Regular Article

Plasminogen activator inhibitor-1 promotes the proliferation and inhibits the apoptosis of pulmonary fibroblasts by Ca$^{2+}$ signaling

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Aim: Our previous investigation demonstrated that plasminogen activator inhibitor-1 (PAI-1) siRNA ameliorated bleomycin (BLM)-induced rat lung fibrosis. The present study was undertaken to explore the effect and the mechanism of PAI-1 siRNA and plasmid pcDNA on the proliferation and apoptosis of cultured fibroblasts from BLM-induced fibrotic lung tissues.

Materials and Methods: The fibroblasts from BLM-induced fibrotic lung tissue were isolated and transfected using PAI-1 siRNA and plasmid pcDNA-PAI-1. The techniques of real time RT-PCR and/or western blot were used to determine the expression of PAI-1, α-smooth muscle actin (α-SMA) (real time RT-PCR only), collagen type-1 and type-3 (real time RT-PCR only), and the levels of caspase-3, ERK and AKT signal molecules. The proliferation of fibroblasts was measured by cell cycle with flow cytometry. The intracellular concentration of Ca$^{2+}$ was examined by confocal laser microscopy.

Results: PAI-1 siRNA downregulated the PAI-1 mRNA expression by 70±7% at 24 h and protein expression by 73.5±10% and 42±3% at 48 h and 72 h compared to Non-specific siRNA group. Flow cytometry showed that the fibroblasts at the G2M+S phase were significantly reduced by 20.56±1.03% after transfecting PAI-1 siRNA and were significantly increased by 43.8±1.21% after transfecting plasmid pcDNA-PAI-1. The mRNA expressions of α-SMA, collagen type-1 and type-3 were downregulated after transfecting the PAI-1 siRNA, whereas increased after transfecting pcDNA-PAI-1. PAI-1 siRNA increased the level of caspase-3, inhibited the expressions of p-ERK and p-AKT protein molecules, while the pcDNA-PAI-1 transfection showed a reversal effect on these expressions. Intracellular Ca$^{2+}$ concentration was decreased after transfecting PAI-1 siRNA, whereas increased after transfecting pcDNA-PAI-1.

Conclusion: PAI-1 promotes the proliferation, transforming into myofibroblasts, collagen synthesis, and inhibits apoptosis of pulmonary fibroblasts by activating Ca$^{2+}$, ERK and AKT signaling pathway. Decreasing PAI-1 expression is an available strategy in inhibiting the progression of pulmonary fibrosis.

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Introduction

Idiopathic pulmonary fibrosis is characterized by chronic lung inflammation, progressive fibroblast proliferation, and excessive extracellular matrix deposition [1,2]. Despite decades of intensive study, no effective treatment has been developed due to poor understanding on the pathological process of the disease [3]. Available evidence suggested that fibroblast converting into myofibroblast played an important role during the exacerbation of pulmonary fibrosis [4]. Fibroblast/myofibroblast apoptosis represented a critical checkpoint in the pulmonary injury and repairment. The resistance of fibroblast/myofibroblast to apoptosis was associated with persistent tissue fibrosis [5]. Therefore, inhibiting the proliferation and promoting the apoptosis of fibroblast may be an available strategy in the treatment of idiopathic pulmonary fibrosis.

Recent investigation focused on the interactions between fibrotic disease and fibrinolysis system. PAI-1 is a primary inhibitor of urokinase-type (uPA) and tissue-type plasminogen activators. In vitro study, PAI-1 promoted proliferation and inhibited spontaneous and induced apoptosis of multiple cell lines [6,7]. But the effect of PAI-1 on fibroblast has been rarely reported. In vivo study, over- or down-regulating expression of PAI-1 with transgenic manipulation exacerbated or alleviated bleomycin-induced lung fibrosis, respectively [8,9]. The inducible lung-specific urokinase expression in mice and aerosolization of uPA in rabbits limited lung fibrosis and improved the survival following lung injury [10,11]. Our previous data indicated that PAI-1 siRNA inhibited alveolitis and pulmonary fibrosis in BLM-treated rats by inhibiting ERK and AKT signalling pathways [12]. In order to explore the antifibrotic mechanisms of PAI-1 siRNA and the role of PAI-1 in the development of pulmonary fibrosis, the present study was undertaken to observe the effect of PAI-1 siRNA and plasmid on proliferation and apoptosis of cultured fibroblasts from
BLM-induced lung tissue. Furthermore, the changes of the intracellular Ca2+ concentration, ERK and AKT signaling pathways are investigated to clarify the association of the molecules during the process.

Materials and methods

**Bleomycin-induced rat lung fibroblasts isolate**

A total of 12 male Wistar rats weighing 130-140 g provided by The Experimental Animal Center of Hebei Medical University, China were used. All rats were housed and manipulated according to the Care and Use of Laboratory Animals (Beijing), and kept under specific pathogen-free conditions. The rats were divided into two groups, sham group and bleomycin (BLM) group. The rats in BLM group were subjected to intratracheal administration of BLM (5 mg/kg, 0.2 ml) to induce lung fibrosis, while the rats in the sham group were administrated with normal saline in the equal-volume. On day 28, rats were sacrificed, and fibrotic lung tissues were harvested for isolating fibroblasts in vitro.

**Cell culture and transfection**

The fibroblasts derived from BLM-induced lung tissues were used to detect the effect of PAI-1 plasmid and to screen the different PAI-1 siRNA sequences. The fibrotic lung tissues were cut into 1 × 1 × 1 mm3 scraps and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, USA), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 15% heat-inactivated fetal calf serum (Gibco, USA). Purified fibroblasts were obtained after being cultured 2-3 passages and identified by immunohistochemistry staining with the antibodies against vimentin (Santa Cruz) and α-SMA (Epitomics, USA) proteins according to the manufacturer’s protocol.

The three pairs of siRNAs against rat PAI-1 mRNA as 219 siRNA, 559 siRNA, and 1061 siRNA and Ns-siRNA (Non-specific siRNA) (synthesized by Guangzhou Ribobio Co, LTD) [13], were transfected into the fibroblasts using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The siRNA sequences above were shown in Table 1. The plasmid with PAI-1 gene (pcDNA-PAI-1) (provided by Dr. Fang Wang) was transfected into fibroblasts and our previous data determined that PAI-1 protein expression was upregulated 277% and 204% at 48 h and 72 h [12]. The effectiveness of siRNAs in inhibiting the PAI-1 expression was evaluated by real time RT-PCR (24 h) western blotting analysis (48 h and 72 h after the transfection). To determine fibroblasts proliferation, cell cycle analysis was measured at 24 h after transfecting PAI-1 siRNA and pcDNA-PAI-1 by flow cytometry according to the manufacturer’s protocol.

**Real time Reverse Transcription Polymerase Chain Reaction**

Total RNA was extracted from lung fibroblasts 24 h after transfection of siRNA and pcDNA-PAI-1 using Trizol reagent (TakaRa, Japan) according to the manufacturer’s protocol. Quantitative real time RT-PCR was performed on a RotorGene 3000A PCR instrument (Corbett, Australia).

**Western blot analysis**

At 48 h and 72 h after transfection of siRNA and pcDNA-PAI-1, the fibroblasts were harvested. The homogenization of samples and the determination of protein concentration were conducted by the Coomassie blue assay. After electrophoresing on 12% SDS-PAGE and transferring to polyvinylidene difluoride (PVDF) filters (Millipore, USA), the samples were incubated with mice anti-PAI-1 antibody (BD, USA), rabbit anti-Caspase-3 antibodies (Santa Cruz), rabbit anti-AKT and anti-ERK antibodies (Bioworld, USA), rabbit anti-p-AKT and anti-p-ERK (cell signal, USA), rabbit against α-SMA (Santa Cruz). The ECL luminescence system was used to detect the primary antibodies. The integral optical density (IOD) of each band was measured using a Gel-image analyzing system (Alpha Image 2200, Alpha, USA).

**Assessment of calcium concentration in fibroblasts**

To investigate the signaling mechanisms of PAI-1 in lung fibrosis, we observed the changes of calcium concentration in cultured fibroblasts by downregulating and upregulating PAI-1 expression. The fibroblasts, which were plated on a 24-well plate at 5 × 10^4 cells/well, were transfected with PAI-1 siRNA or pcDNA-PAI-1 when the cells were at 50–80% confluence. At 24 h and 48 h after transfecting, the cells were added into pollen grains to detect the calcium concentration by confocal laser scanning microscopy.

Fluo-4/AM (Invitrogen, Inc. USA) of 1 μmol/L in dimethylsulfoxide (DMSO) was mixed with F-127 of 1 μmol/L (Sigma Inc. USA), and then the mixture of 500 μl was added into the treated cells, and incubated in the dark at 25 °C for 30 min. Fluorescent probes were excited by 488 nm laser, and emission fluorescence was filtered by a 510 nm filter to eliminate the auto-fluorescence of pollen grains. The fluorescence intensity of pollen cells in standard buffers was measured by Leica SPIII confocal laser scanning microscope (Leica, Inc. German) in ×200 times and converted into the corresponding Ca2+ concentration by Leica confocal software.

**Statistical analysis**

Statistical analysis of values was performed with SAS8.0 software. All data were described as mean ± SD and analyzed by t-test and one-way ANOVA. P < 0.05 was considered significant.

**Result**

**The effect of PAI-1 siRNA on PAI-1 expression**

The treatment with BLM has been proven to induce pulmonary fibrosis in previous study [12]. We successfully isolated the fibroblasts from BLM-induced fibrotic lung tissues. The cells isolated were verified using SYBR Green PCR Kit (TakaRa, Japan). The housekeeping gene GAPDH was used as an internal control, and gene-specific miRNA expression was normalized against GAPDH expression. The primer sequences were summarized in Table 2.

**Table 1**

| 219 siRNA | Sense 5'-GCCACCAUCUCGGAGUAATT-3' |
| 559 siRNA | Anti-sense 5'-UUAUCUCUCAUGUGGCGGCT-3' |
| 1061 siRNA | Sense 5'-GCCACACCGCAGUCACUGCCT-3' |
| Non-specific siRNA | Sense 5'-UUCUCCGAGCGUUGUCAGTT-3' |

**Table 2**

<table>
<thead>
<tr>
<th>Primer sequences for real time polymerase chain reaction</th>
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<tbody>
<tr>
<td>α-SMA Forward 5'-CCCTTCCAGATCCATACAA-3'</td>
</tr>
<tr>
<td>Reverse 5'-CTTCCCTCCACTCCTA-3'</td>
</tr>
<tr>
<td>collagen I Forward 5'-GGTGGTATTACGTCAGCTCC-3'</td>
</tr>
<tr>
<td>Reverse 5'-CATCCAGGCAGTGTCCCT-3'</td>
</tr>
<tr>
<td>caspase-3 Forward 5'-GTCCTACAGGCTGATGTT-3'</td>
</tr>
<tr>
<td>Reverse 5'-GAGGAGAGGAGGAGGAT-3'</td>
</tr>
<tr>
<td>GAPDH Forward 5'-ATCTGTTGCGCAGATGCTT-3'</td>
</tr>
<tr>
<td>Reverse 5'-ACGGTATGACCTGATGAC-3'</td>
</tr>
</tbody>
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to be fibroblasts by the positive stain of Vimentin immunoparticles and negative stain of α-SMA (Fig. 1A, B).

Three candidate siRNA sequences were transfected into fibroblasts to evaluate the effective sequence of siRNA against PAI-1. Real time RT-RCR (Fig. 1C) showed that 559 siRNA and 219 siRNA downregulates PAI-1 mRNA expression by 70% ± 7%, and 25% ± 13% at 24 h respectively, compared to Non-specific siRNA group. Western blotting analysis in Fig. 1D and E showed that the PAI-1 protein expression was downregulated 73.5% ± 10% and 42% ± 3% by 559 siRNA and 47% ± 20% and 29.3% ± 1% by 219 siRNA at 48 h and 72 h respectively, while 1061 siRNA and Non-specific siRNA had no effect on PAI-1 protein expression. These indicated that 559 siRNA most effectively inhibited the PAI-1 protein expression. Therefore, we chose this sequence of siRNA for the experiment in vitro.

Effect of regulating PAI-1 expression on fibroblast proliferation

The assay used flow cytometry showed that the fibroblasts were arrested at the G0/G1 phase, and the fibroblasts at the G2M+S phase were significantly reduced by 20.56 ± 1.03% after transfecting PAI-1 siRNA. Reversely, the fibroblasts at the G2M+S phase were significantly increased by 43.8 ± 2.12% after upregulating PAI-1 expression at 24 h by pcDNA-PAI-1 (Fig. 2).

Effect of regulating PAI-1 expression on profibrotic cytokine

It was shown that the mRNA expressions of α-SMA and collagen type-1 were decreased at 24 h after transfecting PAI-1 siRNA, while their expressions were increased after upregulating the PAI-1 expression by pcDNA-PAI-1. The mRNA expression of collagen type-3 was not affected (Fig. 3).

The effect of regulating PAI-1 expression on the apoptosis of the fibroblasts

The apoptosis of pulmonary fibroblasts was evaluated by determining caspase-3 expression by real time RT-RCR at 24 h (Fig. 4A, B) and by western blot analysis at 48 h (Fig. 4C, D). The results showed that the

Fig. 1. The effect of different PAI-1 siRNAs on the PAI-1 expression in cultured fibroblasts from bleomycin (BLM)-induced fibrotic lung tissues of rats. The microphotographs show the cultured cells in A and immunoparticles of vimentin in B which verified that the cells are fibroblasts. The bar graph of C shows the changes of PAI-1 mRNA assayed by real time RT-RCR at 24 h after the transfection of PAI-1 siRNA. The expression was normalized against GAPDH. The upper panels in D and E are representative of immunoblot bands of the western blot analysis at 48 h (D) and 72 h (E) after the transfection of PAI-1 siRNAs. The lower panels are quantitative presentation of the immunoblots with integral optical density (IOD). The ratios of the IOD of immunoblot of the aim protein to that of β-actin are used for statistics. *p < 0.05 vs Non-specific siRNA (Ns-siRNA) group.

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expression of caspase-3 was induced by PAI-1 siRNA compared with Ns-siRNA groups, while inhibited by pcDNA-PAI-1.

The effect of regulating PAI-1 expression on intracellular Ca²⁺ concentration of the fibroblasts

The assay used confocal laser microscopy showed that Ca²⁺ concentration-associated intracellular fluorescence intensity was significantly decreased at 24 h and 48 h after transfecting PAI-1 siRNA compared with Ns-siRNA groups (Fig. 5A), which indicated that the intracellular Ca²⁺ concentration of the fibroblasts was decreased. Whereas, the intracellular fluorescence intensity was significantly increased after transfecting pcDNA-PAI-1 compared with pcDNA3.1 groups (Fig. 5B), which indicated that the intracellular Ca²⁺ concentration was increased.

The effect of regulating PAI-1 expression on AKT and ERK signal pathways

To investigate the signaling pathways of PAI-1 in lung fibrosis, the expression of AKT, p-AKT, ERK(1/2), p-ERK(1/2) were determined in cultured fibroblasts. Western blot analysis shows that administration of PAI-1 siRNA significantly inhibited the expressions of p-AKT and p-ERK at 48 h and 72 h (Fig. 6A), while the expressions were significantly increased after transfecting pcDNA-PAI-1 at the observed time points (Fig. 6B).
Discussion

The pathogenesis of pulmonary fibrosis remains unclear and controversial [14], and PAI-1 might be a potential pro-fibrotic factor [8,9]. Further, several reports indicated that pulmonary and hepatic fibrosis, allergic asthma and keloid scarring could be treated by inhibiting PAI-1 level [15–18]. Recently, it was found that small molecule PAI-1 inhibitor TM5275 and TM5007 prevented the bleomycin-induced lung fibrotic process in mice [19,20]. Our previous investigation indicated that intratracheal injection of PAI-1 siRNA alleviated alveolitis, and prevented the fibrotic progression of lung in BLM-treated rats [12].

But, the mechanism underlying the process remains unclear. In the present study, we investigated the effect of PAI-1 siRNA and plasmid on proliferation, apoptosis and transformation of cultured fibroblasts from BLM-induced fibrotic lung tissue. We found that downregulating PAI-1 level by PAI-1 siRNA inhibited fibrotic lung fibroblasts

![Graphs showing gene expression and caspase-3 expression](image)

*P<0.05 vs Non-specific siRNA (Ns-siRNA) group. △P<0.05 vs pcDNA3.1 group.

Fig. 3. PAI-1 promotes transformation and collagen synthesis of the cultured fibroblasts from bleomycin (BLM)-induced fibrotic lung tissue of rats assayed by real time RT-PCR.

Fig. 4. The effect of regulating PAI-1 expression on the proliferation and apoptosis in the cultured fibroblasts from bleomycin (BLM)-induced fibrotic lung tissue of rats by real time RT-PCR (A, B) at 24 h and western blot analysis (C, D) at 48 h. *P<0.05 vs Non-specific siRNA (Ns-siRNA) group. △P<0.05 vs pcDNA3.1 group. It can be found that transfection of PAI-1 siRNA significantly promoted the caspase-3 expression in fibroblasts, while the expression was inhibited after upregulating PAI-1 expression with pcDNA-PAI-1.
proliferation by reducing the cells in G2M+S phase and the conversion of the fibroblasts to myofibroblasts, and increased apoptosis of the fibroblasts by upregulating caspase-3 level. While upregulating PAI-1 level by PAI-1 plasmid showed opposite results with the PAI-1 siRNA. These results indicated that PAI-1 promoted the proliferation, transforming into myofibroblasts, collagen synthesis of the fibroblasts, and inhibited apoptosis of pulmonary fibroblasts in the progress of pulmonary fibrosis. Our previous study using MTT assay also showed promoting effect of PAI-1 on fibroblast proliferation [12]. Meanwhile, Chen et al. reported similar phenomenon in vascular smooth muscle cells of SM22-PAI+mice that overexpression PAI-1 promoted proliferation and inhibited the apoptosis by inhibition of caspase-3 [21,22]. Therefore, our present findings provide convincing evidence to indicate the mechanism of PAI-1 siRNA inhibiting pulmonary fibrosis, and strongly suggest, together with our previous observation in vivo, that PAI-1 is an important risk factor in pulmonary fibrosis. Our present data and other report indicated that ERK and AKT activation decreased proliferation and induced apoptosis in a variety of cells including epithelial cells, and vascular smooth muscle cells [33,34]. Our previous data and other report indicated that ERK and AKT...
signal pathways were involved in lung and liver fibrosis respectively [12,35]. In the present study, we observed in cultured fibroblast from fibrotic pulmonary tissue that PAI-1 induced an increase in intracellular Ca$^{2+}$ concentration. The changes of Ca$^{2+}$ were associated with the progression of cell cycle and the activation of ERK and AKT signaling pathways. This is the first time evidence to illustrate that Ca$^{2+}$ signaling and ERK1/2 and AKT protein activation played a central role in fibroblasts proliferation, transformation, and collagen synthesis, and then in the development and progression of pulmonary fibrosis. This is consistent with the observation in PC12 cells, where an increase in intracellular free Ca$^{2+}$ concentration positively regulated Ras signaling, leading to ERK phosphorylation [36].

In summary, all the evidence in the present study suggested that activity of PAI-1 played an essential role in the development of lung fibrosis. Accordingly, diminishing the expression or activity of PAI-1 may constitute a novel prophylactic and therapeutic approach in idiopathic pulmonary fibrosis.

Conflict of interest statement

None declared.

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