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Inhibition of mTOR ameliorates bleomycin-induced pulmonary fibrosis by regulating epithelial-mesenchymal transition

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ABSTRACT

Epithelial-mesenchymal transition (EMT) plays a pivotal role in idiopathic pulmonary fibrosis (IPF). In bleomycin-induced pulmonary fibrosis mice, we observed that inhibition of mTOR (mammalia target of rapamycin) attenuated IPF. Rapamycin suppressed the down-regulation of E-cadherin and up-regulation of fibronectin in bleomycin-induced pulmonary fibrosis mice. In addition, dual immunofluorescence staining for E-cadherin and fibronectin demonstrated that rapamycin pretreatment decreased the proportions of AECs undergoing EMT in bleomycin-induced pulmonary fibrosis, indicating that mTOR inhibition suppressed EMT in *vivo*. In the setting of transforming growth factor (TGF)-β1-induced EMT in AECs, we found that mTOR inhibitor attenuated TGF-β1-induced EMT in AECs. This EMT was characterized by morphology and cell skeleton changes and the expression of EMT phenotype markers. Finally, mTOR blockade decreased S6k and TGF-β1-induced Smad2/3 phosphorylation. Bleomycin induced pulmonary fibrosis and EMT in mice, while mTOR repression inhibited bleomycin-induced pulmonary fibrosis and attenuated EMT *in vivo*. Hence, our study provided evidence of a novel mechanism by which mTOR inhibitor ameliorates pulmonary fibrosis. Suppression of mTOR and EMT may be a target for treatment of pulmonary fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating fibrotic lung disease with a poor prognosis [1]. The mechanism underlying IPF development remains unclear, and there is limited evidence regarding appropriate therapies for this disease. It is widely accepted that alveolar epithelial injury and abnormal wound healing play important roles in its development [2]. Pathologically, IPF is characterized by fibrotic foci, in which myofibroblasts and activated fibroblasts are the main effector cells [3]. These mesenchymal cells develop from epithelial cells via EMT [4].

During EMT, a polarized epithelial phenotype transdifferentiates into an elongated fibroblastoid phenotype. This process is characterized by the loss of epithelial phenotype markers and the acquisition of mesenchymal phenotype markers, as well as cell-cell junction disassembly, and cytoskeletal remodeling. It occurs physiologically during embryonic development and tissue regeneration and pathologically in diseases such as fibrosis and cancer [5–8]. Alveolar epithelial cells (AECs) undergo EMT in response to various stimuli and produce fibroblasts and myofibroblasts [6,7]. Studies have shown that approximately one-third of fibroblasts originate from epithelial cells in the setting of bleomycin-induced pulmonary fibrosis [7]. Therefore, it is therapeutically important to identify interventions that inhibit EMT in pulmonary fibrosis and potentially retard pulmonary fibrosis progression.

Mammalian target of rapamycin complex (mTORC) belongs to PI3K-related kinase family, which consist of two protein subunits, known as mTOR Complex 1 (mTORC1) and 2 (mTORC2) [9]. mTOR exerts multiple functions in cell growth, survival and apoptosis and is involved in multiple diseases, such as cancer, organ fibrosis and aging[10–13]. mTOR pathway is abnormally overactivated in fibrotic diseases, such as lung fibrosis, skin fibrosis, liver fibrosis, cardiac fibrosis and kidney fibrosis [11,12,14–16]. mTOR inhibition appears to exhibit anti-fibrotic and pro-fibrotic function in different animal models of pulmonary fibrosis [17,18]. Besides, the effect of mTOR inhibitor on EMT is also conflict in different types of cells

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[19–22]. In our study, we found that mTOR inhibitor rapamycin attenuated bleomycin-induced pulmonary fibrosis and EMT in FVB mice and TGF-β1-induced EMT in AECs. In addition, rapamycin inhibited the phosphorylation of S6k and smad2/3 in TGF-β1-induced EMT in AECs. Thus, our study indicated that mTOR inhibitor ameliorated IPF by mediating EMT through a smad-dependent mechanism. These suggest a novel explanation for mTOR inhibitor attenuating pulmonary fibrosis.

2. Materials and methods

2.1. Reagents

Bleomycin, recombinant human TGF-β1, and phalloidin were obtained from Invitrogen (Carlsbad, CA, USA). The rapamycin used for animal experiments was obtained from Yunda Biological Technology Company (Beijing, China), and the rapamycin used for cellular experiments was obtained from Sigma Aldrich (Grand Island, NY, USA). Hydroxyproline analysis kit was from Jiancheng Inc. (Nanjing, China). Antibodies against KLF4, E-cadherin, fibronectin, phosphorylated Smad2/3, Smad2/3and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-S6K (Thr389), S6K, mTOR and phosphorylated mTOR were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.2. Bleomycin-induced fibrosis model

Male PVB mice aged 8–11 weeks with an average weight of approximately 25–30 g were intratracheally injected with 5 mg/kg bleomycin or saline on day 0 and then killed on day 21. Each group contained 5–7 mice. Lungs were fixed in formalin, embedded in paraffin and sectioned for further analysis. The lung sections were then subjected to hematoxylin and eosin (H&E) and Masson staining. The Ashcroft scoring system was used to quantify fibrosis severity throughout the entire lung. For immunofluorescence staining, lung tissue samples were embedded in Tissue-Tek OCT compound and snap frozen in liquid nitrogen. All animal care and experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and were approved by the Animal Research Committee of Peking University First Hospital.

2.3. Rapamycin administration

Rapamycin was prepared from a 20 mg/ml stock solution via dissolution in ethanol and dilution to a concentration of 0.8 mg/ml in a solution containing 0.25% PEG and 0.25% Tween-80. To determine if rapamycin could prevent pulmonary fibrosis, FVB mice were pretreated with rapamycin (4 mg/kg, once daily) or vehicle two days prior to receiving bleomycin intratracheally, which was administered for a total of 24 days. Drug dosages were chosen based on previous studies involving mice treated with rapamycin [17, 20]. Control mice were administered saline following rapamycin or vehicle treatment.

2.4. Hydroxyproline analysis

Total lung collagen levels were determined as the instruction described in Hydroxyproline Analysis Kit.

2.5. Cell culture

Human type II alveolar epithelial A549 cells were purchased from ATCC (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and antibiotics at 37 °C in a humidified 5% CO_2 atmosphere.

2.6. Western blotting

Lysis buffer (50 mM Tris-HCL, pH 7.5, 15 mM EGTA, 100 mM NaCl, and 0.1% Triton X-100 supplemented with protease inhibitor cocktail) was used to extract the total protein lysates from the AECs. The proteins were then resolved via SDS-PAGE before being subjected to immunoblotting with primary antibodies and a horseradish peroxidase (HRP)-conjugated secondary antibody and ECL detection (Amersham Biosciences, Fairfield, CT, USA).

2.7. Immunohistochemistry and immunofluorescence

Lung tissue samples were embedded with paraffin using standard methods. The cells were cultured on cover slips and treated as indicated. The sections were incubated with the appropriate primary antibodies at 37 °C for 1 h and 4 °C overnight before being incubated with the appropriate secondary antibodies for 1 h at 37 °C. The sections were viewed via light microscopy. Negative controls were not incubated with the abovementioned primary antibodies.

For F-actin identification, F-actin was stained with a 2% BSA solution containing FITC-conjugated phalloidin for at least 30 min before being incubated with the appropriate secondary antibody (1:1000 dilution) for 2 h in the dark in a humid chamber. Cell nuclei were stained with Hoechst solution. Images were examined via confocal laser scanning microscopy.

2.8. Statistical analysis

Data are expressed as the mean ± SEM. Multi-group comparisons were performed via Student's *t*-test or one-way ANOVA. $P < 0.05$ was considered statistically significant. The non-quantitative results were representative of at least three independent experiments.

3. Results

3.1. Rapamycin attenuated bleomycin-induced pulmonary fibrosis

To explore the possible protective role of mTOR in pulmonary fibrosis *in vivo*, FVB mice were intratracheally injected with bleomycin to establish a pulmonary fibrosis model. Rapamycin was administered via intraperitoneal injections, as previously described [14]. Control mice received vehicle. Lung histology was assessed on day 21 via H&E and Masson staining, which demonstrated the presence of normal pulmonary tissue in the control group and alveolar wall thickening, inflammatory cell infiltration, and increased interstitial collagen deposition in the bleomycin-treated group. Rapamycin pretreatment attenuated alveolitis and pulmonary fibrosis (Fig. 1A, B). We semi-quantitatively assessed lung fibrosis using Ashcoff scores and hydroxyproline analysis and found that the bleomycin-treated group exhibited significantly higher scores than the control group. Rapamycin pretreatment significantly reduced these fibrotic scores and collagen fiber accumulation in the bleomycin-induced pulmonary fibrosis model (Fig. 1C, D).

Fibroblast foci was the main feature of IPF. Immunohistochemistry was performed to study the overactivation of mTOR. The expression of α-SMA, was strongly positive in bleomycin mediated pulmonary fibrosis, indicating of the existence of myofibroblast in IPF. Phosphorylated S6K, a downstream protein of mTOR was

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