European Review for Medical and Pharmacological Sciences 2014; 18: 3199-3204

Artesunate restraining MAPK passage by smad7 to resist pulmonary fibrosis

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Abstract. – OBJECTIVE: This study aims to discuss the function and molecular mechanism of artesunate in resisting pulmonary fibrosis.

METHODS: Artesunate was used to stimulate the HFL-I cell line, which restrains the expression of Smad7 protein. Under different conditions, all treatment factors were checked, including Smad7, p-P38, ERK, and p-JNK protein expressions. Flow cytometry was used to detect the cell cycle. For the silent expression of the p-Smad7 protein, Western blot analysis revealed that Smad7, p-P38, and p-JNK proteins decreased compared with those of the non-treatment group.

RESULTS: No significant changes were observed in Smad7, p-P38, and p-JNK proteins after the cells with silent p-Smad7 protein expression were stimulated by artesunate ($p > 0.5$). No significant changes were observed in the expression of Smad7, p-P38, and p-JNK proteins after using TGF-β1 recombination factor to cells whose p-Smad7 protein expression is silent ($p > 0.5$).

CONCLUSIONS: Artesunate blocks the MAPK cell conduction pathway through Smad7 to restrain idiopathic pulmonary fibrosis.

Key Words:
Artesunate, MAPK, Smad7, TGF-β1, Signal transduction pathway.

Abbreviations

HFL-1 = human lung fibroblasts; TGF-β1 = transforming growth factor beta 1; MAPK = mitogen-activated protein kinase; JNK = c-Jun N-terminal kinase; ERK = extracellular-signal-regulated kinase; shRNA = small hairpin RNA; PAK1 = serine/threonine-protein kinase 1; GFP = green fluorescent protein; HELF = human embryonic lung fibroblast; GAPDH = glyceraldehyde 3-phospathe dehydrogenase; TBS-T = tris buffered saline with tween; TRAF6/TAK1 = TNF receptor associated factor 6/TGFβ-activated kinase1; ALK5 = Activin receptor-like kinase 5; BMPs = bone morphogenetic protein 2: p38:38kDa protein.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a pulmonary interstitial inflammatory disease with no definite pathogen. The main manifestations are tussiculation and progressive dyspnea. In most cases, IPF develops to terminal respiratory failure or death within three to eight years after the manifestations. The morbidity rate of IPF is gradually increasing every year. At present, research on its morbidity mechanism is not clear and lacks objective and decisive prognostic factors or treatment response[1]. Recent studies have shown that artemisinin drugs not only resist malaria, but also have functions in regulation, immunization, and resistance against tumor, infection, and fibrosis, among others[2]. Our previous research showed that treating HFL-I cells with 2.5 mg/L to 160 mg/L artesunate for 48 h greatly restrains cell development in a dose-dependent manner. We employed 10 mg/L artesunate for 24 and 48 h, and used reverse transcriptase-polymerase chain reaction to verify whether the expression of caspase-3 mRNA strengthens with time. We also used western blot analysis to detect whether the protein expression of caspase-3 and the degradation product of pro-caspase-3 strengthen with time. The results showed that artesunate promotes the death of human embryonic lung fibroblast (HELF) by increasing the expression of Caspase-3 mRNA and Caspase-3 protein and, thereby, inducing the death of HFL-I cells. Furthermore, flow cytometry revealed that treating the fibroblast with the cell factor retards the cell cycle. Western blot analysis verified that the pretreatment of artesunate for 30 min can restrain the phosphorylation of Smad3 and Smad7 by the TGF-β1 growth factor. Thus, artesunate restrains fabrication through the TGF-β1/Smad pathway. Previous studies also observed the function of Smad7 in many processes and stages. However, the core factor is still not confirmed during the working

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process of artesunate. The present research shows that Smad7 induces many factors. Signals or chemicals may take Smad7 as a hinge to regulate MAPK signaling pathway. More evidence proved that Smad7 can play the main role in this process and can be used as the framework protein to stimulate JNK or p38 as well as cell apoptosis induced by them[3]. In this experiment, artesunate is used to resist pulmonary fibrosis. We observed the expression of Smad7, p-P38, ERK, and p-JNK proteins inside the cell and identified the molecular mechanism of artesunate as an anti-pulmonary fibrosis. The results of this study may serve as a theoretical basis for the clinical use of artesunate in treating pulmonary fibrosis.

Materials and Methods

Cell Culture

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Company, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Hyclone Company, Logan, UT, USA, article number 30087.02), and incubated at 37 °C under 5% CO_2. The cells were passaged four times before use.

Protein Expression of Artesunate at Different Action Times

Previous experiments showed that 10 mg/mL artesunate is sufficient for cell inhibition. To observe the effect of artesunate on HFL-I (purchased from the Cell Bank of the Chinese Academy of Sciences) at different times and the protein expression of Smad7, TAI-1, p-p38, and p-JNK, 6×10^5 HFL-I cells were inoculated in 25 cm^2 culture vessels and added with artesunate to achieve 10 mg/mL concentration. The cells were collected at 5 min, 15 min, 1h, 12h, 24h, and 48h after the addition of artesunate. The extracted protein was subjected to western blot to measure the protein expression of TGF-β1, Smad3, and Smad7.

Protein Expression at Different Action Times of TGF-β1

Previous experiments showed that 2.5 ng/mL recombination human TGF-β1 cell factor (Perpotech Inc., Rocky Hill, NJ, USA, article number 100-21) stimulates the HELF cell expression of TGF-β1 until the peak is reached. Subsequently, adding the cell factor enabled TGF-β1 expression to be in the platform period. The cells were collected at 5 min, 15 min, 1h, 12h, 24h, and 48h after the addition of the TGF-β1 cell factor. After extracting protein, western blot was used to detect the protein expression of p-Smad7, TAI-1, p38, and JNK.

Restraining of p-Smad7 Protein Expression

The shRNA eukaryon expression vector was constructed for the HFL-I cells. According to the GenBank database and research on *Smad7* gene sequence[4,5], the SODN and ASODN of shRNA target sequence were designed as 5′-GCTGCGGGGAGAAGGGGCGAC-3′ (GenBank AF010193). The SODN is 5′-CGCGTCCCCGCTGCGGGGAGAAGGGGCGACTTCAAGAGAGTCGCCCCTTCTCCCCGCAGCTTTTTGGAAAT-3′, and the ASODN is 5′-CGATTTCCAAAAAGCTGCGGGGAGAAGGGGCGACTCTCTTGAA GTCGCCCCTTCTCCCCGCAGCGGGGA-3′. The shRNA target sequence was annealed after it was composed by the Invitrogen Company and then connected to the pLVTHM supporter. The pLVTHM-sh-Smad7 supporter comprised the connection system: 1 L T4 ligase, 2 L T4 ligase buffer solution, 1 L pLVTHM (MluI + ClaI), and 1 L masterplate after shRNA was annealed. To select a single bacterial colony after bed plank formed monoclonal antibodies, the culture was augmented in a 2YT culture medium with 100 μg/mL AMP and the plasmid was then obtained.

The pLVTHM-sh-Smad7 plasmid was mixed with two kinds of structure plasmid, namely, PAK and MD2G in serum-free DMEM. The plasmids were left for 5 min prior to mixing with lipo2000 at room temperature for 15 min. The mixture was then added with 293 T cells. After 24h, the GFP fluorescence was observed as more than 85%. The virus was then collected after 48h.

After the cell fabric swatch reached 85%, ELISA was performed to detect virus titration concentration. The proportion of the culture medium and the virus was 1:1, and 8 ng/mL polybrene was added to infect the HFL-I cells. GFP fluorescence was observed in 90% of the cells after 24h. The cells were then divided into three groups: virus infection, artesunate stimulation (10 mg/mL), and TGF-β1 stimulation factor (2.5 ng/mL) groups. The cells were stimulated for 24h, and the protein was collected after 48h of virus infection.

Protein Expression After Artesunate and TGF-β1 Stimulation

Artesunate and TGF- 1 stimulation were employed to HFL-I cells after gradual virus infec-

tion to detect the effect on the protein expression of p-Smad7, TAI-1, p38, and JNK. The protein expressions of the HFL-I cells before and after virus infection were compared using artesunate stimulation and TGF- 1 stimulation.

Western Blot

RIPI protein lysis solution (Blue Sky) was used to decompose the HELF cell at a low temperature (4°C) and to extract the cell protein. Protease and phosphorylase inhibitors were added into the protein. Bicinchoninic acid assay was employed to measure protein concentration by injecting 50 μg of the sample and conducting electrophoresis on lauryl sodium sulfate polyacrylamide gel (10% separation gel and 5% gathering gel). The membrane was transferred for 120 min under a constant voltage of 100 V to the polyvinylidene fluoride membrane (Millipore Company, Billerica, MA, USA). The seal was kept close for 2 h at room temperature with 5% defatted milk powder. Antibody (concentration 1:1000) and actin antibody GAPDH (concentration 1:2500) were then added. The membrane was subsequently stored overnight at 4°C temperature. The membrane was washed thrice with TBS-T for 15 min each time. Goat-anti-mouse IgG marked by horseradish peroxidase (concentration 1:3500) was added and mixed by slightly shaking for 1h at room temperature. The membrane was again washed thrice with TBS-T. The product stripe was scanned under BIO-RAD (Hercules, CA, USA) high-sensitivity chemiluminescence imaging system. The Image Lab 3.0.1 software was used to take a photo and analyze the gray level of the strip.

Statistical Analysis

The result is expressed as mean standard deviation ($\bar{x} \pm s$). Each group of data was initially subjected to normality test and one-way analysis of variance. The Student-Newman-Keuls test (q test) was adopted for multiple comparisons; $p < 0.05$ was considered to indicate statistical significance. Each test data were from independent experiments repeated three times.

Results

Smad7 Protein Expression

Western blot test results showed that the cells processed by artesunate at different action times had increasing Smad7 protein expression with action time prolonging within 24h ($p < 0.05$). p-JNK and p-P38 protein decreased with action time prolonging within 24h ($p < 0.05$). Smad7, p-JNK, and p-P38 protein changed accordingly at 5 min after the addition of artesunate. However, the change in protein expression stopped after 24h. As shown in Figure 1, ERK protein expression had no changes at longer action times ($p > 0.05$).

Protein Expression of Smad7, p-JNK, p-P38, and ERK

The results are shown in Figure 2. Human recombination TGF-β1 cell factor disposed the cells at different action times. As for the cells disposed by artesunate at different action times, Smad7 protein expression decreased with action time prolonging within 24h ($p < 0.05$), whereas p-JNK and p-P38 proteins increased with action time prolonging within 24h ($p < 0.05$). Smad7, p-JNK, and p-P38 proteins changed immediately at 5 min after disposal by artesunate. Similar with the stimulation for cells by artesunate, the change in protein expression stopped after 24h (Figure 2), and the ERK protein expression had no changes with action time ($p > 0.05$).

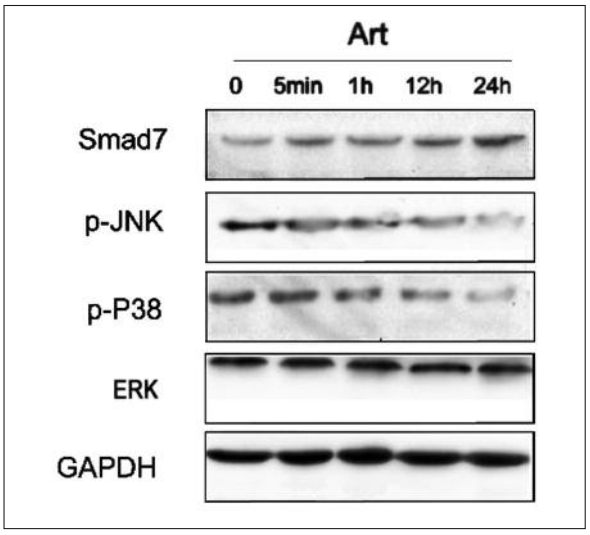

Figure 1. Western blot detection results show that cells processed by artesunate at different action times and Smad 7 protein expression increased with action time prolonging within 24h. p-JNK and p-P38 protein decreased with action time prolonging within 24h. Smad7, p-JNK, and p-P38 proteins changed accordingly at 5 min after processing by artesunate. However, the change in protein expression stopped after 24h. ERK protein expression exhibited no changes at longer action times.

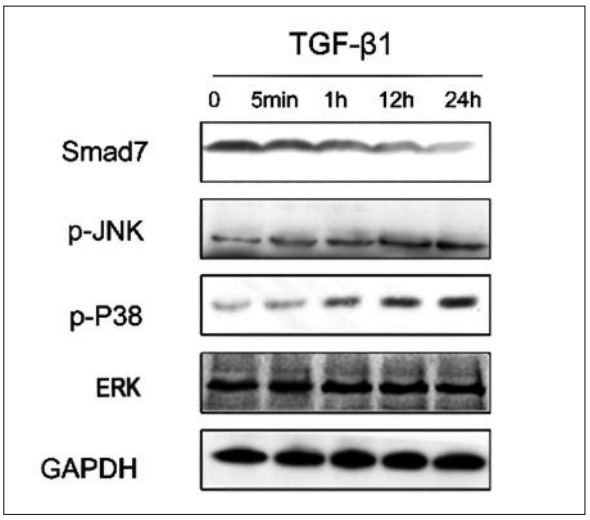

Figure 2. Human recombination TGF-β1 cell factor disposed the cell at different action times. As for the cells disposed by artesunate at different action times, Smad7 protein expression decreased with action time prolonging within 24h, whereas p-JNK and p-P38 proteins increased with action time prolonging within 24h. Smad7, p-JNK, and p-P38 proteins changed immediately at 5 min after disposal by artesunate. Similar with the stimulation of artesunate for cells, the change in protein expression stopped after 24h. Under artesunate's stimulation, the ERK protein expression showed no changes with the action time.

Protein Expression of HFL-I Cell

Compared with the control group, the cells disposed by sh-Smad7 can inhibit the expression of p-Smad7 protein ($p < 0.05$), and the expression of p-JNK and p-P38 decreased ($p > 0.05$), as shown in Figure 3.

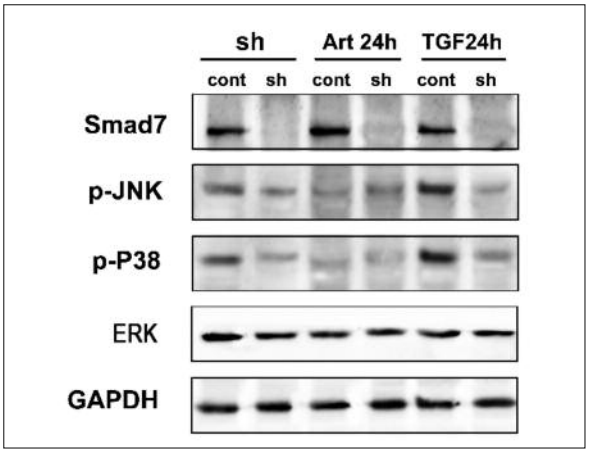

Figure 3. The protein expressions of p-Smad7, p-JNK, ERK, and p-P38 were detected at 24h after using artesunate and human recombination TGF-β1 cell factors to dispose silent Smad7.

At 24h after using artesunate to stimulate the cells disposed by sh-Smad7, the expression of p-Smad7, p-JNK, and p-P38 had no changes ($p > 0.05$) compared with the control group. Their expressions were lower than those of normal HFL-I cell ($p < 0.05$).

For the use of recombination TGF- 1 to stimulate the cells disposed by sh-Smad7 for 24h, the expression of p-Smad7, p-JNK, and p-P38 had no changes ($p > 0.05$) compared with the control group. Compared with the p-Smad7, p-JNK, and p-P38 proteins of the normal HFL-I cells, the protein expression also decreased ($p < 0.05$).

After using artesunate stimulation for cells disposed by sh-Smad7 and using recombination TGF-β1 stimulation for cells disposed by sh-Smad7, the ERK gene change was not found in the protein expression test.

HFL-I Cell Cycle

As presented in Table I, when comparing HFL-I cell treated by artesunate with the control group, the G1 stage increased (81.97%). This result indicates that the growth of most cells was stagnant. When using TGF-β1 recombination cell factor to stimulate HFL-I cell, after 24h, cell cycle detection showed that the G1 stage decreased and the S stage increased (36.72%). This finding indicates that the cell is in the state of growth. Disturbance was used to make the Smad7 gene silent, and then artesunate was used again for stimulation. Compared with the cells that were not disposed, the cells exhibited no changes. However, the cells in the S stage increased (36.78%) after using the TGF-β1 recombination cell factor to stimulate HFL-I cells.

Discussion

During the signal transduction process related with Smad, its process is under the accurate regulation of many levels[6]. Among them, the inhibitory Smad protein is the key negative regulation factor during TGF-β/Smad signal transduction process, which mediates the crosstalk between TGF-β/Smad signal and other signal transduction pathways[7,8]. According to the present biochemical experiments and our previous research results, Smad7 is an inhibitor of the TGF-β superfamily and plays an important role in resisting pulmonary fibrosis[3,9]. Using gene knockout, RNA disturbance, or transgenosis, Smad7 has

Table I. Cell cycle of HFL-I cell treated by artesunate.

Group	G1	G2	S	*p*
Art 24h	81.97	10.12	7.92	< 0.05
TGF-β1 24h	54.20	9.08	36.72	< 0.05
Sh Art 24h	76.55	11.02	12.43	> 0.05
Sh TGF-β1 24h	54.88	8.34	36.78	< 0.05
Control	76.89	11.73	11.37	

Note: p represent compared with the control group.

been recently found closely related to cell proliferation and death and may be the target spot of disease treatment[3,9,10].

MAPK is a series of serine/threonine kinases and is an important signal system that mediates cell response. MAPK includes three passageway proteins: ERK, c-Jun amino terminal kinase (JNK), and p38 pathways. These pathways activate some transcription factors and regulate gene expression[10,11]. Research shows that artesunate can resist fibrosis[2]. Artesunate can inhibit the MAPK level chain in the cytoplasm and restrain the phosphorylation of Smad 2/3 by inhibiting JNK and p38 to resist fibrosis[10].

Our previous research revealed that TGF/Smad pathway plays an important role in resisting pulmonary fibrosis. In recent years, many studies have studied the effect of TGF/Smad pathway on the MAPK pathway[7]. In mouse muscle fibroblasts, the p38 inhibitory factor can inhibit the phosphorylation of the Smad connection area[12,13]. In the human mesangial cell, ERK's inhibitory factor can inhibit the phosphorylation of the Smad connection area and promote the composition of I-type collagen[14]. Artesunate was used to disturb the HELF cells. Research shows that artesunate resists fibrosis through MAPK. Further research verifies that artesunate plays a main role by mediating the Smad protein during the process of resisting fibrosis. Furthermore, artesunate's inhibition of the MAPK pathway through the Smad7 protein has a slight effect on ERK. By contrast, some studies reported that among the three main pathways of MAPK, Smad7 has a limited effect on p38 and mainly goes through JNK and ERK pathways[3,15,16].

Aside from the adjustment of TGF-β/Smad to the expression of Smad7, many inflammation factors have induction functions to Smad7. These signals or chemicals can regulate the MAPK signaling pathway through Smad7[9,17]. Meanwhile, Smad7 can regulate other signaling pathways[7]. TGF-β can activate the MAPK signal, and increasing evidence supports the major role of Smad7 in the whole process. The study shows that Smad7 may act as skelemin that mediates the activation of JNK or p38 and has the function of inducing cell death[11]. As an adaptor, Smad7 can promote the interaction between TRAF6/TAK1 and ALK5, and mediate the process of TGF-β activating p38[10,18]. Moreover, Smad7 plays a regulative role in the process of BMP2 activating TAK1/p38[19]. This experiment proves that artesunate can resist fibrosis through Smad7's function to p38 in the signal conduction pathway.

Conclusions

Our previous study has proven that TGF-β plays an important role in resisting pulmonary fibrosis. In the present study, Smad7 as the main functioning protein of TGF-β mediates TGF-β during cell cycle retardation and cell death, whereas other processes play roles in resisting fibrosis. This function mechanism has been mentioned by some scholars[6,20]. However, the present study is the first to report on the function mechanism of artesunate in resisting fibrosis through Smad7. This study provides the molecular mechanism and serves as a basis for the clinical treatment of fibrosis.

Acknowledgements

This study was supported by Projects of Natural Science Foundation of Guangxi (Grant no: 2011GXNSFA018220) and Scientific Research and Technology Development project (Grant no: 101240084).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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