Anti-pulmonary fibrotic activity of salvianolic acid B was screened by a novel method based on the cyto-biophysical properties

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ABSTRACT

Various methods have been used to evaluate anti-fibrotic activity of drugs. However, most of them are complicated, labor-intensive and lack of efficiency. This study was intended to develop a rapid method for anti-fibrotic drugs screening based on biophysical properties. A549 cells in vitro were stimulated with transforming growth factor-β1 (TGF-β1), and fibrogenesis was confirmed by conventional immunological assays. Meanwhile, the alterations of cyto-biophysical properties including morphology, roughness and stiffness were measured utilizing atomic force microscopy (AFM). It was found that fibrogenesis was accompanied with changes of cellular biophysical properties. TGF-β1-stimulated A549 cells became remarkably longer, rougher and stiffer than the control. Then, the effect of N-acetyl-L-cysteine (NAC) as a positive drug on ameliorating fibrogenesis in TGF-β1-stimulated A549 cells was verified respectively by immunological and biophysical markers. The result of Principal Component Analysis showed that stiffness was a leading index among all biophysical markers during fibrogenesis. Salvianolic acid B (SalB), a natural anti-oxidant, was detected by AFM to protect TGF-β1-stimulated A549 cells against stiffening. Then, SalB treatment was provided in preventive mode on a rat model of bleomycin (BLM)-induced pulmonary fibrosis. The results showed that SalB treatment significantly ameliorated BLM-induced histological alterations, blocked collagen accumulations and reduced α-SMA expression in lung tissues. All these results revealed the anti-pulmonary fibrotic activity of SalB. Detection of cyto-biophysical properties were therefore recommended as a rapid method for anti-pulmonary fibrotic drugs screening.

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1. Introduction

Idiopathic pulmonary fibrosis (IPF) is the most common interstitial lung disease characterized by myofibroblast accumulation, α-smooth muscle actin (α-SMA) over-expression, excessive collagen deposition and extracellular matrix (ECM) remodeling [1,2]. It is a devastating and progressive respiratory disease with a mean survival of 2–3 years from initial diagnosis due to irreversible loss of lung function [3]. Unfortunately, there is still no effective therapy until now. Thus, there is an urgent need to look for effective drugs for IPF. Various models in vitro have been used to evaluate anti-fibrotic activity of drugs by morphological observation and detection of immunological markers [1,4,5]. However, these assays are complicated, labor-intensive, lack of systematic, and cannot provide instantaneous and quantitative information.

Cellular biophysical properties, including cell morphology and mechanical properties, take an important part in many essential cellular functions such as shape, transport, movements, differentiation and signaling, and are also associated with a number of pathological processes [6]. Diseases can change biophysical properties in individual living cells, while the corresponding changes in cyto-biophysical properties also influence the onset and progression of diseases [7,8]. Recent studies suggest that cellular biophysical properties may be applied as novel biological markers of cell phenotypes, reflecting changes in cellular differentiation [9,10]. Therefore, researches on cellular biophysical properties can help to get a better understanding of the pathophysiology and
pathogenesis of diseases.

Atomic force microscopy (AFM) has become a powerful and versatile tool in medical, biological and biophysical researches. AFM reveals novel molecular resolution information about membrane structure, cell organelles and the cytoskeleton [11–14]. Compared with traditional assays, AFM is with many advantages, such as label-free, technical simplicity, real-time and quantitative measurements of direct changes in living cells. This technology opens the door to study the biophysical properties from the cellular surface to deeper parts of the cell.

In this regard, our work focused on developing a new method based on cellular biophysical properties to evaluate anti-fibrotic activity of drugs. A classical model of pulmonary fibrosis was copied by stimulating human alveolar epithelial cells A549 in vitro with Transforming growth factor-β1 (TGF-β1) [15]. The changes of both immunological markers and biophysical properties in A549 cells were characterized. Then, the effect of N-acetyl-L-cysteine (NAC) as a positive drug for IPF [3,4] was measured by both immunological and biophysical markers in TGF-β1-stimulated A549 cells to verify these biophysical indexes. Finally, Salvianolic acid B (SalB), a natural anti-oxidant, was taken as a potential drug to evaluate this new method. Its anti-pulmonary fibrotic activity was screened by biophysical marker and confirmed with bleomycin (BLM) –induced pulmonary fibrosis in rats.

2. Materials and methods

2.1. Cell culture and treatment

The human alveolar epithelial cell line A549 was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 (Hyclone, Logan, UT) containing 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO) in an incubator at 37 °C with 5% CO₂. Cells were rendered quiescent by serum free medium for 24 h, and then pretreated with 3 mM NAC (Sigma) or 40 μM SalB (Shanghai Winherb Medical Technology Co., Ltd, China) for 3 h before stimulation with 5 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN) for another 48 h. A549 cells simulated with PBS only were taken as the control.

2.2. Immunofluorescence cell staining

Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. Non-specific sites were blocked by incubating with 1% BSA (Sigma) in PBS at 37 °C for 1 h. For staining, cells were incubated with following rabbit polyclonal primary antibodies overnight at 4 °C: α-SMA, E-cadherin and vimentin (Proteintech Group, Chicago, IL), and then incubated with FITC-labeled goat anti-rabbit IgG antibody (Proteintech Group) and Hoechst 33258 (Sigma) at room temperature for 45 min [15]. Fluorescent images were taken by Olympus fluorescence microscope.

2.3. Western blotting

Protein lysates (40 μg) were separated by SDS-PAGE, transferred onto Hybond-P PVDF membrane (Millipore, Bedford, MA) and incubated with primary antibodies overnight at 4 °C. Primary antibodies were detected by HRP-labeled IgG conjugates and visualized using ECL-Plus detection kit [16]. Results were normalized to GAPDH (Hangzhou Goodhere Biotechnology Co., Ltd, China).

2.4. AFM imaging

Morphological and ultrastructural assays were performed using a Nanoscope VI Dimension 3100 AFM (Veeco Company, Santa Barbara, CA) and oxide-sharpened Si3N4 tips (MLCT, radius 10–40 nm; Bruker Company, Santa Barbara, CA). Cells were seeded in 70 mm culture disk. For living cell imaging, the triangular cantilever with a normal spring constant 0.01 N/m was employed. The spring constant was calibrated with a Thermal Tune Adapter (Veeco Company). The probe was localized onto the cell surface with the assistance of a CCD camera. The experiments were performed at contact mode. The scan force was 50 pN and the scan rate was 0.3 Hz [6].

2.5. Measurement of mechanical properties by AFM

The elasticity of cells was also detected at contact mode. Measurements were conducted above the nucleus region of the cell to avoid the influence of the underlying substrate. 100 Force-distance curves were obtained from 10 different cells in each group at the ramp rate of 0.5 Hz. Force curves were obtained at the same loading rate and were analyzed by Matlab 7.6.0 [6]. The Hertz model was used to compute the Young’s modulus according to following formula. (F: loading force, υ: Poisson ratio, b: indentation, E: Young’s modulus, R: radius of the curvature of the AFM tip.)

\[ F_{sphere} = \frac{4ER^{1/2}S^{3/2}}{3(1-\nu^2)} \]  
\[ F_{cone} = \frac{2E\delta^2 \tan \theta}{\pi(1-\nu^2)} \]

2.6. Animals

SPF degree Wistar rats (male, 180–200 g, 6–7 weeks old) were provided by Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All the rats were fed in standard rat chows with food and water ad libitum and kept in a temperature-controlled (20–22 °C) animal room with an alternating cycle of 12 h light and dark. All experimental procedures were carried out according to the Guideline for Animal Experimentation of Shenyang Pharmaceutical University, and the protocol was reviewed and approved by the Animal Ethics Committee of the institute.

2.7. Model establishment

Rats were randomly divided into three groups (n = 6) as follows: control, BLM and SalB. Bleomycin sulfate (0.2 ml, 5 mg/kg, diluted in saline solution, Japanese Medicine Co., Ltd, Japan) or equal volumes of saline as control were slowly instilled intratracheally into rats [17]. Rats in the BLM + SalB group were injected intravenously with SalB 20 mg/kg/d from 3 day before BLM instillation to 3 day after bleomycin instillation. All the rats were euthanized on the 28th day after BLM instillation. Lung tissues were either fixed in 4% polymethylaldehyde for histochemical and immunohistochemical analysis or stored in liquid nitrogen for further analysis.

2.8. Histological examination of lung tissues

The lungs fixed in 4% polymethylaldehyde for 24 h were dehydrated in ethyl alcohol, and then embedded in paraffin. 5-μm-thick
sections were used for Hematoxylin and Eosin (H&E) staining to evaluate the severity of lung inflammation and Masson’s trichrome staining to assess the degree of fibrosis [17]. Microscopic images were acquired with the 10 x and 20 x objectives by Olympus fluorescence microscope.

2.9. Immunofluorescence staining of lung tissues

5-μm-thick sections was treated by 0.3% H2O2 for 30 min to quench the endogenous peroxide activity, then incubated with 1% BSA and 0.1% Tween-20 in PBS at 37 °C for 1 h to eliminate nonspecific binding. Following blocking, the sections were rinsed and incubated overnight at 4 °C with the rabbit polyclonal IgG primary antibodies for α-SMA and then incubated with FITC-labeled goat anti-rabbit IgG antibody and Hoechst 33258 at room temperature for 45 min [17]. Fluorescent images were taken by Olympus fluorescence microscope.

2.10. Statistical analysis

All experiments were performed at least in triplicate. The difference between multiple groups was assessed by one way analysis of variance (ANOVA) followed by the Scheffe’s multiple range tests. Central tendency of data were presented as box-plots. Principal Component Analysis (PCA) was performed to select the leading indexes from all biophysical properties. The weight of each biophysical property was represented as initial eigenvalue % of variance. All statistical analysis was performed utilizing SPSS 19.0 and P < 0.05 was considered to be statistically significant in all cases.

3. Results

3.1. TGF-β1-induced fibrogenesis in A549 was confirmed by conventional assays

Morphological changes of cells in different group were observed first by general optical microscopy. Phase contrast images in Fig. 1A showed that 5 ng/ml TGF-β1 induced prominent morphological changes in A549 cells from a cobblestone appearance to a spindle-shaped appearance.

In this study, E-cadherin, α-SMA and vimentin proteins were employed as protein markers to evaluate fibrogenesis [5]. Cell immunofluorescence staining assay showed that the expression of E-cadherin protein was obviously inhibited in TGF-β1-stimulated A549 cells (Fig. 1B), while the levels of α-SMA and vimentin proteins displayed an opposite trend (Fig. 1C and D). We further examined the expression of these proteins by Western blotting analysis. As shown in Fig. 1E and F, TGF-β1 stimulation decreased the expression of E-cadherin but increased the levels of α-SMA and vimentin proteins. These results demonstrated that TGF-β1 induced fibrogenesis in A549 in vitro.

3.2. Dramatic changes of biophysical properties in A549 cells were observed during fibrogenesis

Utilizing AFM, A549 cells were imaged to evaluate the changes of biophysical properties after TGF-β1 stimulation. The whole cell imaging with a scan size of 70 μm showed that A549 cells were remarkably elongated from 32.86 ± 1.67 μm to 48.35 ± 3.38 μm (Fig. 2A-b and A-f). However, the height of these cells did not changed significantly (Fig. 2A-a and A-e). 3D imaging figures of AFM were consistent with the above results (Fig. 2A-d and A-h). The local cell imaging (10 μm of scan size) revealed nano-structure and roughness of cellular surface. As shown in Fig. 2A-c and A-g, the ruffle structures were clearly observed on the surface of TGF-β1-stimulated cells, and Ra and Rq values (the markers of cell roughness) were increased from 76.04 ± 7.01 nm and 93.01 ± 8.10 nm to 164.44 ± 9.13 nm and 199.67 ± 16.28 nm, respectively. We also investigated the alteration in cellular stiffness during fibrogenesis. After 48 h of TGF-β1 stimulation, Young’s modulus of TGF-β1-stimulated A549 cells were dramatically increased from 2.15 ± 0.26 kpa to 10.45 ± 0.78 kpa. All results above indicated that the formation of fibrosis induced by TGF-β1 in A549 cells was accompanied with obvious changes of biophysical properties.

Fig. 1. Morphological and immunological assay for fibrogenesis induced by TGF-β1 in A549 cells. A549 cells pretreated with or without 3 mM NAC were stimulated by 5 ng/ml TGF-β1 for 48 h, and fibrogenesis was confirmed by phase contrast images (A) and detection of marker proteins. (B), (C) and (D) are the results of immunofluorescence staining for E-cadherin, α-SMA and vimentin proteins, respectively. Scale bars: 20 μm, magnification: 40 x. (E): Western blots for expression of E-cadherin, α-SMA, vimentin and GAPDH proteins. (F): Band densitometry analysis of protein expression. Results are presented as mean ± SD, n = 3. *p < 0.05, **p < 0.01 vs. the control; ***p < 0.01 vs. TGF-β1 group.
Fig. 2. Characterization of the biophysical properties in TGF-β1-stimulated A549 cells by AFM. A549 cells pretreated with or without 3 mM NAC were stimulated by 5 ng/ml TGF-β1 for 48 h, and cells were detected by AFM to evaluate the biophysical properties. (A): Ultrastructural characterization. a), e), i) and b), f), j) are the height images and the deflection images of 70 × 70 μm, respectively; c), g) and k) are the deflection images of 10 × 10 μm; d), h) and i) are 3D images of cells. (B): Typical force curves (the upper line) and the histogram of Young’s modulus (the lower line). (C): Box-plots of the biophysical properties. The values of Length, Height, Ra, Rq and stiffness were displayed as box-plots. The inter-quartile range represents the central tendency of data. *P < 0.05, **P < 0.01 vs. the control; #P < 0.05, ##P < 0.01 vs. TGF-β1 group.
including cellular length, roughness as well as stiffness.

3.3. The anti-fibrotic activity of NAC was evaluated by both immunological markers and cyto-biophysical properties in A549 cells

Phase contrast images in Fig. 1A showed that 3 mM of NAC suppressed the alteration of cellular shape induced by TGF-β1. And to a certain extent, the changes of immunological markers during fibrogenesis including E-cadherin, α-SMA as well as vimentin were reversed (Fig. 1B–E). Meanwhile, the changes of biophysical parameters in TGF-β1-stimulated A549 cells were also ameliorated by NAC treatment. The values of length, Ra and Rq were reduced to 37.44 ± 1.82 μm, 120.11 ± 8.37 nm and 134.67 ± 8.44 nm from 48.35 ± 3.38 μm, 164.44 ± 9.13 nm and 199.67 ± 16.28 nm, respectively. Remarkably, the stiffness index showed a broader degree of shift, as demonstrated by significant reduction of the Young’s modulus from 10.45 ± 0.78 kpa to 3.54 ± 0.55 kpa after NAC treatment. These results suggested that biophysical properties could be employed as markers to evaluate the anti-fibrotic activity of NAC in A549 cells.

PCA was performed to compare the weight of each biophysical factor using data of the control and TGF-β1 groups, and each weight was evaluated by calculating initial eigenvalue% of variance. Correspondingly, the initial eigenvalue % of cellular length, height, Ra, Rq and stiffness were 0.594%, 0.440%, 1.277%, 16.404% and 81.285%, respectively. It suggested that the stiffness parameter should be a first principal factor contributing the most to the variance among all biophysical parameters.

3.4. SalB protected TGF-β1-stimulated A549 cells against stiffening

As a potential anti-pulmonary fibrosis drug, the anti-fibrotic activity of SalB was evaluated by this new method first. A549 cells pretreated with or without 40 μM SalB were stimulated by 5 ng/ml TGF-β1 for 48 h. Then AFM was utilized to detect Young’s modulus of cells in different groups. The result showed that SalB treatment significantly reduced Young’s modulus of TGF-β1-stimulated A549 cells from 7.87 ± 1.40 kpa to 3.70 ± 1.16 kpa, which suggested that SalB could protect A549 cells against stiffening during fibrogenesis (Fig. 3A, P < 0.05). SalB therefore was supposed to be anti-fibrotic.

3.5. SalB ameliorated bleomycin-induced pulmonary fibrosis in preventive mode

BLM-induced pulmonary fibrosis was performed on rats to evaluate the anti-fibrotic activity of SalB. Fig. 3B illustrated the histopathological changes in lungs detected by H&E staining and Masson’s trichrome staining. Lungs in control group showed normal alveolar spaces and normal thickening of alveolar septa. The BLM treatment led to significantly abnormal morphologies including thickening of the alveolar/bronchial walls, collapse of alveolar spaces and interstitial infiltration by inflammatory cells. However, rats treated with both BLM and SalB showed remarkable suppression of the BLM-induced inflammatory cellular infiltration as evidenced by the decrease in the cellular infiltrates and thinned alveolar septa (Fig. 3B upper line). Besides, SalB treatments strongly inhibited the extent and intensity of collagen compared to
BLM group of rats which displayed a remarkable increase in lung collagen deposition (Fig. 3B middle line). Immunofluorescence staining of lung sections illustrated in Fig. 3B lower line showed a decreased amount of α-SMA expression in the lung tissues after SalB treatment compared with rats in BLM group. Western blotting demonstrated a similar result in Fig. 3C. All above suggested that SalB treatments provided protection against BLM-induced pulmonary fibrosis which revealed the anti-pulmonary fibrotic activity of SalB.

4. Discussion

IPF is a particularly distressing medical condition partially due to lack of effective drugs. The aim of this study was to develop a new method for rapid screening of anti-fibrotic drugs.

The cell cytoskeleton is responsible for the determination of both cell shape and mechanical properties [11,13]. Every cell line has a specific shape and size which hold a specialized function. If cells cannot maintain their inherent shape, their functions are compromised. Therefore, rearrangement of cytoskeletal structure can probably change cell behavior and phenotype. In our investigations, TGF-β1 stimulation of A549 cells induced dramatic changes in cytoskeleton organization. As the down-regulation of E-cadherin during fibrogenesis, rearrangement of cytoskeleton occurred with loss of cytokeratin expression and acquisition of α-SMA and vimentin [11,18], which was revealed by immunofluorescence analysis. This rearrangement gives cells a centripetal striction which stretches cells in lengthways [18], and the increasing vimentin stress fibers assembled parallel to the long axes of cell bodies [19], converting cobblestone-like epithelial cells into a spindle-like appearance emphasized by our quantitative analysis of cellular morphology. Since the cellular intermediate filaments network altered from a cytokeratin-rich mode to a vimentin-rich mode [20,21], vimentin particles assembled into vimentin filaments and moved towards the cellular surface, which mediated the formation of the ruffles and increased the cellular roughness [18,22]. The synthesis of ECM induced by TGF-β1 also contributed to the cellular roughness. Meanwhile, vimentin filaments filling the entire cytoplasm during fibrogenesis stiffened cells. These reports suggested a close relationship between fibrogenesis and cellular biophysical properties, such as cellular shape, surficial roughness and stiffness.

Our research observed that TGF-β1-stimulated A549 cells became remarkably longer, rougher and stiffer than the control during fibrogenesis. The effect of NAC on ameliorating fibrogenesis in TGF-β1-stimulated A549 cells could be verified by not only protein markers but also cellular biophysical properties. Correspondingly, we supposed that cellular biophysical properties, especially cellular stiffness, could be used as markers for anti-fibrotic drugs screening. The protection of SalB for TGF-β1-stimulated A549 cells against Stiffening suggested SalB might be strongly anti-fibrotic and its anti-fibrotic activity was confirmed by BLM-induced pulmonary fibrosis model. Using AFM, anti-fibrotic activity of drugs could be evaluated in vitro through a quantitative analysis of cellular biophysical properties in only minutes rather than hours needed for biochemical assays. Therefore, measurement of cellular biophysical properties was recommended to be a rapid method to screen anti-fibrotic drugs.

SalB is one of the major water-soluble compounds of Salvia miltiorrhiza (Danshen), which was one of the most popular Chinese herbs listed in the Chinese Pharmacopoeia and has been used for the treatment of ischemic cardiovascular and cerebrovascular diseases clinically in China for centuries [23,24]. As the most bioactive component of salvianolic acid extracted from Danshen, SalB shows higher free radical scavenging activity than vitamin C, and its antioxidant capacity is in agreement with its protective effects against cell injury from oxidative stresses [25]. SalB has been reported to protect against liver fibrosis in animals and patients [26,27], as well as to reduce experimental renal interstitial fibrosis in rats [28]. In this study, the SalB was screened to be strongly anti-pulmonary fibrotic which might also be due to its antioxidant capacity. The follow-up research demonstrated the protection of SalB against oxidative stresses not only in TGF-β1-simulated A549 and MRC5 cells, but also in BLM-induced pulmonary fibrosis model (the data is not shown). The further mechanism is still under research. Taken together, we found that pulmonary fibrogenesis was accompanied with alterations of cellular biophysical properties, such as cellular shape, surficial roughness and stiffness. SalB was screened to be strongly anti-fibrotic by cellular stiffness measurement and its anti-fibrotic activity was demonstrated by BLM-induced pulmonary fibrosis model. Accordingly, measurement of cellular biophysical properties could be applied as a rapid method for anti-fibrotic drugs screening. Future investigation will focus on the factors influencing cellular biophysical properties to get better understanding in pathogenesis of pulmonary fibrosis and develop new strategy for IPF prevention.

Conflict of interest

There is no conflict of interest.

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