Wang-Bi tablet, a patented Chinese medicine, maintains the balance of Th1/Th2 in mice with collagen-induced arthritis

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Abstract

OBJECTIVE: To investigate the pharmacological mechanism of Wang-Bi tablets (WBTs), a Chinese patented medicine, in rheumatoid arthritis (RA) using mice with collagen-induced arthritis (CIA).

METHODS: A mouse model of CIA was induced using bovine type II collagen. WBT treatment was administered and efficacy was evaluated. The levels of interferon-γ (IFN-γ), interleukin-2 (IL-2), and interleukin-4 (IL-4) were examined using an enzyme-linked immunosorbent assay, and the proportions of Th1 and Th2 were detected using flow cytometry. T-bet and GATA-binding protein 3 (GATA3) expression were demonstrated using Western blot analysis.

RESULTS: Paw swelling and the arthritis index decreased significantly following WBT treatment. Histopathological analysis revealed markedly alleviated damage to synovium tissue in the WBT and methotrexate treatment groups. WBT regulated the production of IFN-γ, IL-2, and IL-4 and modulated Th1 and Th2 cell populations, which might have been induced by the attenuation of Th1 and Th2 cell differentiation through a decrease in the expression of T-bet and an increase in the expression of GATA3 in the synovial tissue in CIA mice.

CONCLUSION: These results indicate that WBT may produce a therapeutic effect on CIA through maintaining the balance of Th1/Th2 cells, which could result in a decrease in the autoinflammatory disorder observed in RA.

Keywords: Arthritis, rheumatoid; Collagen; Th1-Th2 balance; Wang-Bi tablet

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease that leads to joint destruction, joint stiffness, and loss of function. The pathogenesis of RA is not well understood. The underlying mechanism of RA involves various inflammatory cells, including T and B cells, macrophages, and natural killer cells, which infiltrate joints and induce cell proliferation in the synovial lining. The abnormal differentiation of T helper cells (Th), also known as CD4+ T cells, plays a pivotal role in the pathogenesis of RA. Briefly, autoreactive T cells may recognize a number of autoantigens, induce Th1 cell differentiation, and disturb the balance between Th1 and Th2 cells. The perturbed ratio of Th1 and Th2 cells may induce the autoimmune response that contributes to the pathogenesis of RA and trigger synovial inflammation, which contributes to cartilage and bone destruction.
Several drugs are used in the treatment of RA, such as disease-modifying antirheumatic drugs (DMARDs), the anchor drug methotrexate (MTX), hormonal drugs, and new biologic agents. These drugs dramatically enhance the success of RA management, but the side effects and economic burden of these treatments remain significant challenges for patients. In traditional Chinese medicine (TCM), RA is referred to as Bi pattern, Liijie and Wangbi, which causes blockade of Qi and blood circulation because of the exterior/interior pathogenic wind, cold, damp and/or heat that obstruct the channels and collaterals. TCM has a long history of treating RA with Chinese herbal medicines. Many classic Chinese herbal medicines have been developed into Chinese patented medicines. For example, the Wang-Bi tablet (WBT) was approved by authorities as a nationally protected medicine, and it is widely used for RA treatment in the clinic in China. The formula of WBT is derived from a clinical prescription of Prof. Jiao Shude, the National TCM master. It consists of 17 Chinese medicinal materials according to the Pharmacopoeia of the People’s Republic of China 2010, including Shudihuang (Radix Rehmanniae Preparata), Dihuang (Radix Rehmanniae), Chuanxuduan (Radix Dipsaci Asperoidis), Fuзи (Radix Aconiti Lateralis Preparata), Duhuo (Radix Angelicae Biserratae), Gusuibu (Rhizoma Drynariae), Guizhi (Ramulus Cinnamomi), Yinyanghuo (Rhizoma Anemarrhenae), Shenjincao (Radix Paeoniae Alba), Gouji (Flos Carthami), Zaojiaoci (Gleditsiae Semen), Weilingxian (Radix Saposhnikoviae), Chuanxuduan (Radix Rehmanniae), Gouji (Flos Carthami), Ot Capnace seu Oris (Yang Gu is from farmed goats and sheep).

Some published clinical results demonstrated that WBT ameliorated RA symptoms, decreased the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level, and improved joint pain and morning stiffness. However, the pharmacological activity and molecular mechanisms of WBT in the treatment of RA are not clear. The present study established a collagen-induced arthritis (CIA) model in mice to evaluate the effects of WBT treatment on the inflammatory response. We also investigated the possible anti-inflammatory mechanisms underlying the balance between Th1 and Th2 cells to elucidate the possible immune-regulatory effects of WBT treatment in the CIA mouse model. The present study identified the specific cellular targets involved in the anti-autoimmune effects of WBT.

**METHODS**

All procedures in this study were performed in accordance with the Guidelines for Experimental Animal Care and Use from the Animal Care Committee of Beijing University of Chinese medicine (BUCM), Beijing, China. The Animal Care Committee of BUCM, Beijing, China approved the protocol.

**Materials**

WBT (registered No. Z20044066) was obtained from the GMP-approved Liaoning Good Nurse Pharmaceutical Co., Ltd., Liaoning, China. MTX was obtained from the pharmaceutical factory of Xinyi, Shanghai (No. H31020644). Freund’s incomplete adjuvant, formic acid and bovine type II collagen were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunoabsorbent assay (ELISA) kits, including interferon-γ (IFN-γ, ab100689), interleukin-2 (IL-2, ab47591), and interleukin-4 (IL-4, ab221833), were ordered from Abcam (Cambridge, MA, USA). Anti-T-bet/Tbx21 (4B10, ab91109) and anti-GATA3 antibodies (ab106625) were ordered from Abcam. The flow cytometry antibodies anti-mouse-CD4-FITC, anti-mouse-IFN-γ-PE, and anti-mouse-IL-4-PE were ordered from BD Bioscience (San Jose, CA, USA). Additional reagents were obtained from the Laizi Biotechnology Co. (Beijing, China).

**Animal handling procedure**

Male DBA/1 mice (aged 6–8 weeks) were purchased from Beijing Charles River Laboratory Animal Technology Co., Ltd. (Beijing, China) and raised in the animal center of Beijing University of Chinese Medicine. The Animal Care and Ethics Committee in the Beijing University of Chinese Medicine reviewed and approved the experimental procedures before the animal experiments were performed (No. 20180114). Arthritis was induced as previously described. Briefly, mice were intradermally injected at the base of the tail with 100 μg of bovine type II collagen in 0.05 M acetic acid emulsified in an equal amount of incomplete Freund’s adjuvant. The mice were given a booster of the same preparation 7 d after the primary immunization.

**Experimental groups and drug treatment**

The following experimental groups (n = 10 per group) were used: (a) a normal control (NC) group [distilled water, intragastric administration (i.g.)]; (b) a CIA group (distilled water, i.g.); (c) an MTX group (MTX-treated, 2 mg/kg, once every 3 d, i.g.); and (d) a WBT group (WBT-treated, 0.8 mg/kg, once daily, i.g.). Drug treatment began after booster immunization and lasted for 21 d. The dose of WBT was based on the clinical dosage (6 g once daily, 60 kg body weight).

**Arthritis assessment**

CIA mice were assessed for disease severity every 7 d after booster immunization. Arthritic severity scores were expressed as a mean arthritic index on a 0–4 scale, according to published literature. After 21 d of administration, mice were anesthetized via intraperitoneal injection with 1% pentobarbital sodium (0.4 mL/100 g, i.p.). Blood was collected from the heart via puncture. Mice were sacrificed using cervical dislocation. Joint
and synovial tissues were harvested for subsequent experiments. Joint tissue histopathology was assessed using HE staining. Inflammation, pannus, cartilage damage, and bone damage were scored on scales from 0-3.11

**Enzyme-linked immunosorbent assay (ELISA)**
Serum levels of IFN-γ, IL-2, and IL-4 were measured using ELISA kits, according to the manufacturer’s instructions. Absorbance was read at 450 nm using a microplate reader. Samples and standards were analyzed in triplicate.

**Flow cytometry for Th1 and Th2 detection**
Lymphocyte cell suspensions from mice in each group were collected into tubes using standard methods. Cells (approximately 1 × 10⁶) were washed twice in PBS and labeled with CD4-FITC. The double-labeled cells were blocked, fixed, and permeabilized using a fixation/permeabilization kit and further stained with IFN-γ-PE and IL-4-PE antibodies. The stained cells were washed with PBS, measured using flow cytometry (Becton Dickinson), and analyzed using the CellQuest software package. Gates were established using forward and side scatter to exclude cellular debris. The CD4+ cell subpopulation was sorted using CD4 staining, and cells expressing IFN-γ-PE and IL-4-PE were analyzed.

**Western blot analysis**
Total protein was extracted from synovial tissue using a protein extraction buffer. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated with primary antibodies, including anti-mouse-T-bet and anti-mouse-GATA3, and incubated with HRP-conjugated secondary antibodies. Densitometry plots of the protein expression levels were normalized to tubulin and expressed relative to the levels in the NC group.

**Statistical analysis**
All data were presented as the mean ± standard deviation. Data were processed using SPSS 13.0 (SPSS Inc., Released 2005. SPSS Statistics for Windows, Version 13.0. Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by the Tukey-Kramer test was conducted to test for differences between groups. A P < 0.05 was considered statistically significant.

**RESULTS**

**Effect of WBT on clinical score and histopathological changes in CIA mice**
Following booster immunization, the clinical CIA scores were determined weekly. The results showed that the swelling score significantly increased in CIA mice after 7 d (Figure 1A). The WBT and MTX treatments significantly reduced arthritis severity scores 14 and 21 d after booster immunization. Histological evaluation of joint tissue revealed synovial hyperplasia and pannus formation in CIA mice, and MTX and WBT treatment alleviated these effects (Figure 1B). Pannus formation was reduced, and cartilage surfaces were smoother in the MTX and WBT treatment groups in CIA mice.

**Effect of WBT on IFN-γ, IL-2, and IL-4 levels in CIA mice**
To investigate the expression of proinflammatory cytokines secreted by Th1 and Th2 cells, we quantified the production of Th1-type cytokines IFN-γ and IL-2 and the level of Th2-type cytokine IL-4 in sera of different groups. The results showed that the levels of IFN-γ and IL-2 increased markedly (P < 0.01), and IL-4 levels decreased significantly in CIA mice compared with the NC group (P < 0.01). Furthermore, the levels of IFN-γ and IL-2 decreased, and the level of IL-4 increased in the WBT group compared with the CIA group (P < 0.05 and P < 0.01). However, there were
no significant differences in the levels of these cytokines between the MTX and WBT groups (Figure 2).

**Effect of WBT on the proportion of Th1 and Th2 cells in peripheral blood of CIA mice**

The results of flow cytometry showed that the population of Th1 cells increased significantly (4.6 ± 1.2), and the population of Th2 cells had the same increasing trend (3.0 ± 0.6) in CIA mice compared with NC mice. However, the population of Th1 and Th2 cells was downregulated in the MTX and WBT groups compared with the CIA group (Figure 3A, 3B). The ratio of Th1/Th2 cells in CIA mice (Th1/Th2 = 1.36) was markedly higher than in NC mice (Th1/Th2 = 0.89) (Figure 3C). But the ratio of Th1/Th2 cells in the WBT group was lower (Th1/Th2 = 1.06) than in the CIA group. There was no significant difference in the Th1/Th2 ratios between the WBT and MTX groups (Th1/Th2 = 1.16).

**Protein expression of T-bet and GATA3**

Protein expression of T-bet and GATA3 was determined using Western blot analysis. T-bet expression in the CIA group was markedly lower than in the NC group (P < 0.01) (Figure 4A, 4B). Levels of T-bet in the MTX and WBT groups were lower than in the CIA group (P < 0.01). The level of GATA3 expression in the CIA group was significantly lower than in the NC group (P < 0.01) (Figure 4C, 4D). However, expression levels of GATA3 in the MTX and WBT groups were higher than in the CIA group (P < 0.01). The ratio of T-bet/GATA3 was calculated, and the increase in the ratio of T-bet/GATA3 in the CIA group was markedly lower than in the MTX and WBT groups (Figure 4F).

**DISCUSSION**

A CIA model in DBA/1 mice was used to evaluate the therapeutic effects of WBT on the inflammatory response and focused on T cell dysfunction (imbalance in the Th1/Th2 cell ratio) to further examine the mechanism of WBT in this process. CIA is a well-recognized animal model for RA research, and the proliferation and differentiation of T cells in CIA mice reflect similar pathological changes observed in RA patients. The pharmacodynamic results showed that paw swelling and the arthritis index decreased significantly in the WBT group, which suggests that WBT alleviated the degree of pathology in joint tissue.

Chronic inflammation in the joints and an imbalance between different Th cell subsets are triggered in this pathology. Naive CD4+ T helper cells differentiate into Th1 cells, Th2 cells, Th17 cells, and regulatory T cells (Treg). Th1 cells are characterized by the production of IFN-γ and IL-2, and the Th2 cell lineage is characterized by the production of IL-4, IL-5, IL-13, and IL-10. Published reports suggest that the production of IL-2 and interferon γ (IFN-γ) by Th1 cells increases significantly, and RA patients show an imbalance between Th1 and Th2 subsets in their peripheral blood.

The differentiation of Th1 and Th2 effector cells is governed by master regulator transcription factors and transcription activators, such as T-box transcription factor TBX21 (T-bet) in Th1 cells and GATA-binding protein 3 (GATA3) in Th2 cells. The expression of T-bet/GATA3 reflects the relationship between Th1/Th2 differentiation and the pathogenesis, development, and prognosis of some autoimmune diseases.

The present study found that WBT downregulated the production of IFN-γ and IL-2 and suppressed the differentiation of Th1 cells via reducing the expression of T-bet and increasing the expression of GATA3. The ratio of T-bet/GATA3 was markedly lower than in the CIA model group, which suggests that WBT produces some therapeutic effects on CIA via regulation of T-bet and GATA3 protein expression and balances the ratio of Th1 and Th2 cells. Paradoxically, studies have demonstrated contrary results. For example, overexpression of the T-bet gene (T-bet-tg mice) did not increase IFN-γ expression in a
Data are presented as mean ± standard deviation (n = 3). Significant differences compared with NC group were designated as *P < 0.01; significant differences compared with CIA group were designated as **P < 0.01.

Figure 3 Flow cytometry detection of Th1 and Th2 cells in peripheral blood in different groups
A: Th1 subset profile; B: Th2 subset profile; C: percentage of Th1/Th2 cells in peripheral blood. A1, B1: NC: normal control (distilled water); A2, B2: CIA: collagen induced arthritis (distilled water); A3, B3: MTX: methotrexat (2 mg/kg, once every 3 d); A4, B4: WBT: Wang-Bi tablet (0.8 mg/kg, once every a day). Daily by gavage from the day when the first sign of arthritis was observed for 21 d. Data are presented as mean ± standard deviation (n = 3).

Figure 4 Protein expression of T-bet and GATA3 in synovial tissue.
A: T-bet Western blot; B: relative expression of T-bet; C: GATA3 Western blot; D: relative expression of GATA3; E: ratio of T-bet/GATA3. NC: normal control (distilled water); CIA: collagen induced arthritis (distilled water); MTX: methotrexat (2 mg/kg, once every 3 d); WBT: Wang-Bi tablet (0.8 mg/kg, once every a day). Daily by gavage from the day when the first sign of arthritis was observed for 21 d. Data are presented as mean ± standard deviation (n = 3). Significant differences compared with NC group were designated as *P < 0.01; significant differences compared with CIA group were designated as **P < 0.01.
CIA model, and T-bet-tg mice did not develop CIA or produce anti-collagen antibodies. The biological function of T-bet is complex, and many more transcription factors may be involved in Th1 and Th2 cell differentiation. Therefore, more in-depth studies on Th cell differentiation and the regulatory mechanisms of WBT will be performed in future studies.

Our study had some limitations; for example, the differentiation characteristics of naïve CD4+ T helper cells were not investigated in the present study. We are carrying out relevant research to further reveal the mechanisms of Th1 and Th2 cell differentiation.

In conclusion, our findings suggest that WBT can maintain the balance of Th1 and Th2 cells, which may reduce the expression of T-bet and increase the expression of GATA3 in synovial tissue in CIA mice. This may provide the mechanism underpinning WBT’s beneficial effects in RA.

REFERENCES