A Study on the Effect of IL-17A on Phenotypic Transformation of Fibroblasts in Bleomycin-Induced Pulmonary Fibrosis in Mice and Its Mechanism

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Abstract: Objective: In this study, lung fibroblasts were cultured and identified in mice lung fiber model with bleomycin. Under the induction of IL-17A, lung fibroblasts were gradually transformed into myofibroblasts in pulmonary fibrosis, and the specific induction effect of IL-17A in pulmonary fibrosis was analyzed, which could provide ideas for the prevention and treatment of clinical pulmonary fibrosis. Methods: To investigate the transcriptional expression of bleomycin-induced fractional pulmonary fibrosis in different pulmonary fibrosis processes. The 14-day mice model was taken as the research object, and the pulmonary fibrosis model was established by induction of myogenesis. After 14 days of modeling, lung tissue was removed, and after centrifugation and repeated adherent treatment, lung fibroblasts could be cultured at the origin. After three generations of culture, the morphological changes of lung fibroblasts could be observed under a microscope. Indirect immunofluorescence was used to establish the expression of vimentin, and IL-17 was used to stimulate primary cultured lung fibroblasts to detect the expression and specific localization of a-SMA in cells. Western blotting was used to stimulate the expression of lung fibroblast protein by IL-17A at different time points. Results: The typical characteristics of primary culture lung fibroblasts were obtained. After purification and culture, lung fibroblasts were obtained in morphology. The morphology of the 3rd and 4th generation cells was relatively uniform, showing long carboxyform. 1-2 nucleoli can be observed by microscope, which have distinct cell boundary and are lined up like fish schools. The results of indirect immunofluorescence showed that the vimentin staining in the third generation cells was positive, and the plasma was dark red. There were collagenous fibrous septa between the cells, which might make them develop into lung fibroblasts. A-SMA immunofluorescence results showed that in the absence of IL-17A induction, A-SMA signal was relatively weak in the lung fibroblasts of the control group and was in the cytoplasm, while after IL-17A induction, A-SMA signal was stronger in the lung fibroblasts of mice and the whole cells presented spindle structure. Western bletting showed that lung fibroblasts were stimulated by IL-17 in the 0h group. Compared with the 1h, 2h, and 4h groups, the expression of A-SMA in lung fibroblasts was significantly increased in the 1h, 2h, and 4h groups. The fibroblasts were very low in the 2h and 4h groups. There was no significant difference in the expression of AS MA signal. Compared with 0h, protein contents of p-IKB-a and p-p65 were higher in lung fibroblasts at 1h, 2h and 4h. Protein expressions of Acti, 1P6, IKB-a and P65 were different in lung fibroblasts, but there was no significant difference. However, there was no significant statistical difference in the expression of these proteins in lung fibroblasts at different times. Conclusion: By differential centrifugation and repeated adhesion, bleomycin-induced lung fibroblasts can be isolated and purified, and more cell production can be obtained. The staining vimentin was strongly positive after identification by indirect immunofluorescence. The stimulation of IL-17A could gradually transform non-fibroblasts into myofibroblasts and play an important role in pulmonary fibrosis. Therefore, through experimental studies, it was found that IL-17A stimulated F-kB signal and then increased the expression of P-IKB-a and P-P65 proteins, and transformed non-phosphorylated proteins into phosphorylated proteins, thus transforming lung fibroblasts into myofibroblasts and playing a role in pulmonary fibrosis.

Keywords*:* IL-17A; Pulmonary Fibrosis; Fibroblasts; Transformation

1. Introduction

Interstitial pulmonary disease is a group of pulmonary diseases with varying degrees of fibrosis and inflammation, and is also a disease of idiopathic pulmonary fibrosis. The clinical manifestations are dyspnea, dry cough and other symptoms, and the imaging manifestations are diffuse interstitial and parenchymal injury. The mortality and morbidity of this disease are high. However, there is no effective treatment for this disease and the cure rate is relatively low. Therefore, the focus of current clinical research is to explore the pathogenesis of pulmonary fibrosis and find effective therapeutic drugs[1].

2. Research materials and methods

2.1 Animal sample sources

In this study, a total of 20 SPF male mice with an average age of 7 weeks and a weight of 20 grams were selected and purchased from an animal experiment center of a university. During the feeding process, the temperature and humidity of the mice were required to be kept at 20℃ and 65% to ensure free water intake.

2.2 Reagents and instruments

The experimental materials used in this experimental study included IL-17A reagent, bleomycin powder, fetal bovine serum or anti-mouse antibodies, trypsin, 3% barbital solution, penicillin, streptomycin, recombinant mice, RIPA, BSA, PMSF, goat anti-rabbit IGg-HRP, and P-mouth DF membrane. The instruments used in the research include animal laboratory instruments, such as scalpel, gauze, transcendence table, alcohol lamp, scales, centrifuge, water bath, carbon dioxide incubator, etc.

2.3 Research Methods

How to select research objects? For the previous studies on the expression of IL-17RAMRNA in the lung tissues of PF mice induced by bleomycin in different stages of PF formation, the model mice on the 14th day could be selected as the research objects. The method and process of making mice PF model induced by bleomycin were consistent with previous experiments.

When drawing concrete materials, the culture bottle was wrapped with gelatin, and after one night culture, the gelatin was taken out. Meanwhile, 2 ml of the culture solution was poured into the culture bottle, and then place the culture bottle on super worktable and take out the lung tissue of the mice by dissecting the mice. In dealing with the specific operation, it can adopt routine cervical dislocation method to avoid death. Then it shall use ethanol for surface sterilization. The skin on the chest of mice could be disinfected with iodine first and then cleaned with alcohol. The left hand can pinch the neck and back of the mouse to expose the skin of the chest of the mouse, while the right hand can cut the skin of the mouse. After using alcohol disinfection, it can cut the ribs along the lower end of the sternum of the mice and cut the sternum transversally in the middle of the incision of the mice to take out the lung tissue, and then put the tissue in the double antibody culture flask. The primary lung tissue of mice was cultured, and the lung tissue was cut into multiple lung lobes through PPS buffer solution, and the excess blood vessels and bronchus at the hilum of the mice were subtracted, and the pleura was removed[2]. Then it shall transfer the lung tissue into the culture bottle containing penicillin. After repeated PPS cleaning, lung tissue fibers can be cut to a cubic millimeter size tissue block. And then double anti PBS suspension was used. After the tissue naturally sank, it shall abandon supernatant for three times repeatedly, until the supernatant keep clear. Then use 0.1% of the pancreatic enzyme to clean the tissue and add 1 ml of 0.1% trypsin for each mouse. After being digested at 37℃ for 20 minutes, most of the tissues can be digested into suspension. After adding the same amount of digestive solution, 10% DMEM containing FBS can be added to stop digestion after a period of time. After being beaten evenly, the cells are filtered to obtain cellular blood centrifugation. And then add 35ml of DMEM to the sediment. After 5 minutes' centrifugation, collect the supernatant and sediment respectively. Among them, LF is mainly distributed in the supernatant, and a few are epithelial cells. After centrifugation, the supernatant was discarded. Then use an incubator containing 5% carbon dioxide to culture the tissue at 37℃ for 40 minutes, and remove the unattached cells in time, among which the attached cells were mainly LF. 0.5ml of culture solution was replaced for overnight culture, and 2ml of culture solution was added on the second day for further

culture. The sediments are epithelial cells, and a few contains LF. The sediments can be removed by centrifugation for 5 minutes, and then add it into 10%FBS of DMEM culture solution. After resuspend the sediments, transfer it into a 25ml culture bottle. Then use the same method to continue culture, the most unattached cells are epithelial cells. After absorption and discarding, abandon the supernatant by centrifugation at low temperatures and resuspend the sediments in the culture bottle to remove unattached cells by repeatedly beating two or three times, among which LF are the attached cells. After adding culture solution into culture bottle for overnight culture, the structure and growth of the cells can be observed by telescope. At the same time, the survival of cells was determined by staining. After three days of isolation and purification, the cells were close to fusion state, and then they were connected into a network structure. When the cells reached 90% fusion, the subculture could be carried out according to a ratio of 1:2.

2.4 Observation Indicators

First, identify the lung fibroblasts of the mice. The cell slides of primary culture and third-generation culture can be selected and fixed with 4% paraformaldehyde for indirect fluorescence immunochemical staining of vimentin. Second, observe the structural changes of lung fibroblasts, and then observe LF form after cell culture with an inverted microscope. Third, after IL-17A stimulation, observe the expression of A-SMA signal and its specific localization. Fourth, observe phenotypic changes under the IL-17A simulation at different time points and then analyze transcriptional indexes and expression of transcriptional suppressor genes and activation of target genes[3].

3. Research Results

First, observe the morphology of lung fibroblasts(see Figure 1). When observing the structure of LF in vitro culture through inverted microscope, it can be found that in 30 minutes' meta-generation culture, the cells can grow adhere to the wall, but part of the hematopoietic cells may suspend in the culture solution. After 24 hours of culture to obtain new culture solution, remove suspending cells to improve the growth number of the attached cells, which are round or polygonal in shape. Such cells are mainly transparent and have relatively big cell nucleus and 2-3 nucleoli, which were in line with the structural morphological characteristics of Fb. After further culture for 36~48 hours, the cells entered the logarithmic phase, and the FB cells increased and were arranged radially or helically. After 72 hours of culture, most of the cells were found to be spindle-shaped, accompanied by some quasi-circular or circular structures. It can be observed through the microscope that local cells mainly grew in scattered forms. After one week of culture, attached cells showed long fusiform and abundant cytoplasm. 1~2 nucleoli and relatively clear cell boundaries could be observed through the microscope, which arranged like fish schools.

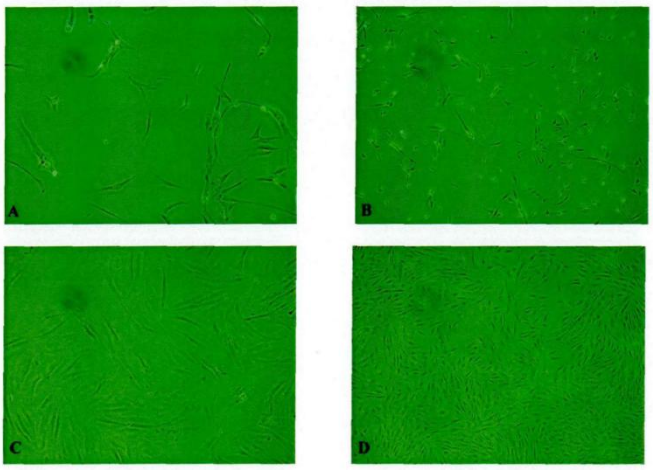

Figure 1. Morphology of lung fibroblasts

Identify lung fibroblasts (see **Figure 2**). After the observation of the vimentin cultured in the third generation by laser confocal microscope, since the protein staining of the vimentin cultured in the third generation showed strong positive with dark red cytoplasm and collagenous fibrous septa between the cells, it could be confirmed as LF.

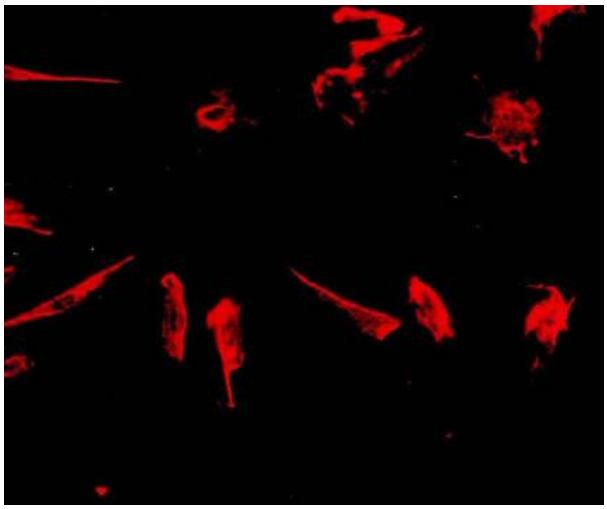

Figure 2. Identification of lung fibroblasts

According to the results of A-SMA immunofluorescence (**see Figure 3**), it was found that after the stimulation of IL-17A, the LF cells in childhood had a strong S-AM fluorescence signal, which was in the plasma envelope. After the stimulation of IL-17A, the mice had a strong A-SMA fluorescence signal, and the whole cells presented a spindle state. After the stimulation of IL-17A, the expression of different proteins in lung fibroblasts could be detected by using Western bloting at different time points, including p65, P-p65, IKB-a, p-IKB-a and ACTI.

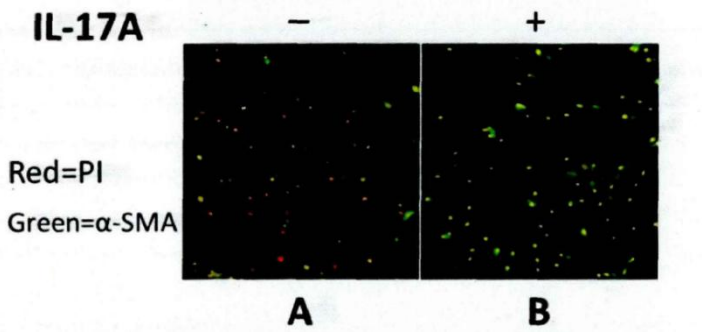

Figure 3. The results of A-SMA immunofluorescence

First, select LF with good growth condition, adjust its density to a certain range and inoculate it in the new solution, and the cells will grow adherently the next day. IL-17A was added to the serum-free culture solution after 24 hours, and then cells were collected after 0, 1, 2, and 4 hours respectively, and the cells were quantified by lysis. Western blotting assay was used to detect the expression of different proteins, including a-SMA, ACTL, p65, P-p65, IkB-a, and P-IkB-a (see **Table 1**). The results showed that the expression of a-SMA in LF of the 0 h group was weak. After 7 hours of IL-17A stimulation, the expression of a-SMA in LF in 1 hour, 2 hours and 4 hours was higher than that in 0h mice group. Compared with that in 1-hour group, the expression of a-SMA in 2h and 4h group was increasing. There was no significant difference in the expression of A-SMA in LF between the 2h and 4h group. In other words, after LF was induced by IL-17A, A-SMAs increased in 1h, and reached the peak in 2h, and then maintained high expression in 4h. The results of optical density and image table scanning showed that the protein expression levels of P-IkB-a and P-p65 in LF cells at 1h, 2h, and 4h after IL-17A stimulation were significantly higher than those at 0h. The protein expression of p-IKB-A and p-p65 was not significantly different between LF in 2h and 4h. After the stimulation of IL-17A, the protein expression of P65 and P-IkB-a in mouse LF was higher at different time points, and there was no significant difference in the expression of the two proteins at different time points. Once again, the expression of P65 and p-IKB-A proteins in mouse LF cells was not affected by IL-17A induction. However, after the stimulation of IL-17A, the phosphorylation of IkB-a and P65 protein reached the peak within 1 hour. The expression was lower in 2 hours group and higher in 4 hours group than that in 0 hour group. In addition, there was no significant change in the expression of Actl protein in LF cells after IL-17A stimulation at different time points, and the

expression of this protein was consistently low[4].

Table 1. Expression of a-SMA, ACtl, p65, P-p65, IkB-A and P-IkB-a proteins at different times

	N	0h	1h	2h	4h
α-SMA	20	0.290±0.007	0.445±0.010*	0.706±0.014*Δ	0.700±0.016*Δ
P-p65	20	0.248±0.009	0.570±0.011*	0.490±0.024*Δ	0.484±0.025*Δ
p65	20	0.944±0.015	0.950±0.016	0.943±0.017	0.942±0.015
P-IκB-α	20	0.368±0.012	0.718±0.013*	0.466±0.020*Δ	0.461±0.020*Δ
IκB-α	20	0.283±0.013	0.286±0.012	0.282±0.012	0.281±0.014
Actl	20	0.265±0.044	0.261±0.018	0.266±0.023	0.275±0.021

4. Discussion and conclusion

In vitro and in vivo studies have shown that GF-β1 plays an important role in the transition from Fb to MFb. According to studies, it can promote the transition of Fb from MFb through smads signal, but the smads signal pathway is not the only one involved in the transition from Fb to MFb. Hashimoto showed that GF-β1 could also promote MFb transformation through JNK. Studies have shown that both TGF-β1 and MAPK signal pathways can participate in the regulation and expression of a-SMA in lung fibroblasts. MAPK signal pathway is composed of ERk and P38MAPK, and other cytokines can utilize this signal pathway and participate in the phenotypic transformation process from Fb to MFb. In addition, the MAPK pathway can also be activated by mechanical tension to promote the regulation of non-tissue fibrosis. IL-17A plays an important role in the formation of PF, which is influenced by a variety of cytokines. Therefore, the following speculation can be proposed: IL-17A can participate in the formation of PF during the transformation from FB to MFB. In this study, the Western bloting experiment showed that IL-17A played an important role in promoting the formation of PF, and its A-SMA indirect immunofluorescence experiment was consistent with previous conclusions. It can be speculated that IL-17A can increase the protein expressions of P-p65 and P-IkB-a in cells through the activation of NF-KB signal pathway, and transform non-phosphorylated proteins into phosphorylated proteins, thus promoting the gradual transformation of fibroblasts into integrated fibroblasts, and finally forming pulmonary fibrosis.

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