Artesunate ameliorates lung fibrosis via inhibiting the Notch signaling pathway

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Abstract. The present study aimed to determine the underlying molecular mechanism of the antifibrotic effect of artesunate in pulmonary fibrosis (PF). Primary lung fibroblasts were isolated from the lung tissues of rats, and treated with artesunate (8 µg/ml) and transforming growth factor (TGF)-β1 (5 ng/ml). For in vivo experiments, the rats were administered bleomycin intratracheally, followed by daily intraperitoneal artesunate injections for 27 days. Western blotting, and immunohistochemical and immunofluorescent staining were used to assess the expression of key components of the Notch signaling pathway, including α-smooth muscle actin (α-SMA) and type IV collagen. Artesunate (8 µg/ml) was identified to inhibit TGF-β1-induced α-SMA and collagen protein expression, and repress the Notch signaling pathway, in primary lung fibroblasts. Downregulation of α-SMA and collagen by artesunate was associated with inhibition of the Notch signaling pathway. The daily intraperitoneal injection of artesunate (1 mg/kg) in rats was determined to inhibit bleomycin-induced overexpression of α-SMA and type IV collagen proteins, and inhibit the Notch signaling pathway, in lung tissues. In conclusion, the results of the current study indicate that artesunate inhibits the TGF-β1-induced differentiation of rat primary lung fibroblasts into myofibroblasts and ameliorates bleomycin-induced PF. In addition, the results of the present study suggest that the underlying molecular mechanism for these effects of artesunate is repression of the Notch signaling pathway.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive, severely debilitating disease with a high mortality rate (1). However, there are currently no effective treatments for IPF. The clinical features of IPF include chronic (>6 months) exertional shortness of breath, a dry cough, inspiratory bibasilar crackles and finger clubbing (2,3). Although the risk factors for IPF remain unclear, a prevailing hypothesis suggests that injuries in the alveolar epithelium trigger pathophysiological alterations, including increased production of transforming growth factor (TGF)-β, which induces the differentiation of fibroblasts into myofibroblasts and the excessive deposition of extracellular matrix (ECM) (4). The alteration of fibroblasts and the ECM serves a major role in the development of fibrosis, and creates a profibrosis positive feedback loop (5). The differentiation of fibroblasts into myofibroblasts, which are fibroblasts that contain α-smooth muscle actin (α-SMA) and other contractile elements, has been identified as a key event in the development of IPF and other profibrotic conditions (6,7).

The neurogenic locus notch homolog protein (Notch) signaling pathway is highly conserved and serves a key role in cellular proliferation, specification and differentiation (8). In mammals, there are four types of Notch receptor (Notch1-4) and five Notch receptor ligands (Jagged1, Jagged2, delta-like 1, delta-like 3 and delta-like 4). Notch receptors are activated by ligand binding, upon which the Notch intracellular domain (NICD) is released and translocates into the nucleus, where it interacts with transcriptional repressors to modulate the expression of target genes that include the well-characterized transcription factor hairy enhance of split (Hes).

Previous studies have reported that the Notch signaling pathway is associated with human fibrotic diseases, including pulmonary fibrosis (PF) (9,10). Artesunate, the recommended first-line treatment for severe malarias (11), was recently demonstrated by our group to inhibit the proliferation of lung fibroblast by promoting apoptosis and reducing collagen secretion (12). In addition, artesunate was determined to downregulate the expression of TGF-β1, mothers against decapentaplegic homolog 3, heat shock protein 47, α-SMA and collagen type I (12).

The present study aimed to evaluate the effect of artesunate on the TGF-β1-induced differentiation of lung fibroblasts into myofibroblast. In addition, the effect of artesunate on bleomycin-induced pulmonary fibrosis in rats was investigated. Furthermore, the potential underlying molecular mechanisms of the effects of artesunate on these processes was explored.
Materials and methods

**Materials.** Antibodies for cleaved Notch (N1CD, cat. no. ab52301), α-SMA (cat. no. ab5694) and Hes-1 (cat. no. ab108937) were purchased from Abcam (Cambridge, MA, USA). Antibodies for Jagged1 (cat. no. Sc-8303) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies for β-actin (cat. no. PR0255), horseradish peroxidase (HRP)-conjugated anti-mouse or -rabbit immunoglobulin G antibodies (cat. no. ZB-2301 and ZB-2305), and Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G antibody (cat. no. ZF-0311) were obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). The γ-secretase inhibitor DAPT was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Human recombinant TGF-β1 was purchased from Peprotech, Inc. (Rocky Hill, NJ, USA). Penicillin/streptomycin, fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Bleomycin hydrochloride for injection was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan). Artesunate was purchased from Guilin Pharmaceutical (Shanghai) Co., Ltd. (Guilin, China). The DAB kit (cat. no. ZLI-9017) was obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China) according to the manufacturer's protocol. Other reagents were purchased from Sigma-Aldrich (Merck KGaA).

**Lung fibroblast culture.** Lung fibroblasts were isolated from the rats described below according to a previous method (13). The cells were cultured at (37°C, 95% humidity and 5% CO₂) in DMEM supplemented with 10% FBS. The identity of fibroblast cells was verified at passage four by testing the expression of vimentin (1:200 dilution; cat. no. 5741; CST, Inc., Danvers, MA, USA) by immunofluorescence staining. Cells at passage 5 were used for further experiments.

The primary fibroblasts were synchronized by incubation (at 37°C in 5% CO₂) in serum-free DMEM for 24 h and then randomly divided into five groups as follows: The control group, incubated in serum-free DMEM; the TGF-β1 group, incubated in DMEM containing 5 ng/ml TGF-β1; the Notch inhibitor (DAPT) + TGF-β1 group, incubated in DMEM containing 5 ng/ml TGF-β1 and 10 µM DAPT; the artesunate + TGF-β1 group, incubated in DMEM containing 5 ng/ml TGF-β1 and 8 µg/ml artesunate; and the artesunate control group, incubated in DMEM containing 8 µg/ml artesunate at 37°C for 24 h.

**Animal experiment protocols.** Male Sprague Dawley rats (n=24) weighing 180-250 g at 8 weeks old were obtained from the Center for Experimental Animals at Guilin Medical University (Guilin, China). The rats were housed in specific pathogen-free conditions at 20-24°C and humidity 30-50% with a 12-h light/dark cycle and ad libitum access food and water. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Guilin Medical University (Guilin, China).

For in vivo study, the rats were randomly divided into the following four groups: The control group (n=6), which received intratracheal administration of 0.9% NaCl solution on day 1, followed by daily intraperitoneal injections of 0.9% NaCl solution (1 ml) for 27 days; the bleomycin group (n=6), which received intratracheal administration of 0.9% NaCl solution at day 1, followed by daily intraperitoneal injections of 0.9% NaCl solution (1 ml) for 27 days; the artesunate group (n=6), which received intratracheal administration of 0.9% NaCl solution at day 1, followed by daily intraperitoneal injections of artesunate (100 mg/kg) for 27 days; and the bleomycin + artesunate group (n=6), which received intratracheal administration of bleomycin (5 mg/kg) at day 1, followed by daily intraperitoneal injection of artesunate (100 mg/kg) for 27 days. All animals were euthanized by pentobarbital overdose (100 mg/kg) at the end of treatment period, and lung tissues were quickly removed and processed for further analysis.

**Masson's trichrome staining.** Masson's trichrome staining was performed to observe lung fibroblast collagen secretion. Lung fibroblasts (0.3x10⁶/well) were plated in 6-well plates with or without TGF-β1 (5 ng/ml) at 37°C in 5% CO₂ for 24 h, and the cultured cells were washed 3 times for 1 min each with ice-cold PBS and fixed at 4°C with 4% paraformaldehyde for 30 min. Then lung fibroblasts were stained with Masson trichrome staining kit manual at 20-24°C for 5 min and subsequently examined with a light microscope (Olympus Corporation, Beijing, China).

**Immunofluorescence staining.** Lung fibroblasts (7,000 cells per well) were plated on 6-well chamber slides with or without TGF-β1 (5 ng/ml) at 37°C for 24 h, then the culture medium was then aspirated, and the slides were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 20 min. Cells were permeabilized with 0.05% Triton X-100 at 4°C for 10 min and blocked with 3% bovine serum albumin (BSA; cat. no. OR0015; Leagene Co., Ltd, Beijing, China) for 1 h. The slides were incubated at 4°C overnight with primary antibodies directed against α-SMA (dilution, 1:100 in 3% BSA) or against vimentin (1:200 dilution; cat. no. 5741; CST, Inc.). Subsequently, the slides were washed with PBS and incubated with secondary anti-rabbit Texas green-conjugated antibodies at 4°C (dilution, 1:200 in 3% BSA). Slides were then washed in PBS for 20 min. Two drops (10-15 µl) of ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Inc.) was added to each well prior to the addition of coverslips. The slides were then examined by fluorescence microscopy.

**Western blot analysis.** The lung tissue was homogenized in radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor cocktail (cat. no. 04693159001; Roche Diagnostics, Basel, Switzerland) containing 1 mmol/l phenylmethylene-sulfonyl fluoride (PMSF) using a tissue grinder. Cultured cells were lysed on ice in ice-cold RIPA buffer with a protease inhibitor cocktail containing 1 mmol/l PMSF for 20 min. Proteins from lung tissue and fibroblast were prepared as previously described (12,13). A total of 20 µg protein lane was loaded into each lane and separated via 10% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane, which was blocked with 5% non-fat milk in Tris-buffered saline (TBS) at room temperature for 1.5 h. The membranes were then incubated at 4°C overnight with cleaved Notch (NICD 1:1,000 dilution; cat. no. ab52301), α-SMA (1:1,000 dilution;
cat. no. ab5694), Hes-1 (1:800 dilution; cat. no. ab108937), Jagged1 (1:500 dilution; cat. no. Sc-8303) and Notch1 (1:400 dilution; cat. no. 4380), followed by three washes with TBS containing Tween-20 (5 min/wash). The membrane was subsequently incubated with secondary antibodies, horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G antibodies (1:5,000 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., cat. no ZB‑2301 and ZB‑2305, respectively) at 20‑24˚C for 1.5 h. Protein bands were detected using Enhanced Chemiluminescent reagent and imaged with a ChemiDoc MP Imaging system (Bio‑Rad Laboratories, Inc., Hercules, CA, USA).

Immunohistochemical staining. Tissues were fixed with formalin at 20‑24˚C for 24 h and embedded with paraffin, then sliced into 5 µm thick sections, which were deparaffinized in xylene followed by rehydration in a graded series of alcohols. Antigen retrieval was performed through pressure cooking (at 121˚C) in citrate buffer solution for 3 min. Slides were rinsed with PBS and then incubated in 3% hydrogen peroxide solution for at 20‑24˚C for 20 min. After rinsing with PBS three times, the slides were incubated with 10% fetal bovine serum (cat. no. 10437028; Gibco; Thermo Fisher Scientific, Inc.) in PBS for 30 min to block nonspecific binding. The slides were then incubated with primary antibodies directed against Jagged1 (1:400), NICD (1:200), Hes-1 (1:200), α-SMA (1:200) or Type-IV collagen (1:400 dilution; cat. no. ab6586; Abcam) at 4˚C overnight, followed by three washes in PBS (5 min/wash). Subsequently, the slides were incubated with biotinylated goat anti-rabbit polyclonal secondary antibody (cat. no. XIT-9901; Maixin-Bio, Fuzhou, China) at room temperature for 30 min, followed by three washes in PBS (5 min/wash). The slides were then stained with the DAB kit at 20‑24˚C for 5‑10 min according to manufacturer’s instruction. Finally, the slides were dehydrated, cleared with xylene and mounted with neutral gum. Images of tissue sections were captured using a BX53 digital light microscope (Olympus Corporation, Tokyo, Japan).

ECM-deposition assay. Lung fibroblasts (0.3x10^6/well in 6-well plate) were stimulated by incubation with TGF-β1 (5 ng/ml) for 24 h at 37˚C with or without artesunate (8 µg/ml) or DAPT (10 µmol/l), then in serum-free DMEM at 37˚C for 48 h. Collagen secretion and deposition (collagen type I-V) were determined using the Sirius Red Collagen Detection kit (cat. no. 9062; Chonrex, Inc., Redmond, WA, USA) according to the manufacturer’s protocol.

Statistical analysis. Statistical tests were performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA). Results are presented as the mean ± standard error. The statistical significance of differences between groups was determined using one-way analysis of the variance with a post hoc Tukey’s range test. P<0.05 was considered to indicate a statistically significant difference.

Results

TGF-β1 induces the differentiation of rat primary lung fibroblasts. To assess whether TGF-β1 serves a role during the differentiation of primary lung fibroblasts into myofibroblasts, the expression of α-SMA, a myofibroblast marker and all type of collagen, was detected (Fig. 1). This revealed that protein levels of α-SMA (Fig. 1A and B) and collagens (Fig. 1C and D) were increased in TGF-β1-treated fibroblasts compared with the control group, indicating that TGF-β1 induces the differentiation of fibroblast into myofibroblasts.
TGF-β1 activates the Notch signaling pathway in primary lung fibroblasts. The Notch signaling pathway is an important signaling pathway in PF (8). To investigate the role of the Notch signaling pathway in the TGF-β1-induced differentiation of fibroblasts into myofibroblasts, the protein expression of Jagged1, Notch1, NICD and Hes-1 was measured. This revealed that all were increased in TGF-β1-treated fibroblasts compared with the control group (Fig. 2). Furthermore, DAPT, a γ-secretase inhibitor and inhibitor of the Notch signaling pathway, significantly suppressed the TGF-β1-induced overexpression of α-SMA (P<0.01; Fig. 3). These findings indicate that the Notch signaling pathway is associated with the TGF-β1-induced differentiation of fibroblasts to myofibroblasts.

**Artesunate inhibits the differentiation of primary lung fibroblasts.** Based on our previous finding that artesunate exerts an antifibrotic effect in rats with bleomycin-induced PF (12), and the finding of the current study that the expression of α-SMA and collagens was upregulated in TGF-β1-treated fibroblasts (Fig. 1), it was hypothesized that artesunate may play a role in the differentiation of lung fibroblasts. To test this hypothesis, the effect of artesunate on the expression of α-SMA and collagens in TGF-β1-treated lung fibroblasts was examined. The protein expression of α-SMA (Fig. 3A) and the amount of collagens secreted in culture medium (Fig. 3B) were significantly decreased after treatment with artesunate. In addition, the effect of artesunate was only slightly less compared with that of DAPT (Fig. 3). These findings suggest that artesunate is able to inhibit the differentiation of fibroblasts into myofibroblasts.

**Artesunate inhibits the Notch signaling pathway in primary lung fibroblasts.** The aforementioned experiments indicated that the Notch signaling pathway serves a role in the TGF-β1-induced differentiation of fibroblasts to myofibroblasts, and that artesunate is able to inhibit this. To evaluate whether the inhibitory effect of artesunate on this differentiation is via the Notch signaling pathway, the protein levels of Notch1, Jagged1, NICD and Hes-1 in lung fibroblasts treated with artesunate was measured. The expression of these proteins was markedly decreased in lung fibroblasts following treatment with Art or DAPT compared with the control group (Fig. 4), suggesting that artesunate inhibits the Notch signaling pathway.
Artesunate suppresses the expression of collagens, α-SMA and certain components of the Notch signaling pathway in the lung tissues of rats with bleomycin-induced PF. Bleomycin induces inflammation and collagen deposition in the lungs of rats, and a previous study by our previously groups demonstrated that artesunate can ameliorate these pathological alterations in cultured fibroblasts (12). In the present study, an in vivo experiment was performed to confirm these findings. Immunohistochemistry (Fig. 5A) and western blotting (Fig. 5B) were employed to detect the expression of collagen, α-SMA and certain key components of the Notch signaling pathway in the lung tissue from rats with bleomycin-induced PF treated with artesunate. The results demonstrated that the protein levels of collagen, α-SMA, Jagged1, Notch, NICD and Hes-1 were markedly increased following exposure to bleomycin, indicating that the Notch signaling pathway is activated by bleomycin. However, the protein levels of collagen, α-SMA, Jagged1, Notch1, NICD and Hes-1 were decreased in the lung tissues from rats treated with artesunate, or bleomycin and artesunate, suggesting that artesunate inhibits the Notch signaling pathway.

Discussion

The results of the present study indicate that artesunate inhibits the TGF-β1-induced expression of collagen and α-SMA in lung fibroblasts, and that artesunate reduces bleomycin-induced PF in rats. In addition, artesunate was identified to downregulate key components of the Notch signaling pathway in vitro and in vivo, including Jagged1, Notch1, NICD and Hes-1.

PF is a chronic lung disorder characterized by the dysregulated recruitment, proliferation and differentiation of fibroblasts, excessive deposition of ECM and abnormal lung remodeling (4). Fibroblasts serve an important role in the pathogenesis of PF, and several factors influence their proliferation and synthesis of ECM (14). Myofibroblasts, a marker of fibrotic diseases, secrete ECM, collagens and α-SMA, which leads to the loss of alveolar function (4,14). Numerous profibrotic cytokines, including TGF-β1, platelet-derived growth factor and tumor necrosis factor α, serve roles in the differentiation of fibroblasts into myofibroblasts. The present study demonstrated that TGF-β1 could induce the differentiation of fibroblasts into myofibroblasts, in addition to activating the Notch signaling pathway.

There are few reports on the role of the Notch signaling pathway in the differentiation of lung fibroblasts into myofibroblasts. The Notch signaling pathway is a highly conserved pathway, which is essential to normal embryonic development, cellular proliferation, specification and differentiation, and is associated with fibrotic disease (8). The findings of the present study aid in the better understanding of the pathophysiology of lung fibrosis, in addition to suggesting that the Notch signaling pathway is a potential therapeutic target for this disease. Since overexpression of Hes1 enhances the promoter activities of α-SMA and collagen type 1 α2 (15), therefore TGF-β1 may function, at least partially, via the Notch signaling pathway to upregulate Hes1 expression and promote α-SMA expression.

The results of the present study are consistent with previous reports that Notch deficiency has a significant inhibitory effect on the response to bleomycin-induced PF (10,16), suggesting that cellular signaling pathway crosstalk serves an essential role in the pathogenesis of PF. Our group recently reported that artesunate, a drug used for the treatment of severe malaria in adults and children worldwide, may inhibit the development of PF (12,17). Previous experiments by our group demonstrated artesunate can induce the apoptosis of fibroblasts, inhibit TGF-β1-induced epithelial-to-mesenchymal transition and downregulate the expression of TGF-β1 in an animal model of PF (12,18). The present study provided additional evidence revealing the potential underlying molecular mechanism of the antifibrotic effect of artesunate. However, further experiments are required to test whether artesunate functions via the same mechanism in other types of pulmonary cells.
In conclusion, the current study demonstrated that artesunate effectively inhibits the Notch signaling pathway and the TGF-β1-induced differentiation of primary lung fibroblasts into myofibroblasts in vitro, and ameliorates PF in vivo. The results of the present study indicate that the Notch signaling pathway serves a role in the differentiation of fibroblasts into myofibroblasts, and may be a potential novel therapeutic target for PF.

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