen-Pi-Tang not only has an antioxidative effect but also anti-inflammatory effects derived from Rhei Rhizoma and Glycyrrhizae Radix. Therefore, these crude drugs may play an important role in the attenuation of gliosis in ALS model mice.

In conclusion, we demonstrated that Wen-Pi-Tang delayed disease onset in ALS mice by ameliorating astrocytic and microglial activation and proliferation. Although this effect is important for not only FALS but also SALS, further investigation is needed to clarify the effects and mechanisms of each crude drug or compound that comprises Wen-Pi-Tang. Moreover, elucidating the role of each crude drug in the effect on the ALS pathology may lead to the discovery of new drugs from natural products for ALS treatment.
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REFERENCES