Notch signaling pathway mediates the immunomodulatory mechanism of Yangfei Huoxue decoction alleviating bleomycin-induced pulmonary fibrosis in rats

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OBJECTIVE: To investigate the immunomodulatory mechanism by which Yangfei Huoxue decoction (YHD) alleviates bleomycin (BLM)-induced pulmonary fibrosis (PF) in rats.

METHODS: Rats were randomly divided into two time-point groups (day 14 and 28), and each time-point group comprised the following six subgroups: control, BLM, dexamethasone (DXM), YHD high dose (YHD-H), YHD middle dose (YHD-M), and YHD low dose (YHD-L). Haematoxylin and eosin and Masson staining, flow cytometry, enzyme-linked immunosorbent assay, Western blotting and UPLC-QT of analyses were examined.

RESULTS: The results showed that YHD reduced the degree of alveolar inflammation and fibrosis; downregulated the expression of CD28, CD80, CD86, Delta-like 1, Notch2, and Notch3; and upregulated the proportions of Th1/Th2 and Tc1/Tc2. The seven components of YHD were detected.

CONCLUSION: The current study indicates that YHD mainly functions by regulating the immune system and that the molecular mechanism may be related to the regulation of the Notch signaling pathway.

Keywords: Pulmonary fibrosis; Immunity; Receptors, Notch; Signal transduction; Th1-Th2 balance; Yangfei Huoxue decoction

INTRODUCTION

Pulmonary fibrosis (PF) is a process of epithelial cell injury and abnormal wound repair that occurs in the absence of preceding inflammation; these processes are associated with the proliferation of mast fibroblasts and T lymphocytes, excessive accumulation of the extracellular matrix, and formation of fibroblastic foci, which eventually replace the normal lung tissue. The pathophysiology of PF features a paradigm involving injury, loss of the epithelial cell barrier with aberrant re-epithelialization, fibroblast activation and unregulated myofibroblast deposition of extracellular matrix (ECM) components. Previously, the long-held belief was that chronic inflammation played an essential role in the pathogenesis of PF. However, increasing evidence suggests that inflammation does not play a pivotal role in PF and that potent anti-inflammatory therapy is ineffective. Accumulating evidence suggests that immunological mechanisms may participate in the perpetua-
tion and maintenance of fibrotic responses in this disease. Immune reactions mediated by immune cells and cell cytokines are involved throughout the process of PF.7 These cytokines and with their receptors play biological roles through various signaling pathways to mediate the development of PF.

PF is a progressive irreversible illness. Although early diagnosis and early initiation of treatment are highly important for long-term clinical outcomes, none of the available drugs have satisfactory effects. Given this background, Traditional Chinese Medicine (TCM) is becoming increasingly accepted as a novel therapeutic approach. Yangfei Huoxue decoction (YHD) has long been used for the treatment of lung diseases, such as asthma, lung cancer, and PF, and produces much better clinical effects.8 Our previous experiments proved that YHD plays a key role in improving inflammation and angiogenesis in PF;8,9 however, immune regulation has not been studied.

The Notch signaling pathway is involved in the regulation of immune cell development, such as differentiation and maturation of T lymphocytes, B cells, and DC cells. Many scholars have verified that this pathway is widely involved in the development of fibrotic diseases. However, whether the Notch pathway-mediated immune response can be an important factor in the development of PF is unclear. In this study, we focused on the immune regulation of the Notch signaling pathway by YHD.

MATERIALS AND METHODS

Preparation of YHD

Huangqi (Radix Astragali Mongolici), Beishashen (Radix Glehniae), Wuweizi (Fructus Schisandrae Chinesis), Danshen (Radix Salviae Miltiorrhizae), Hu Zhanggen (Radix Polygoni Cuspidati), Chuanxiong (Rhizoma Chuanxiong) and Guguiyuan (Ramulus Euonymi) were prepared at a proportion of 20:20:8:15:15:12:12. All TCM herbs were purchased from the Outpatient Department of GuoYiTang, Nanjing University of Traditional Chinese Medicine. Each herb was identified and authenticated by Gong Jiening. Per the Pharmacopoeia of China (Pharmacopoeia of the People’s Republic of China, vol. 1, 2010 edition), exact amounts of the component herbs were weighed according to the classic percentages. The mixture was soaked in distilled water for 30 min and boiled in eight volumes of water (v/w) for 1 h in an herb-extracting machine twice. Then, the decoction was merged twice, and YHD was concentrated to 0.432 g/mL. The concentrations of the three decoctions are expressed as the total dry weight of the crude herbs per millilitre of the decoction.

Animals and induction

Sprague-Dawley rats (n = 96, half of which were males, while the other half were females) weighing 180-200 g were purchased from Shanghai Slake Laboratory Animal Co., Ltd. (Shanghai, China) (200700057754). All rats had ad libitum access to water and standard chow and were housed at a constant room temperature [(20±2) °C] and humidity (50±10%). All experiments were approved by the Experimental Animal Ethics Committee of Capital Medical University. The animals were randomly divided into two time-point groups (day 14 and 28), which comprised the following six subgroups (8 rats per subgroup): normal saline (control) group, bleomycin (BLM) group, bleomycin (BLM) + dexamethasone [Shanghai xinyi pharmaceutical factory Co., Ltd. (No. 015140802)] DXM group, BLM + high-dose YHD (YHD-H) group, BLM + medium-dose YHD (YHD-M) group, and BLM + low-dose YHD (YHD-L) group. The rats were treated with a single dose of BLM (5 mg/kg body weight) through a tracheal cannula inserted directly into the trachea, except for the rats in the normal group, which were treated with NS (0.9%). BLM was diluted to a concentration of 5 mg/mL and administered on day 1 of the experimental period. Then, DXM (0.000405 g·kg⁻¹·d⁻¹), YHD-H (18.36 g·kg⁻¹·d⁻¹), YHD-M (9.18 g·kg⁻¹·d⁻¹), and YHD-L (4.59 g·kg⁻¹·d⁻¹) were administered by gavage daily. On days 14 and 28, all lung tissues and blood samples were collected for the following experiments. The instruments used are as follows: IX51 optical microscope (Olympus, Japan), Lkb-v microtome (BROMMA, Sweden), RM2145 paraffin slicer (Leica, Germany), CAP 3202S electronic balance (sidris, Germany), BSA 124S analytical balance (sidris, Germany), Low-speed automatic balancing centrifuge dm5-3 (Beijing times beli centrifuge Co., Ltd.), Xw-80a micro-vortex mixer (Shanghai huxi analytical instrument factory), Oscillator pcb-11 (Eppendorf, Germany), Flow cytometry (BD Biosciences, USA).

Histological analysis

For the histological analysis, the lungs were harvested on day 14 and 28, fixed with 4% formalin and embedded with paraffin. Then, the tissues were sectioned and stained with haematoxylin and eosin (HE, Wuxi, China), Biological staining agent, Shanghai sanaisi reagent Co., Ltd. (No. 20130218) to examine the degree of fibrosis. The grading of inflammation and fibrosis was performed blindly using a semiquantitative scoring system according to the criteria described by Szapiel et al.10 The extent of inflammation was graded according to the HE staining and scored from 0 to 5, where 0 was defined as normal, and 1, 2, 3, 4, and 5 were defined as the presence of inflammation involving 10%, 10%-30%, 30%-50%, 50%-80%, and > 80% of the lungs, respectively. Collagen deposition was assessed with Masson’s trichrome staining in which the collagen fibres were stained blue.
**Flow cytometry**

On day 14 and 28, 1-2 mL of peripheral blood was harvested from each rat, and the Th1/Th2 and Tc1/Tc2 ratios were determined using flow cytometry to analyze the intracellular cytokine staining of IFN-γ and IL-4 in blood CD3+ and CD8+ T lymphocytes. The ratio of CD3+ IL-4+ T lymphocytes to CD3+ IFN-γ+ T lymphocytes and the ratio of CD8+IL-4+T cells to CD8+ IFN-γ+ T lymphocytes were determined. The cellular blood (200 μL) was diluted 1:1 with RPMI 1640 medium [semmar faisher biochemical products (Beijing) Co., Ltd., (No. sh30809,01b)], incubated at 37 °C in 5% CO₂ for 6 h and stained with CD3-TC (tricolour-conjugated, APC Mouse Anti-Rat CD3,0.1 mg. BD Bioscience. Lot: 2320854) and CD8-FITC (fluorescein isothiocyanate-conjugated 3687, PerCP Mouse Anti-Rat CD8a,0.1 mg. BD Bioscience. Lot. 3291747) monoclonal antibodies (BD Bioscience) in the dark at room temperature for 15 min. After two washes with phosphate-buffered saline, the cells were resuspended and fixed with 100 μL of reagent A (FIX & PERM.CELL FIXATION & PERME-ABILIZATION KIT.1×5 mL Reagent A.1×5 mL Reagent B. Nordic MuBio.Gas-04). The cell population was detected by flow cytometry.

**Enzyme-linked immunosorbent assay (ELISA)**

Peripheral blood samples (1 mL) were obtained from the rats on day 14 and 28 and allowed to stand for 1 h. After centrifugation at 1509.3 × g for 10 min, the plasma was retained and stored at −80 °C. The plasma levels of CD28, CD80, and CD86 were examined using the corresponding ELISA kits (Nanjing SenBeijia Biological Technology, Nanjing, China) according to the manufacturer’s recommendations.

**Western immunoblotting**

The extraction of protein was performed as follows: the lung tissue samples were harvested on day 14 and 28. With the lysis in RIPA buffer (Beyotime) and centrifugation for 20 min, the proteins were extracted from the cells. The Bradford method was used to detect the quality of the proteins. SDS-PAGE was used to separate equal amounts of proteins, and then, the proteins were transferred onto PVDF membranes. The membranes were incubated with primary antibodies against actin [mouse anti-rat monoclonal antibody (ab6276) was purchased from Abcam (batch number: XP69729.1)], Delta-like 1 (DLL1) [Thermo Scientific (Lot#Q320173315)], Notch2 [Notch2 (D67C8) XPTM Rabbit mAb,Cell Signaling Technology (Lot# 4530S)], or Notch3 [Notch3 (m-134): sc-5593,rabbit polyclonal IgG, Santa Cruz Biotechnology (Lot# 5438)] at 4 °C for 24 h, following by incubation with the secondary antibodies at room temperature for another 1 h. The bands were visualized with an ECL reagent (Millipore Biotechnology Inc.) and quantified using Image Lab 6.0 analysis software.

**LC-MS conditions**

YHD was extracted by sonication with 1 L ethanol/water (70: 30, v/v) for two cycle (1 h per cycle) at room temperature. The combined extracts were filtered, condensed, and reconstituted with 50 mL methanol before the analysis. Standard Cyanidin, Ferulic Acid, Polydatin, Calycosin 7-O-glucoside, Litospermic acid B, Schisandrin, and Isoimperatorin were prepared in methanol to yield concentrations of 5 μg/mL. After centrifuging at 20,000 x g for 10 min, 4 mL supernatant was injected into UPLC-QToF for analysis. The separation and analysis of the samples were performed on a Waters ACQUITY UPLC system (Waters, Milford city, MA, USA) equipped with a binary solvent system, an automatic sample manager and a column compartment. The sample separation was achieved on an ACQUITY BEH C18 column (1.7 μm, 2.1 × 100 mm, Waters, UK) at 40 °C. The flow rate was 0.3 mL/min. The mobile phase comprised 0.1% formic acid in water (A) and acetonitrile (B) with the following gradient programme: 0-2 min, 5%- 5% B; 2-15 min, 5%-40% B; 15-22 min, 40%-95% B; 22-26 min, 95%-95% B; 26-26.1 min, 95%-5% B; and 26.1-30 min, 5%-5% B. The injection volume was 4 μL per run.

The MS data were recorded using a Waters Xevo G2-XS QToF system (Waters, Milford city, MA, USA) equipped with an electrospray ion source (ESI). The following settings were applied: ionization voltage: 1 kV for the positive scan and 2.5 kV for the negative mode; sampling cone voltage: 40 V; source offset: 50; source temperature: 120 °C; desolvation temperature: 400 °C; and desolvation gas flows: 600 L/h. The collision energy of the low energy function was set at 6 V, and the ramp trap collision energy of the high energy function was set at 20-40 V. Leucine-enkephalin was used as an external reference for real-time calibration. The data analysis was performed by using MassLynx V4.1 software.
Statistical analysis
All data are expressed as the mean ± standard deviation (x ± s). One-way analysis of variance was performed, followed by the least significant difference method, for multiple comparisons to compare the differences among the groups. Statistical significance was achieved at P < 0.05. The statistical analysis was performed using SPSS software version 13.0 (IBM, California State University, Fresno, CA, USA).

RESULTS
BLM-induced PF inflammation and fibrosis in the lungs
HE and Masson staining were performed to examine inflammation and fibrosis in the lungs (Figure 1). Fourteen days after BLM was administered, the histological examinations showed infiltration of numerous inflammatory cells in the lung, and the level of inflammation reach a peak (Figure 1A). On day 28, infiltration was decreased, and pulmonary inflammation was gradually decreased (Figure 1B). These observations show that during the process of PF, alveolitis transforms from heavy to light. As shown in Figure 1E, compared with the BLM group, the DXM, YFD and especially the YHD-H groups exhibited alleviated inflammation (P < 0.05). However, on day 14, the extracellular matrix began to accumulate, and the alveolar wall was constantly thickened (Figure 1C). On day 28, a large level of fibrous connective tissue proliferation and PF were evident and reached their peak (Figure 1D). Similarly, YFD and especially the YFH-I groups exhibited better effects than the BLM group (P < 0.05).

Balance of Th1/Th2 and Tc1/Tc2 cells in the blood
During the process of PF, the balance of Th1/Th2 and Tc1/Tc2 cells is disrupted (Figure 2A-F). As IFN-γ and IL-4 are typical representatives of Th1, Tc1, Th2, Tc2, we performed flow cytometry to test the ratio of IFN-γ/IL-4 to represent the ratios of Th1/Th2 and Tc1/Tc2. In the experiment, IFN-γ expression in the BLM group was lower than that in the control group, and IL-4 expression was higher than that in the control group (P < 0.05). The ratios of Th1/Th2 and Tc1/Tc2 in the whole blood from the BLM group were significantly lower than those in the control group (P < 0.05), and on day 28, all YHD groups showed higher ratios than the BLM group (Figure 2E, 2F). YHD tended to balance the ratios of Th1/Th2 and Tc1/Tc2 and alter the formation of inflammation and fibrosis.

Figure 1 YHD alleviates BLM-induced PF inflammation and fibrosis in the lungs on day 14 and 28
A1-A6: HE staining on day 14 (x 400); B1-B6: HE staining on day 28 (x 400); C1-C6: Masson staining on day 14 (x 400); D1-D6: Masson staining on day 28 (x 400); E: HE staining scores; F: Masson staining scores. YHD: Yangfei Huoxue decoction; BLM: bleomycin; DXM: dexamethasone; YHD-H: YHD high dose; YHD-M: YHD middle dose; YHD-L: YHD low dose; PF: pulmonary fibrosis; HE: hematoxylin and eosin. *P < 0.05, compared with control group.
ELISA kits were used to measure the CD28, CD80 and CD86 levels in the plasma (Figure 2G-I). The expression levels of CD28, CD80 and CD86 in the BLM group were significantly higher than those in the control group after 14 and 28 d ($P < 0.05$); however, the YHD groups exhibited significantly lower levels ($P < 0.05$). As shown in Figure 2G-I, the levels in the YHD group are almost equal to those in the control group, indicating that our medicine greatly improved the immune system.

Figure 2 YHD balances the ratios of Th1/Th2 and Tc1/Tc2 cells in the blood and inhibits the levels of CD28, CD80 and CD86 in the plasma on day 14 and 28

A1-A6: ratio of Th1/Th2 on day 14; B1-B6: ratio of Th1/Th2 on day 28; C1-C6: ratio of Tc1/Tc2 on day 14; D1-D6: ratio of Tc1/Tc2 on day 28; G-I: relative protein level of CD28, CD80, and CD86. YHD: Yangfei Huoxue Decoction; BLM: bleomycin; DXM: dexamethasone; YHD-H: YHD high dose; YHD-M: YHD middle dose; YHD-L: YHD low dose; PF: pulmonary fibrosis. *$P < 0.05$, compared with BLM group.
Notch signaling pathway in the lung

The Notch receptors Notch2 and Notch3 and the Notch ligand DLL1 were assessed by Western blotting (Figure 3). Compared with the control group, the expression level of DLL1 in the lung tissues from the model group was significantly higher. Additionally, the expression level of Notch2 in the BLM group was significantly higher than that in the normal group on day 14 and 28, and the expression of Notch3 in the BLM group was significantly higher than that in the normal group only on day 28, suggesting that the DLL1/Notch2 and DLL1/Notch3 signaling pathways in the lung tissues may be inhibited during the early stage of PF and activated during the later stage of PF. YHD (especially in the high and medium dose groups) achieved great results, and the expression levels of DLL1, Notch2 and Notch3 were significantly decreased and occasionally even lower than those in the control group. As Notch signaling pathways are widely involved in PF, this finding suggests that YHD could interfere with the Notch signaling pathway to regulate the immune response to further improve PF.

Chemical constituents of YHD

The chemical constituents of YHD were identified using UPLC-QT of mass spectrometry by comparing the retention time and mass-to-charge of authentic standards (Figure 4). Figure 4 shows the total ion chromatogram of YHD in both the positive (A) and negative (B) scan modes, and the seven components, including cianidanol, ferulic acid, polydatin, calycosin, 7-O-glucoside, lathosterol acid B, schisandrin, and isoimperatorin, were successfully identified. Less than 5 ppm were observed between the measured mass and theoretical mass, further corroborating these results.

DISCUSSION

PF progression involves CD4+ and CD8+ T cell-mediated immune regulation by which the early Th1 and Tc1 cell-mediated acute inflammatory immune response gradually shifts to a Th2 and Tc2 cell-mediated humoural immune process. Many studies have demonstrated that during the development of PF, a Th2-driven immune/inflammatory response is significantly enriched and that the Th2 interleukin cytokines are increased. The balance of Th1/Th2 and Tc1/Tc2 determines the occurrence and development of diseases; when Th2 and Tc2 cells and their secreted cytokines predominate, PF becomes more serious. Our study showed that the Th1/Th2 balance was shifted towards Th2 in rats exposed to BLM and that YHD played essential roles in protecting the rats against BLM-induced PF by regulating the Th1/Th2 balance. Dendritic cells (DCs), which are standard antigen-presenting cells (APCs), are members of the innate immune system and respond to dangers by immediately generating protective cytokines. During antigen presentation, DCs upregulate the biosynthesis of co-stimulatory receptor molecules, such as CD86 and CD80. These activated DC receptor molecules bind cognate

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Figure 3 Protein expression levels of DLL1, Notch2, and Notch3 were assessed by Western blotting
A: expression levels of DLL1, Notch2, and Notch3 on day 14; B: expression levels of DLL1, Notch2, and Notch3 on day 28; C-E: relative protein expression of DLL1, Notch2, and Notch3 on day 14 and 28. 1: control; 2: BLM; 3: DXM; 4: YHD-H; 5: YHD-M; 6: YHD-L. YHD: Yangfei Huoxue Decocction; BLM: bleomycin; DXM: dexamethasone; YHD-H: YHD high dose; YHD-M: YHD middle dose; YHD-L: YHD low dose. *P < 0.05, compared with BLM group.
CD28 receptors present on the T cell membrane, which triggers DCs to secrete cytokines. Growing evidence shows that resident cells, such as fibroblasts and epithelial cells, can accumulate large numbers of DCs in chronically inflamed lung tissues. Our study showed that the expression levels of CD28, CD80, and CD86 in the YHD group were markedly decreased; moreover, the high-dose group and 28-day time point groups showed the best reductions.

The Notch signaling pathway plays a key role in the processes of cell proliferation, differentiation and apoptosis. Vertebrates have four identified Notch receptors (Notch 1 to 4) and five ligands belonging the Delta-like (DLL1, DLL3 and DLL4) and Jagged (Jdg1 and Jdg2) families. Studies have implicated Notch signaling in immune system processes, such as T-B lineage commitment and DC and thymic T cell development. Some scholars have found that DLL1 induces DC differentiation and that the Notch-DLL1 interaction can promote Th1 cell differentiation. Altogether with these data, our results support that the functional axis of DLL1/Notch2 and/or Notch3 could be an immunotherapeutic target for PF. In our experiments, we showed that the expression levels of DLL1, Notch2 and Notch3 were significantly decreased and occasionally even lower than those in the control group, suggesting that our Chinese herbal medicine, i.e., YHD, can inhibit the Notch signaling pathway to regulate the immune response in PF.

TCM is based on the notion that the pathogenesis of PF involves Qi and Yin deficiencies and collateral block circulation. A growing number of studies have verified that Chinese herbs and their extracts achieve good experimental results in immune regulation in PF. We chose YHD, whose effects include nourishing Qi and Yin, activating blood circulation, and achieving significant clinical efficacy, to treat PF. YHD comprises two parts, Huangqi (Radix Astragali Mongolici), Beishen (Radix Gilehniae), and Wuweizi (Fructus Schisandraceae Chinensis), whose main effect is supplementing Qi and nourishing Yin, and Danshen (Radix Salviae Miltiorrhizae), Huzhanggen (Radix Polygoni Cuspidati), Chuanxiong (Rhizoma Chuanxiong) and Guijianyu (Ramulus Euryphi), whose main effect is activating blood circulation. In the study, our Chinese medicine groups exhibited better outcomes than the BLM group, indicating that YHD may be a new option for PF patients.

Moreover, the seven components of YHD, including cianidanol, ferulic acid, polydatin, calycosin 7-O-glucoside, lithospermic acid B, schisandrin, and isoimperatorin, were successfully identified by UPLC-QT of mass spectrometry. Numerous studies have shown that these components have a regulatory effect on immunity; for example, cianidanol possesses sufficient potential to modulate immune activity by cellular and humoral mechanisms. Ferulic acid supresses the Th2 immune response, schisandrin A exerts a protective mechanism through the activation of autophagy, etc.

Overall, the Chinese medicine prescription YHD can regulate the immune response to treat PF, and the molecular mechanism may be related to the Notch signaling pathway. These findings may contribute to future metabolic and pharmacokinetic studies of this prescription. However, in this study, only animal studies were performed. Thus, in our following study, we may focus on the role of the seven components of YHD and the Notch signaling pathway in PF through in vitro cell experiment and human clinical studies.
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