Neuroinflammation, Oxidative Stress, and Neurogenesis in a Mouse Model of Chronic Fatigue Syndrome, and the Treatment with Kampo Medicine

Qiang He, Mio Sawada, Naruhiro Yamasaki, Sumiyo Akazawa, Hisakazu Furuta, Hiroaki Uenishi, Xiangjin Meng, Takeshi Nakahashi, Yasuhiro Ishigaki, and Junji Moriya

Department of General Internal Medicine, Kanazawa Medical University; Uchinada, Kahoku, Ishikawa 920–0293, Japan; and Division of Molecular and Cell Biology, Kanazawa Medical University; Uchinada, Kahoku, Ishikawa 920–0293, Japan.

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The diagnosis of chronic fatigue syndrome (CFS) is mainly symptom-based, and the etiology is still unclear. Here, we evaluated the pathological changes in the brain of a mouse model of CFS and studied the effects of Kampo medicine. A mouse model of CFS was established through six repeated injections of Brucella abortus (BA) every two weeks for a period of 12 weeks. Neuroinflammation was measured by estimating interleukin (IL)-1β, IL-6, and interferon-gamma (IFN-γ), and oxidative stress by nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE) 6 weeks after the last injection. Hippocampal neurogenesis was evaluated through K-67, doublecortin (DCX), and 5-bromodeoxyuridine (BrdU) assays. The effects of Kampo medicines (Hochuekkito (TJ-41) and Hachimijiogan (TJ-7)) on neuroinflammation during CFS were studied. The wheel-running activity of mice was decreased by about 50% compared to baseline at 6 weeks after the last BA injection. The levels of IL-1β, IL-6, 3-NT, and 4-HNE were increased in both the cortex and the hippocampus of CFS mice at 6 weeks after the last BA injection. Hippocampal neurogenesis was unchanged in CFS mice. Treatment with TJ-41 and TJ-7 reduced the expressions of IL-1β, IL-6, and IFN-γ in the hippocampus but not in the cortex. The results of the present study indicate that neuroinflammation and oxidative stress play important roles in the pathogenesis of CFS. The data further suggest that treatment with TJ-41 and TJ-7 could help reduce the inflammation associated with CFS in the hippocampus, but failed to improve the symptoms in CFS mice.

Key words chronic fatigue syndrome; mouse model; neuroinflammation; oxidative stress; neurogenesis; Brucella abortus

INTRODUCTION

Chronic fatigue syndrome (CFS) is a complicated disorder characterized by extreme fatigue that cannot be explained by any underlying medical condition. Most of the symptoms, such as loss of memory or concentration, unexplained muscle or joint pain, headaches, unrefreshing sleep, and extreme exhaustion, are neural and psychiatric, suggesting an involvement of disorders in neuronal–endocrine–immune interactions. Although the term CFS first appeared over 30 years ago, the diagnosis of this illness is still based on symptoms, and the etiology is still unclear. An agreement on the etiology and pathophysiology of CFS has not been reached yet. A multifactorial etiology is accepted by most researchers. Disturbance in immunity and the involvement of oxidative and nitrosative stress (O&NS) are reported by various researchers to be associated with the onset of CFS. When the peripheral immune system is activated it produces a series of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. This pro-inflammatory signal triggers the production of the same cytokines by glial cells in the brain. After being stimulated by the presence of cytokines, microglia also produces and releases other pro-inflammatory molecules and nitric oxide (NO). Elevated NO leads to increased superoxide and peroxynitrite. This in turn leads to oxidative stress and further induce the production of proinflammatory cytokines. All these factors may finally contribute to neurodegeneration and reduced neurogenesis, which are common pathological changes in many diseases.

Kampo medicines Hochuekkito (TJ-41) and Hachimijiogan (TJ-7) are believed to relieve the symptoms in individuals with poor physical strength who tend to get tired easily, and improving wheel activity, decreasing oxidative stress, and improving cognitive function.

During the past 10 years, we have developed a CFS animal model using six injections of heat-killed Brucella abortus (BA) antigen, which could extend the duration of fatigue that mimics the real chronic course of human CFS. Running wheel activity is suppressed for at least 6 weeks after the last injection, which helps us to test treatments. The disadvantage is that a large number of mice (about 1/3) die during the six injections.

The aim of this research is to: 1) improve the animal model to reduce the death rate; 2) confirm neuroinflammation, oxidative stress, and neurogenesis in the brain of CFS animals; and 3) study the effects of Kampo medicine.
MATERIALS AND METHODS

Animals and Living Conditions Female BALB/c mice (20–24 g, 8 weeks old) were procured from CLEA Japan (Tokyo, Japan). The animals were housed 5 per cage on 12 h light/dark cycle with food and water available ad libitum. Temperature was maintained 22–23°C. Running wheel cages included a running wheel (23 cm in diameter), counters, cribs, and water taps (a detailed description of this cage is provided in our prior research).31) The mice were free to stay in the rest area or enter the running wheel. Our research protocol was approved by the Animal Experimental Committee of Kanazawa Medical University.

Induction of the CFS Model Heat-killed BA ring test antigen was obtained from the National Veterinary Services Laboratories in the United States. In order to obtain an appropriate concentration of BA, the stock solution was centrifuged and resuspended in saline. The CFS animal model was induced by six repeated injections of BA antigen solution (0.2 mL per mouse) intraperitoneally every 2 weeks. The first was induced by six repeated injections of BA antigen solution Laboratories in the United States. In order to obtain an ap layer or enter the running wheel. Our research protocol was approved by the Animal Experimental Committee of Kanaza-

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5-Bromodeoxyuridine (BrDU) was given to mice orally; 0.8 mg/mL solution was made, and 4% sucrose was added in order to neutralize the unpleasant taste of BrDU. Drinking water was changed to BrDU solution at the 15th week for 7 d. Mice were killed 6 weeks after the last injection (at the end of the 16th week).

Kampo Medicine Intervention TJ-41 and TJ-7 were obtained from Tsumura & Co. (Tokyo, Japan). TJ-41 contained a mixture of spray-dried hot water extracts of 10 medicinal plants: Astragali radix (16.7%), Atractyloides lancea rhizome (16.7%), Ginseng radix (16.7%), Angelicae radix (12.5%), Bupleuri radix (8.3%), Zizyphi spina (8.3%), Aconiti tuber pericarpium (8.3%), Glycyrrhizae radix (6.3%), Cimicifugae rhizoma (4.2%) and Zingiberis rhizoma (2.0%). TJ-3 is an extract from a mixture of Rehmanniae radix, Corni fructus, Dioscoreae rhizome, Alismatis rhizome, Hoelen, Mentha corticalis, Cinnamomi cortex, and Aconiti tuss. The daily dosage of each drug was 50 kg). Intervention with TJ-41 and TJ-7 started from the 12th week and lasted for 4 weeks until the end of the research.

Brain Fixation and Histology Mice were anesthetized with an overdose of chloral hydrate (Nacalai, Japan). A cannula was inserted into the left ventricle. Mice were perfused with saline (about 20 mL) and about 50 mL of 4% paraformaldehyde/phosphate-buffered saline (PBS) solution. Both the saline and the paraformaldehyde solution were ice-cold. Then the brain was taken out, post-fixed in 4% paraformal-
1:3000), Anti-IL-6 (Abcam, ab7737, 1:1000), Anti-Interferon gamma (IFN-γ, Abcam, ab133566, 1:3000), Anti-Nitrotyrosine (3-NT, Santa Cruz, sc-32757, 1:50), and Anti-4-HNE (R&D, MAB3249, 1:100) at 4°C overnight. This was followed by secondary antibody incubation (m-IgGκ BP-HRP, Santa Cruz Biotechnology, sc-516102, 1:4000; Goat anti-Rabbit IgG HRP, Abcam, ab6721, 1:3000) at room temperature for 1 h. Full film scans of the Western blot data were obtained with Fusion FX7 (Vilber Lourmat, France). The expressions of proteins were quantified by measuring band intensities using ImageJ software (NIH, Bethesda, MD, U.S.A.). The band intensity values of the protein of interest were adjusted to that of actin and standardized to the control group (but not for 3-NT and 4-HNE, since no bands were detected in the control group).

Statistical Analysis All data were expressed as the mean ± standard error of the mean (S.E.M.) and were analyzed by Student’s independent t-test. All statistical analyses were performed with SPSS 22 software. Significance was reached at values of p < 0.05.

RESULTS

Running-Wheel Activities During the injections, only two mice in the research group died. Both of them died after the 6th injection. At the baseline, the running wheel activities did not differ between the CFS and control groups (18217.9 ± 3184.9 vs. 17963.6 ± 2238.0). After the first two injections, the running wheel activity of CFS dropped markedly until the end of the 16th week, remaining at only 51.8% of the baseline. There were no significant differences between the Kampo group and the CFS vacant group with regard to running wheel activities (Fig. 1).

Immunohistochemistry Staining There were no significant differences among the CFS, Kampo, and control groups in KI-67, DCX, and NeuN/BrdU staining (Figs. 2a, b, c, e). 4-HNE positive cells could be seen all over the brain in CFS vacant and Kampo group, but not for the Control group (Fig. 2d).

Results of Western Blot After repeated injections of BA, the expressions of IL-1β, IL-6, 3-NT, and 4-HNE were significantly elevated in both the cortex and the hippocampus. Expression for 3-NT and 4-HNE was absent both in the cortex and the hippocampus in the control group. Treatment
with TJ-41 and TJ-7 resulted in decreased expression of IL-1β, IL-6, and IFN-γ in the hippocampus but not in the cortex compared to the CFS vacant group, while no reductions of 3-NT and 4-HNE could be confirmed either in the cortex or in the hippocampus. Among the decreased expressions of IL-1β, IL-6, and IFN-γ in hippocampus, only IL-6 was decreased remarkably (Fig. 3).

**DISCUSSION**

In the current study, we decreased the dosage of BA to 25% in the first injection which resulted in significant reduction of mice death rate. Only 2 animals died (10%) out of 20 mice in the CFS group. This could be due to the decreased immune response or hypersensitivity reaction from the reduced dosage. Decreased dosage of BA would have helped the animals to adapt with the altered immune system and to withstand the additional dosage.

A large number of CFS patients endure recurrent, persistent, or subacute bacterial and viral infections. To mimic the infections, we performed six repeated intraperitoneal injections in mice. Then we noticed increased expressions of cytokines (IL-1β, IL-6) and oxidative stress markers (3-NT, 4-HNE) in both the cortex and the hippocampus of CFS
mice. This could be due to the intrusive bacteria that caused activation of Toll-like receptors (TLRs). The activated TLR-4 complex leads to the upregulated transcription of nuclear factor-kappaB (NF-κB) and the production of pro-inflammatory cytokines. Second, the cytokine message transmission from the peripheral to the central nervous system (CNS). The mechanisms governing the entry and exit of immune cells/cytokines in and out of the CNS used to be poorly understood. However Louveau et al. recently found functional lymphatic vessels in the CNS that are connected to the deep cervical lymph nodes. This finding suggests that peripheral inflammatory signals can overcome the blood-brain barrier and communicate with the CNS.

The evidence supporting the existence of neuroinflammatory processes in individuals with CFS also includes the findings of Nakatomi and Barnden. Activated microglia and dysfunctional astrocytes were identified by positron emission tomography (PET) and magnetic resonance imaging (MRI). The activated microglia releases a wide range of neurotoxins, including pro-inflammatory cytokines and NO. Although at physiological concentrations NO is a major neuromodulator, elevated levels of NO promotes neuroapoptosis. Increased NO levels and superoxide elevate the generation of peroxynitrite anions, which are very toxic. Peroxynitrite can trigger many kinds of pathologic abnormalities, including depletion of glutathione, lipid peroxidation in membranes, nitration of protein tyrosine residues, and damage to DNA.

The neuroinflammation and O&NS interact and reinforce each other, and could contribute to the onset and retain of CFS. Although it is generally believed that increased cytokines and NO negatively regulate hippocampal neurogenesis, no decreased neurogenesis was identified by K-67, DCX, and BrdU staining) in the hippocampus of CFS mice in our study, which suggests that decreased neurogenesis might not be a pathologic pathway of CFS. This result supports the consensus that loss of newborn neurons is common in depression but not important to CFS. Decreased neurogenesis is mostly pathologic pathway of CFS. This result supports the consensus that loss of newborn neurons is common in depression but not important to CFS. Decreased neurogenesis was identified (by BrdU staining) in the hippocampus of CFS mice in our study, which suggests that decreased neurogenesis might not be a pathologic pathway of CFS. This result supports the consensus that loss of newborn neurons is common in depression but not important to CFS. Decreased neurogenesis was identified (by BrdU staining) in the hippocampus of CFS mice in our study, which suggests that decreased neurogenesis might not be a pathologic pathway of CFS. This result supports the consensus that loss of newborn neurons is common in depression but not important to CFS.

The results of the present investigation support the theory that TJ-41 and TJ-7 could help to decrease the levels of inflammatory cytokines. However, TJ-41 and TJ-7 could not improve the symptoms of CFS that might be related to their inability to decrease oxidative stress and to reduce the increased levels of cortical cytokines. In the current study, we could not examine the expression of additional cytokines and oxidative stress markers. Future studies could focus to elucidate the mechanisms that debilitate neural functions and screen more potent drugs that could decrease neuroinflammation and oxidative stress in brain.

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**Conflict of Interest** The authors declare no conflict of interest.

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