The Japanese Herbal (Kampo) Medicine Hochuekkito Attenuates Lung Inflammation in Lung Emphysema

Hideaki Isago, a,b Akihisa Mitani, a,b Shiho Kohno, b Saki Nagoshi, b Taro Ishimori, b Minako Saito, b Hiroyuki Tamiya, b Naoya Miyashita, b Takashi Ishi, b Hirota Matsuzaki, b Yutaka Yatomi, a Takahide Nagase, b and Taisuke Jo c

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a systemic inflammatory disorder. It often causes weight loss, which is considered a poor prognostic factor. A Japanese herbal Kampo medicine, Hochuekkito (TJ-41), has been reported to prevent systemic inflammation and weight loss in COPD patients, but the underlying biological mechanisms remain unknown. In the present study, we investigated the role of TJ-41 in vivo using a mouse model of lung emphysema. We used lung epithelium-specific Taz conditional knockout mice (Taz CKO mice) as the lung emphysema model mimicking the chronic pulmonary inflammation in COPD. Acute inflammation was induced by intratracheal lipopolysaccharide administration, simulating COPD exacerbation. Mice were fed a diet containing 2% TJ-41 or a control diet. Taz CKO mice showed increased numbers of inflammatory cells in the bronchoalveolar lavage fluid compared to control mice. This effect was reduced by TJ-41 treatment. In the acute exacerbation model, TJ-41 mitigated the increased numbers of inflammatory cells in the bronchoalveolar lavage fluid and attenuated lung inflammation in histopathological studies. Additional in vitro experiments using the human macrophage cell line U-937 demonstrated that lipopolysaccharide-induced tumor necrosis factor-alpha expression was significantly downregulated by TJ-41. These results suggest that TJ-41 has anti-inflammatory effects in lung emphysema both in the chronic phase and during an acute exacerbation. In conclusion, our study sheds light on the anti-inflammatory effects of TJ-41 in lung emphysema. This establishes its potential as a new anti-inflammatory therapy and a preventive medicine for exacerbations during the long-time maintenance of COPD patients.

Key words Hochuekkito; chronic obstructive pulmonary disease; mouse model; lung emphysema; kampo medicine

MATERIALS AND METHODS

TJ-41 TJ-41 bulk powder for prescription use was supplied by Tsumura & Co. (Tokyo, Japan). TJ-41 is composed of spray-dried hot water extracts of 10 natural herbs, including Astragali radix (16.7%), Atractyloclis lancea rhizoma (16.7%), Ginseng radix (16.7%), Angelicae radix (12.5%), Bupleuri radix (8.3%), Zizyphi fructus (8.3%), Aurantii nobilis pericarpium (8.3%), Glycyrrhizae radix (6.3%), Cimicifugae rhizoma (4.2%), and Zingiberis rhizoma (2.0%). Plant materials were authenticated by identification of external morphol-
ogy and marker compounds for plant specimens according to the methods of the Japanese Pharmacopeia and company standards. Extract quality was standardized based on the good manufacturing practice as defined by the Ministry of Health, Labour, and Welfare of Japan. Detailed chemical profiling of TJ-41 determined by 3D-HPLC can be obtained from previous studies. For animal experiments, 2% TJ-41 were added into rodent diet MF (Oriental Yeast Co., Ltd., Japan). The concentration of the feed used in this study was based on previous study. The assumed daily dosage of TJ-41 was about 3000 mg/kg body weight (with mouse body weight assumed 20 g and daily consumption of diet assumed 3 g). For in vitro experiments, TJ-41 bulk powder was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL. After over night incubation at 37°C, this solution was centrifuged and filtered using 0.22-µm filters. DMSO was used as the control.

As a mouse model of emphysema, we used Taz-flox/flox; SPC-pcre+ (Taz CKO) mice, which were generated as described previously. Throughout the study, Taz-flox/fox mice were used as control mice. Mice were bred in specific pathogen-free conditions.

In the chronic pulmonary emphysema model, 7-week-old male Taz CKO and control mice were fed for 12 weeks either a diet containing 2% TJ-41 or a control diet. At 19 weeks of age, mice were sacrificed and subjected to bronchoalveolar lavage fluid (BALF) analysis and whole-lung total RNA extraction.

In the acute exacerbation model, 8-week-old Taz CKO and control mice were fed a diet containing 2% TJ-41 or a control diet. At 10 weeks of age, all mice were treated with intratracheally administered LPS (L3024; Sigma-Aldrich, U.S.A.; 5 µg/g body weight) in 100 µL phosphate-buffered saline (PBS) under isoflurane-anesthesia. One week later after LPS administration, at 11 weeks of age, mice were sacrificed and were subjected to BALF analyses, whole-lung total RNA extraction, as well as histological analysis.

Histological Analysis The murine lungs were fixed by intratracheal injection of 10% buffered formalin at a constant pressure of 25 cm H2O for at least 24 h. Specimens were embedded in paraffin, cut into sections, and stained with hematoxylin–eosin.

BALF Analysis To collect BALF, mice were sacrificed, and, following a tracheostomy, a blunted 18-gauge needle was placed in the trachea. BAL was performed by injecting 1 mL of PBS into the needle and retrieving it three times in each mouse. Retrieved BALF was collected and centrifuged at 450 × g for 10 min. The supernatants were collected and subjected to enzyme-linked immunosorbent assays (ELISAs) to quantify inflammatory cytokines (see Supplementary Method). The cell fraction was resuspended in 1 mL PBS, and cell numbers were counted by a LUNA cell counter (Logos Biosystems, Korea). The leukocyte fraction was determined using Diff-Quik staining (Sysmex, Japan).

Quantitative Real-Time PCR (RT-PCR) To extract total RNA, cultured cells were centrifuged in 1.5 mL tubes and lysed in TRIzol (Invitrogen, U.S.A.). The collected mouse lung tissue was homogenized in TRIzol (Invitrogen) using an MS-100 bead homogenizer (Tomy, Japan). First-strand cDNA was synthesized using ReverTraAce (Toyobo, Japan). Quantitative real-time RT-PCR was performed using the Thermal Cycler Dice® Real Time System III and TB Green Fast qPCR Mix (TaKaRa Bio, Japan), according to the manufacturer’s instructions. Sequences of the primers are presented in Supplementary Tables 1 and 2. Quantification was performed in duplicate and normalized to the Gapdh mRNA level by the delta-delta CT method.

Cell Culture and LPS Stimulation U-937 cells, human macrophage cell line, were provided by the Japanese Collection of Research Bioresources Cell Bank, and cells were used up to the 20th passage. U-937 cells were grown in RPMI-1640 with l-glutamine medium (WAKO, Japan) containing 10% fetal bovine serum (FBS). Prior to LPS stimulation, 5.0 × 105/mL cells were seeded into 12-well dishes in FBS-free RPMI-1640 with l-glutamine medium for starvation, and TJ-41 solution or DMSO (volume was adjusted to 1 µL/well) was administered. After incubation for 24 h, LPS or PBS was added at a final concentration of 1 µg/mL. After another 3 h treatment, cells were retrieved, and total RNA was harvested using TRIzol (Invitrogen).

Study Approval All experiments were approved by the Ethics Committee for Animal Experiments (University of Tokyo, Tokyo, Japan). We did not use human specimens in this study.

Statistics Data are expressed as means ± standard errors of the mean (S.E.M.) unless otherwise stated. p-Values less than 0.05 denoted statistical significance. All statistical analyses were conducted using the JMP Pro 15.1 software.

RESULTS

TJ-41 Attenuates Chronic Lung Inflammation in Lung Emphysema To examine the TJ-41 effects on chronic lung inflammation in emphysema, we administered TJ-41 to Taz CKO mice or control mice for 12 weeks. Mice were treated by oral administration of a diet containing 2% TJ-41 or a control diet (Fig. 1A). No significant changes in body weights were observed among groups (Fig. 1B).

We also analyzed BALFs to evaluate inflammatory cell infiltration 12 weeks after TJ-41 administration. As we have shown previously, Taz CKO mice showed increased numbers of inflammatory cells in the BALF compared to control mice. In Taz CKO mice groups, TJ-41 treatment significantly reduced the presence of inflammatory cells, compared with the control diet (Fig. 1C). In both groups, more than 90% of these inflammatory cells were macrophages (data not shown).

Next, we extracted whole-lung total RNA and analyzed mRNA expressions of major inflammatory cytokines, including Cxcl2, which is reportedly attenuated by TJ-41 administration in LPS-induced lung inflammation. However, the expression levels of all examined cytokines (interleukin (IL)-6, tumor necrosis factor (TNF)-alpha, Cxcl1, Cxcl2) did not show any systematic changes (Fig. 1D). In summary, TJ-41 showed anti-inflammatory effects on lung inflammation in the chronic emphysema mouse model, likely independent from IL-6, TNF-alpha, Cxcl1, and Cxcl2 activity.

TJ-41 Protects Mice from LPS-Induced Inflammation in Lung Emphysema In practice, COPD exacerbations are mainly caused by bacterial or viral infections. Thus, in this animal model, LPS administration is considered a useful model to simulate an exacerbation. To mimic the effects of TJ-41 in patients with COPD exacerbation, we administered LPS to Taz CKO mice and control mice with or without TJ-41.
In the control diet groups, Taz CKO mice had significantly lost weight one week after LPS administration compared to control mice. In the TJ-41-treated groups, no difference was observed between Taz CKO mice and control mice (Fig. 2B). The BALF cell count showed that TJ-41 administration mitigated in Taz CKO mice the increase in the number of inflammatory cells (Fig. 2C), which were mainly composed of neutrophils and macrophages (Supplementary Fig. 1). We analyzed the mRNA expression of major inflammatory cytokines in the whole lung, but no significant differences between groups were observed (Fig. 2D). We also compared levels of these cytokines in BALF, but no significant differences were observed (Supplementary Fig. 2).

The histopathology revealed that after LPS administration, alveolar thickening and infiltration, which are features of pulmonary inflammation, were pronounced in the lungs of Taz CKO mice. This was not observed in the lungs of control mice. Interestingly, the LPS-induced inflammation was attenuated in the TJ-41-fed group (Fig. 3). According to these pathological examinations, the TJ-41 administration attenuated the acute pulmonary inflammation in emphysema model mice.

**TJ-41 Administration Attenuates LPS-Induced TNF-alpha Expression in Human Macrophages in Vitro** The results of these in vivo studies suggested that TJ-41 may suppress lung inflammation. To confirm whether TJ-41 in-
hibits the production of inflammatory cytokine in inflammatory cells, we performed RT-PCR analyses using the human macrophage cell line U-937. TJ-41 dissolved in DMSO was administered, followed by LPS treatment. We determined the final concentration of TJ-41 as 5µg/mL, because at higher concentrations TJ-41 induced cell death in preliminary studies. The RT-PCR results demonstrated that TNF-alpha expression was significantly downregulated by TJ-41 administration, whereas IL-8 expression was not changed (Fig. 4). We further assessed whether this effect was observed in the water-soluble components of TJ-41, however, water-soluble components of TJ-41 did not attenuate the expression of IL-8 and TNF-alpha (Supplementary Fig. 3).

DISCUSSION

This is the first study that demonstrates that TJ-41 attenuates lung inflammation, both in the chronic phase and the acute exacerbation phase of a murine emphysema model. In this study, we used Taz CKO mice as a mouse model of lung emphysema. Taz CKO mice spontaneously develop lung emphysema and exhibit elevated levels of inflammatory

Fig. 2. TJ-41 Attenuates LPS-Induced Lung Inflammation in a Mouse Model of Lung Emphysema

(A) Experimental schedule of the acute exacerbation model. 8-week-old Taz CKO mice and control mice were fed a diet containing 2% TJ-41 or a control diet. At 10 weeks of age, all mice were treated with intratracheally administered LPS. At 11 weeks of age, mice were sacrificed and subjected to analyses. (B) Body weight loss between the time of LPS administration and sacrifice. One-way ANOVA test followed by Bonferroni’s multiple comparison test, n = 5–8 in each group. (C) BALF analysis showing that the TJ-41 administration mitigates the LPS-induced increase in pulmonary inflammatory cells of Taz CKO mice. One-way ANOVA test followed by Bonferroni’s multiple comparison test, n = 5–8 in each group. (D) Whole-lung mRNA expression levels of major inflammatory cytokines in the acute exacerbation model. None of the differences reached statistical significance in the one-way ANOVA test, n = 5–8 in each group. Data are presented as the mean ± S.E.M.
cells in the lungs of adult animals. The porcine pancreatic elastase model is also widely used as a murine COPD model, but it requires the intratracheal administration of elastase and, thereby, causes excessive inflammation and hemorrhage after elastase administration. Thus, we chose Taz CKO mice as a suitable model to analyze the chronic inflammation in lung emphysema and the relatively mild anti-inflammatory effect of herbal medicine.

In the chosen chronic inflammation model, the administration of TJ-41 did not alter the body weights in both control and Taz CKO mice. In patients with COPD, it has been reported that TJ-41 treatment combined with rehabilitation increases the body weight. Our result was in line with the previous study by Tajima et al. They demonstrated in an animal model that TJ-41 treatment causes no significant improvement in body weight loss. Further studies with different feeding protocols may be required to mimic the effect of TJ-41 on body weight as shown in human patients with COPD.

In both the acute and the chronic model, TJ-41 treatment clearly mitigated the number of inflammatory cells in the BALF, confirming that TJ-41 has anti-inflammatory effects in pulmonary inflammation. We also observed that TJ-41 administration reduced LPS-induced lung inflammation in Taz CKO mice by pathological analyses, evoking the possibility that TJ-41 might attenuate the exacerbation of emphysema. These results are in line with several studies reporting the preventive effects of TJ-41 in murine lung disease models, including influenza virus infection, LPS-induced lung injury, and bleomycin-induced pulmonary fibrosis models. Suppression of pulmonary inflammation is observed in these studies, as seen in our present study in a mouse model of lung emphysema. Similar to previous reports in other murine lung disease models, we did not observe decreased expression of major inflammatory cytokines and chemokines, such as IL-6 or Cxcl2 neither in whole-lung nor in BALF samples from TJ-41-treated mice. One of the possible explanations is the timing of each analysis. We retrieved samples one week after LPS administration to observe the effects of TJ-41 on the recovery from acute lung inflammation. To evaluate inflammatory cytokines involved in acute inflammation, we may need to study samples retrieved at shorter time points.

In this study, we showed that TJ-41 suppressed LPS-induced TNF-alpha increase in a human macrophage cell line. To the best of our knowledge, this is the first study that demonstrates the anti-inflammatory effects of TJ-41 on leukocytes in vitro. In the airway of COPD patients, the numbers of alveolar macrophages are markedly increased, and with their capability of secreting various inflammatory cytokines, alveolar macrophages are regarded as orchestrators of COPD. The present study demonstrated that TJ-41 could suppress the numbers of lung macrophages in an animal model, as well as the LPS-induced expression of TNF-alpha in a human macrophage cell line. These effects may account for the findings of previous clinical studies.

In this study, we focused on analyzing the anti-inflamma-
tory effect of TJ-41 as a Kampo medicine, and we did not examine to attribute its effect to certain known compounds. This is because TJ-41 is a compounding of several natural herbs that are digested and metabolized in vivo, which makes it difficult to identify active ingredients. As candidate compounds, several ingredients of TJ-41, such as quercetin and betulinic acid in Zizyphi fructus, Astragalin in Astragali radix, ferulic acid in Angelicae radix, ginsenoside Rbl in Ginseng radix, saikosaponin A in Bupleuri radix, and 6-shogaol in Zingiberis rhizoma, are reported to have anti-inflammatory effects. Interestingly, we observed that water-soluble components of TJ-41 did not attenuate the expression of LPS-induced TNF-alpha at the same concentration of DMSO-dissolved compounds (Supplementary Fig. 3A). As water-dissolved TJ-41 is less toxic for U-937 cells, we tested the higher concentrations, however water-soluble components of TJ-41 themselves increased inflammatory cytokines (Supplementary Fig. 3B). These results suggest the possibility that poorly water-soluble components of TJ-41 are involved in the anti-inflammatory effect of TJ-41. However, further research is needed to understand the biological mechanisms of TJ-41 effects, particularly in relation to the attenuation of macrophage infiltration in the lungs and the reduction in cytokine production by macrophages.

In conclusion, we demonstrated that TJ-41 attenuates pulmonary inflammation in a mouse model of lung emphysema. This sheds light on the potential of long-time maintenance therapy with TJ-41 as a new therapeutic strategy in patients with COPD.

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Conflict of Interest HI, AM, HT, NM, TI, HM, and TJ receive research funding from Tsumura & Co. (Tokyo, Japan) for research related to this study. TJ has received financial contributions from Tsumura & Co. because he has academic affiliations with the Department of Health Services Research, Graduate School of Medicine, The University of Tokyo supported by Tsumura & Co. Tsumura & Co. played no role in this study. The authors report no other conflicts of interest in this work.

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

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