Research Article

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Serum containing drugs of Gua Lou Xie Bai decoction (GLXB-D) can inhibit TGF- β 1-Induced Epithelial to Mesenchymal Transition (EMT) in A549 Cells

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Abstract: The present study explores the mechanism of resistance to pulmonary fibrosis by observing the possible effects of serum containing drugs prepared from Gua Lou Xie Bai decoction (GLXB-D) on transforming growth factor beta 1 (TGF- β 1) induced Epithelial-mesenchymal transition (EMT) of A549 human alveolar epithelial cells. The inhibition rate was observed with the help of thiazolyl blue tetrazolium bromide (MTT) in 24 h and 48 h treated cells. Inverted microscope and transmission electron microscope (TEM) were used to study the changes in the morphology and ultrastructure of the cells. The expressions of E-cadherin and Vimentin were comparatively analyzed by Western blotting, while the expressions of Collagen I and III were analyzed by ELISA. The data obtained indicated that the expression of epithelial marker E-cadherin was decreased, while the expressions of EMT markers such as Vimentin and Collagen I and III were increased in 24 h after TGF- β 1 induction. However, the serum containing drugs of GLXB-D were found to inhibit the TGF- β 1 induced proliferation of cells, increase the expression of E-cadherin and decrease the expression of Vimentin, collagen I and III. In conclusion, the serum containing drugs of GLXB-D effectively reduced pulmonary fibrosis, mainly via the reversal of EMT induction by TGF- β 1. Thus, it can be considered as a potential candidate for the development of better treatment methods for pulmonary fibrosis.

Keywords: pulmonary fibrosis; TGF- β 1; EMT; GLXB-D.

1 Introduction

Pulmonary fibrosis (PF) is a chronic lung disease which is characterized by progressive interstitial fibrosis and progressive dyspnea. The severity of the disease is further enhanced due to its poor prognosis and high rates of morbidity and mortality [1]. Furthermore, the exact etiology and pathogenic mechanisms of the disease are yet to be fully understood. Referring to the ancient literature based on traditional Chinese medicine, many modern scholars have ascribed pulmonary fibrosis as “lung impotent”, “lung paralysis”, “gasp syndrome” and “collateral diseases” [2,3]. The rates of incidence of pulmonary fibrosis in various regions across the world are increasing year by year, especially due to the increasing levels of environmental pollution. It is, therefore, imperative to get further insights into the process of pathogenesis of the disease and develop more effective prevention and treatment methods. Among the various pathogenic factors of PF, epithelial-mesenchymal transition (EMT), which plays a crucial role in the manifestation of pulmonary fibrosis, has attracted a lot of attention [4]. More specifically, the mechanism underlying TGF- β 1-induced EMT has become the hotspot of pulmonary fibrosis research. TGF- β 1 is a multifunctional cytokine that regulates tissue morphogenesis and differentiation. It also controls various other processes including cell proliferation, differentiation, apoptosis, and extracellular matrix production. TGF- β 1 has been implicated as a “master switch” in the induction of fibrosis in many tissues, including the lungs [5]. During the process of EMT, E-cadherin, the epithelial markers, lose their polarity and decrease in expression; while, the mesenchymal markers, namely vimentin, fibronectin, collagen I and III increase in expression and the cytoskeleton gets reorganized to spindle form [6]. E-cadherin is a calcium-dependent transmembrane glycoprotein which is expressed in most epithelial cells. It is responsible for cell polarity and tissue structure determination. Vimentin is an intermediate

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filament protein that is ubiquitously expressed in normal mesenchymal cells. It is responsible for maintaining cellular architecture and tissue integrity. It also plays crucial roles in tumorigenesis, EMT and metastatic spread of cancer [7].

Gua Lou and Xie Bai, are the two most recommended Chinese herbs for relieving chest stuffiness. As per the “Synopsis of Golden Chamber,” they can be used for the treatment of “obstruction of qi in the chest” and “lung paralysis”. They are considered the most classic traditional Chinese medicines that can function as expectorants, for removing obstructions inside lungs. Our previous research studies were mostly focused on the possible “in vivo” intervention effects of GLXB-D on pulmonary fibrosis. The results obtained from these studies indicated that GLXB-D can significantly reduce rat pulmonary fibrosis [8-11], especially due to its ability to inhibit TGF- β 1 induced EMT in A549 cells. However, the exact mechanism of action of these traditional medicines remains unclear [12]. The present study was designed to investigate the effects of serum containing drugs of GLXB-D on “in vitro” TGF- β 1 induced EMT of A549 cells, and further expound the anti-fibrosis mechanism of GLXB-D.

2 Methods

2.1 Drugs and reagents

Recombinant human TGF- β 1 was purchased from PEPROTECH Company (Rocky Hill, USA). Gua Lou (160201) and Xie Bai (20150801) were obtained from Third affiliated hospital, Henan University of Traditional Chinese Medicine. Other chemicals and reagents used in the study include: BCA Protein Assay Kit (Beyotime Biotechnology Company, Shanghai, China), rabbit-anti-E-cadherin primary antibodies (Proteintech Group, Inc. Wuhan, China), rabbit-anti-Vimentin, HRP-conjugated goat anti-rabbit IgG secondary antibody (Boster Biotechnology Company, Wuhan, China), Human Collagen I (AK0016JUL09014) and III (AK0016JUL09013) ELISA Kit (Elabsience Biotechnology Co., Ltd). Osmic acid, plumbum and uranium were obtained from Ted Pella Inc, CA, USA.

2.2 Instruments

Thermo Scientific Series CO₂ incubator (DYCZ-40, Thermo, USA); iMark microplate reader (Thermo, MA, USA); Ultra-

thin slicing machine (LEICA, Germany) and Transmission electron microscopy (JEM-1400, JEOL, Japan).

2.3 Cell line and culture conditions

The A549 human alveolar epithelial cell line was provided by KeyGen Biotechnology Co., Ltd (Nanjing, China). The cell line was grown in 1640 medium (Beijing Solarbio science&technology Co. Ltd., China) supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA). The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

2.4 Experimental animals

New Zealand male rabbits, each weighing 2.5–3.0 kg were purchased from Jinfeng laboratory animal Co. Ltd (SCXK20140006, Jinan, China). All animals were allowed free access to diet and water in a room with temperature (16–26°C), relative humidity (40%–70%) and 12:12 h light dark cycle. This study has been approved by the ethical committee of the Animal Experimental Center of Henan University of Chinese Medicine.

2.5 GLXB-D preparation

Gua Lou (*Trichosanthes kirilowii* (Maxim)) and Xie Bai (*Allium macrostemon* (Bge.)) were mixed in a ratio of 2:1, soaked 30 min and decocted two times, 25 min and 15 min. The soup thus formed was filtrated and decocted for 1 h. The final solution obtained was concentrated to 1.73 g/mL and kept in 4°C.

2.6 Drug serum preparation

The New Zealand male rabbits used in the study were randomly divided into two groups, the blank control group and GLXB-D group (n = 6 per group), weight was not different between groups. In the first seven days, all rabbits were given pellet feed and adequate amounts of water, after which they were fasted for 10–12 h just before the treatment. The GLXB-D group rabbits were intragastrically administered with GLXB-D formulation, while, the control group received the same volume of saline. Each rabbit was treated with 30 mL clinical equivalent dose for 1 week, twice daily. After the last treatment for 2 h, all rabbits were narcotized with the help of administration of 10% chloral hydrate saline solution and collected aortaventralis blood

under aseptic conditions. The blood was standed for 2 h at room temperature and centrifuged at 3000r/min for 15 min. The supernatant drug serum and the blank control serum were collected and inactivated by placing them in a water bath at 56°C for 30 min and then filtered through a microporous membrane (0.22 μ m).

2.7 Cells culture and experimental groups

A549 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), in an incubator with 5% CO₂ at 37°C. The A549 cells (5×10⁴ cells/ mL) were first seeded on culture bottles and were randomly divided into 5 groups, including control group, TGF- β 1 induced group and GLXB-D groups with variable concentrations (5 ng/mL TGF- β 1 + 5%, 10% and 15% GLXB-D drug serum). To the control group was added 10% rabbit blank control serum, and the TGF- β 1-induced group had added 5 ng/mL TGF- β 1+10% rabbit blank control group serum.

2.8 Morphological observation and quantification analysis

The morphological changes in A549 cells treated with GLXB-D drug serum for 24 h and the control group were observed directly under an inverted microscope. Furthermore, the roundness of cells was comparatively analyzed with the help of Image Pro Plus 6.0 software [13].

2.9 Cell growth inhibition assay

A549 cells in the logarithmic phase were seeded on 96-well plates with 8×10³ cells/well in 200 μ L medium. The wells were divided into five groups of four each based on the type of treatments as specified above. The control 1640 medium was matched with 5 ng/mL TGF- β 1 and different concentrations of GLXB-D drug serum (5%, 10%, 15%) in a final volume of 200 μ L. After incubation for 24 h and 48 h, 20 μ L MTT (4.8 mmol/L) was added to each well. The mixture medium was sucked out after 4 h, and DMSO (150 μ L/well) was added. The optical density values (OD value) of the wells were detected at 490 nm. The inhibitory rate of the treated samples was calculated with the following equation:

$$\text{Inhibitory rate (\%)} = (1 - \text{OD}_{\text{treatment}} / \text{OD}_{\text{control}}) \times 100\%.$$

2.10 Western blot and hybridization of E-cadherin and Vimentin

All cells treated for 24 h were collected and total protein was extracted from them with cell lysis buffer. The protein concentration was calculated with the help of the BCA protein concentration determination kit. Equal quantities of the isolated protein (40 μ g) from each group were electrophoresed on 8% separating gel and 5% stacking gel. The separated proteins were then transferred to a PVDF membrane which was blocked by incubating it with 5% skimmed milk for 2 h at room temperature along with shaking. This was followed by washing, after which the membrane was incubated overnight at 4°C in the presence of primary antibody against E-cadherin and Vimentin. The antibodies were used in the following dilutions: E-cadherin- 1:2000; Vimentin- 1:500; GAPDH- 1:1000. This step was followed by washing, after which the membrane was incubated with secondary antibody: HRP-conjugated goat anti-rabbit IgG (1:50000) at 37°C for 2 h on a shaker. The bands thus formed due to hybridization were visualized by chemiluminescence (ECL) and the grey value was analyzed with the help of BandScan software. Target proteins levels were normalized by GAPDH.

2.11 Collagen I and III measurements by ELISA

Collagen I and III in cell culture supernatants of the cultures treated for 24 h and 48 h were quantified using the Human Collagen I and III ELISA Kit. The procedure followed was according to the manufacturer's protocol.

2.12 Ultrastructure analysis of A549 cells

The ultrastructure of A549 cells treated with the fresh 1640 medium matching with 5 ng/mL TGF- β 1 and different concentrations of GLXB-D drug serum (5%, 10%, 15%) for 24 h was analyzed with the help of a Transmission Electron Microscope (TEM). The cells were first fixed with cold 4% glutaraldehyde solution for 4 h. They were then scraped and centrifuged; and fixed with new 4% glutaraldehyde again. The fixed samples were then rinsed with PBS and fixed with 1% osmic acid for 90 min. The fixed cells were then dehydrated with acetone by using it in order of low to high concentration. The cells were then polymerized with 812 epoxy resin, sliced and double dyed by lead and uranium. TEM was used to assess the changes in the ultrastructure of these processed A549 cells.

Figure 1: Changes in the morphology and roundness values of A549 cells stimulated by different concentrations of GLXB-D drug serum and TGF-β1 for 24 h (×200). (A): control group; (B): 5ng/mL TGF-β1 induced group; (C): 5% GLXB-D drug serum group; (D): 10% GLXB-D drug serum group; (E): 15% GLXB-D drug serum group. Comparison with control group: * $P < 0.05$; comparison with the TGF-β1 induced group: # $P < 0.05$.

2.13 Statistical analyses

Statistical analysis was done with the SPSS 17.0 software. Data were described as mean $\pm$ SD and analyzed by one-way ANOVA. Differences were considered to be significant if $P < 0.05$.

3 Results

3.1 Morphological observation and quantification analysis

As observed under an inverted microscope, the morphology of A549 cells in control group was circular, polygonal or cobble-shaped. The space between cells was found to be close (Figure 1A). It was also observed that when A549 cells were induced by TGF-β1, the morphology of the cells changed and they became long and spindle-shaped. Furthermore, some cells were found to produce pseudopodia which increased the intercellular distance (Figure 1B). Compared with the control group, the

roundness of cells was markedly decreased ($P < 0.05$). However, in GLXB-D drug serum groups, the morphology of cells was shorter and rounder than the TGF-β1 induced group, even the cobble-shape of the cells were found to be restored. Compared with the TGF-β1-induced group, the roundness of cells was markedly increased ($P < 0.05$) (Figure 1C, D, E).

3.2 The effects of different concentrations of GLXB-D drug serum and TGF-β1 on A549 cells proliferation

To verify the effect of GLXB-D drug serum on cell proliferation, A549 cells' viability was assessed with the help of MTT assay at 24 h and 48 h of treatment. The OD value of cells induced by TGF-β1 showed no significant differences when compared with control group ($P > 0.05$). However, statistically significant differences were observed in the GLXB-D drug serum groups (5%, 10%, 15%) ($P < 0.05$). It was evident that extension of time and the increase of concentration of GLXB-D caused increase in the cell proliferation inhibition rate in a dose- and time-

Table 1: Inhibitory effects of different concentrations of GLXB-D drug serum on A549 induced by TGF-β1 n=3, x±s .

Groups	24h		48h	
	OD value	Inhibition ratio (%)	OD value	Inhibition ratio (%)
control group	0.609±0.023	-	1.109±0.046	-
5 ng/mL TGF-β1 induced group	0.617±0.015	-	1.046±0.087	-
5% GLXB-D drug serum	0.547±0.041*	11.3%	0.926±0.023*	16.5%
10% GLXB-D drug serum	0.476±0.068*	21.9%	0.843±0.044*	24.0%
15% GLXB-D drug serum	0.437±0.025*	28.3%	0.713±0.034*	35.7%

Note: Comparison with control group: * $P<0.05$

dependent manner (Table 1). The proliferation inhibition rate of 35.7% in 15% GLXB-D drug serum group was the highest observed in the study.

3.3 Expression of Epithelial cell marker E-cadherin and Interstitial cell marker Vimentin

It was observed that A549 cells induced by TGF-β1 had significantly lower ($P<0.05$) levels of expression of E-cadherin, an epithelial cell marker. Conversely, the expression of Vimentin, an interstitial cell marker was considerably increased in the TGF-β1 induced A549 cells. However, when the cells were treated with GLXB-D (24h) the expression of E-cadherin was increased ($P < 0.05$) and the expression of Vimentin was reduced ($P < 0.05$) (Figure 2A, B).

3.4 The expressions of human Collagen I and III

The concentration of collagen I and III were quantified in culture supernatants of cells that were treated for 24 h to 48 h. We found that the expression of Collagen I in the TGF-β1 induced group was increased as compared to the control group ($P < 0.05$) (Table 2). Furthermore, when compared with the TGF-β1 induced group, the expressions of Collagen I and III in the GLXB-D drug serum groups were all decreased ($P < 0.05$). As shown in Table 2 and Table 3, the expressions of Collagen I and III decreased in a dose-dependent manner (5%-15% GLXB-D drug serum).

3.5 Changes in Cell ultrastructure

After A549 cells were induced by TGF-β1 for 24h, the ultrastructure changes in cells were observed directly with TEM. Many lamellar bodies, mitochondria and

Figure 2: Western blot results showing the expression levels of A549 cells treated with different concentrations of GLXB-D drug serum and TGF-β1 for 24 h . (A): The protein expression straps of E-cadherin and Vimentin; (B): The grey value ratio of expressions of E-cadherin and Vimentin. (1) control group; (2) 5ng/mL TGF-β1 induced group; (3) 5% GLXB-D drug serum group; (4) 10% GLXB-D drug serum group; (5) 15% GLXB-D drug serum group.

rough endoplasmic reticulum were found in cytoplasm of cells of the control group while, many microvilli were distributed on the cell surface (Figure 3A). After the cells were induced by TGF-β1 for 24 h, TEM images revealed vacuolar degeneration of lamellar bodies in cytoplasm. Furthermore, it was also observed that the number of lamellar bodies was significantly reduced (Figure 3B). These cytological changes indicate that cells induced by

Figure 3: Representative transmission electron microscopic pictures of the ultrastructure of cells (Lead and uranium double staining, ×30000). A: control group; B: 5ng/mL TGF-β1 induced group; C: 15% GLXB-D drug serum group. Lamellar bodies (→), Microvilli (←), Mitochondria (↓), Rough endoplasmic reticulum (↑).

Table 2: The effects of different concentrations of GLXB-D drug serum on the expression of Collagen I in A549 cells (n=3, x±s).

Group	n	24h	48h
control group	3	9.664±0.091	19.182±0.157
5ng/mL TGF-β1 induced group	3	10.688±0.065*	20.745±0.094*
5% GLXB-D drug serum	3	10.315±0.103*#	18.455±0.146*#
10% GLXB-D drug serum	3	10.032±0.145*#	17.930±0.078*#
15% GLXB-D drug serum	3	9.162±0.126*#	15.274±0.086*#

Note: Comparison with control group: **P*<0.05; Comparison with the TGF-β1 induced group: #*P*<0.05

Table 3: The effects of different concentrations of GLXB-D drug serum on the expression of Collagen III of A549 cells (n=3, x±s).

Group	n	24h	48h
control group	3	0.878±0.012	1.176±0.097
5ng/mL TGF-β1 induced group	3	6.350±0.065*	11.77 2±0.038*
5% GLXB-D drug serum	3	3.957±0.129*#	8.479±0.083*#
10% GLXB-D drug serum	3	3.730±0.037*#	7.015±0.127*#
15% GLXB-D drug serum	3	2.817±0.025*#	4.544±0.129*#

Note: Comparison with control group: **P*<0.05; Comparison with the TGF-β1 induced group: #*P*<0.05.

TGF-β1 for 24h may have undergone EMT. Interestingly, the number and morphology of lamellar bodies in GLXB-D drug serum groups was close to normal (Figure 3C).

4 Discussion

PF is a chronic interstitial pathological condition of the lungs which is characterized by increased proliferation of fibroblasts and accumulation of extracellular matrix (ECM) [1]. The process of EMT has been recognized to play an indispensable role in the process of fibrosis. Conversely, inhibition of EMT may thus help in treating lung fibrosis progression [14]. Though occurrence of EMT in lung tissues have already been demonstrated in several in vivo experiments, meanwhile, the same in type II alveolar epithelial cells have been studied in “in vitro” studies [15]. Furthermore, some studies found that TGF-β, a key fibrogenic cytokine, plays an important role in the process of stimulating pulmonary interstitial matrix deposition [16,17]. In normal mammalian epithelial cells, the TGF-β is a main facilitator of EMT in pulmonary fibrosis in renal tubular epithelial cells, corneal epithelium cells and alveolar epithelial cells [18]. Our findings illuminated that the phenotypic changes of A549 cells induced by TGF-β1 can be clearly observed both under inverted microscope and TEM.

EMT is considered as a complex process of cellular reprogramming in which the cells phenotype changes from epithelial to interstitial during which the motility and invasion ability of cells gets strengthened. It has previously been established that during this process, the special “cell-cell” junctions are gradually weakened, suppressed, shifted and even eliminated. The main molecular changes that were concurrent with the above mentioned cytological modifications decreased in the expression of epithelial markers, such as E-cadherin and Keratin; and increased in the expression of mesenchymal markers, such as N-mucins and Vimentin [19]. In addition, collagen protein is the extracellular matrix protein which can be synthesized

and secreted by epithelial cells and fibroblasts. It is well known that the excessive expression of collagen can cause multiple organ pathological fibrosis. Some research shows that the morphology of A549 cells induced by TGF- β 1 was changed from epithelial form into myofibroblast form due to a decrease in the expression of E-cadherin and an increase in the expression of Vimentin and Collagen I and III [20]. GLXB-D is a preparation consisting of *Trichosanthes kirilowii* (Maxim) and *Allium macrostemon* (Bge.) in a weight ratio of 2:1 [21]. Previously, we have showed that GLXB-D protected rats from pulmonary fibrosis [8-11]. In the present study, the results obtained from MTT assay analysis showed that GLXB-D could inhibit the TGF- β 1 induced proliferation of cells. It was also observed that increase of serum containing drugs concentration causes an increase in the inhibition rate. The ultrastructure changes in cells induced by TGF- β 1 suggest that the specific structures of epithelial cells begin to disappear. Moreover, the number of mitochondria and rough endoplasmic reticulum were increased. Furthermore, the rough endoplasmic reticulum (RER) was observed to expand and fill with floccus which indicates an increase in collagen production. Study of morphology and ultrastructure of cells treated with GLXB-D drug-containing serum groups indicated that the cells became shorter and even got restored to their previous cobble-shaped. Additionally, the lamellar bodies were also restored to their earlier form and number. This indicates that different concentrations of GLXB-D serum containing drug can inhibit the EMT process induced by TGF- β 1.

5 Conclusions

Our study confirmed that GLXB-D can maintain the stability of cell morphology and prevent the transformation of epithelial cells to fibroblast cells. The effects of GLXB-D on cellular proliferation and pulmonary fibrosis may be related to inhibiting the TGF- β 1 signaling pathway by down-regulating expressions of E-cadherin and up-regulating Vimentin and Collagen I and III expression. These findings raise the possibility that GLXB-D is a potential effective drug for the treatment of pulmonary fibrosis.

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