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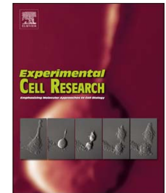

Elongation factor-2 kinase acts downstream of p38 MAPK to regulate proliferation, apoptosis and autophagy in human lung fibroblasts

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a chronic, fatal and progressive fibro-proliferative lung disease, and fibroblast-to-myofibroblast differentiation is a crucial process in the development of IPF. Elongation factor-2 kinase (eEF2K) has been reported to play an important role in various disease types, but the role of eEF2K in IPF is unknown. In this study, we investigated the role of eEF2K in normal lung fibroblast (NHLF) proliferation, differentiation, apoptosis, and autophagy as well as the interaction between eEF2K and p38 MAPK signaling through in vitro experiments. We found that the inhibition of eEF2K markedly augmented cell proliferation and differentiation, suppressed apoptosis and autophagy, and reversed the anti-fibrotic effects of a p38 MAPK inhibitor. Together, our results indicate that eEF2K might inhibit TGF-β1-induced NHLF proliferation and differentiation and activate NHLF cell apoptosis and autophagy through p38 MAPK signaling, which might ameliorate lung fibroblast-to-myofibroblast differentiation.

1. Background

Idiopathic pulmonary fibrosis (IPF) is a chronic, fatal and progressive fibro-proliferative lung disease of unknown cause that leads to breathlessness and ultimately to respiratory failure and death. IPF, which is characterized by aberrant deposition of the extracellular matrix (ECM), leading to extensive lung remodeling [2], has a median survival of 2–3 years and shows a steady incidence in older adults [1]. The disease paradigm currently centers on repetitive injury to the alveolar epithelium through the release of molecules, which results in the proliferation of resident fibroblast-to-myofibroblast differentiation, aberrantly regulated ECM deposition and, in turn, dysfunctional repair and remodeling [2]. To date, drugs targeting various molecules, such as pirfenidone and nintedanib, have been clinically used [3], but this treatment approach has limitations. Most progressive IPF patients cannot completely recover unless they undergo lung transplantation.

Among a variety of pro-fibrotic cytokines, transforming growth factor beta 1 (TGF-β1) appears to be a key factor in the development of pulmonary fibrosis through the regulation of myofibroblast differentiation and proliferation and ECM deposition [4]. TGF-β1-mediated biological activities are regulated by intracellular signaling comprising both SMAD-dependent and SMAD-independent pathways and including mitogen-activated protein (MAP) kinase signaling. There are three major groups of MAPKs: ERK1/2, JNK/SAPK and p38 MAPK. Our previous study showed that JNK/SAPK and p38 MAPK are activated upon TGF-β1-stimulated myofibroblast differentiation [5]. In particular, p38 MAPK has been shown to ameliorate pulmonary fibrosis [6] and is thus an attractive therapeutic target in fibrosis [7]. The anti-fibrotic function of p38 MAPK inhibitors is believed to result from the induction of apoptosis [8].

Eukaryotic elongation factor-2 (eEF2) kinase (eEF2K) is a member of the calmodulin kinase family of proteins and acts as a negative modulator of protein synthesis. The only known substrate of eEF2K is eEF2, which facilitates GTP-facilitated ribosomal movement from sites A to P in protein translation. Because the phosphorylation of eEF2 impairs its ribosomal binding, eEF2K inhibits eEF2 and thus slows the elongation rate. Recent studies have demonstrated that eEF2K expression and activation are increased in the pancreas and breast tumor tissues [9]. In addition, in vascular smooth muscle cells from hypertension and pulmonary hypertension (PH), activating eEF2K facilitates cell proliferation and viability [10,11]. Both apoptosis and autophagy are processes of programmed cell death. eEF2K might modulate the expression of some apoptotic proteins, such as Bcl-XL, to inhibit the apoptotic process in cancer [12] and can regulate autophagy

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under environmental or metabolic stresses, including nutrient deprivation and growth factor inhibition, thereby promoting cancer cell survival [13–15]. eEF2K activity is regulated by a phosphorylation reaction that occurs downstream of various signaling pathways, such as the mTORC1, AMPK, ERK1/2 and p38 MAPK pathways [16–19].

However, the expression of eEF2K in IPF has not been reported. In this study, we demonstrate that the inactivation of eEF2K through p38 MAPK results in the dephosphorylation of eEF2 at Thr 56 and eEF2K at Ser 359, and these effects result in increased TGF-β1-induced NFL proliferation and differentiation and reduced cell apoptosis and autophagy.

2. Methods

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone (Logan, UT, USA), and FBS was obtained from Gibco (MA, USA). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Invitrogen (Waltham, MA, USA). TGF-β1 and anti-α-smooth muscle actin, anti-LC3, anti-p62 (SQSTM1), anti-eEF-2, anti-phospho-eEF-2 (Thr 56), anti-eEF2K, anti-phospho-eEF2K (Ser 359), anti-p38 MAPK, anti-phospho-p38 MAPK (Thr180/tyr182), anti-cleaved caspase-3, anti-phospho-HSP 27 (Ser 82), anti-GAPDH, and anti-rabbit IgG (H + L) antibodies as well as the F(ab')2 fragment (Alexa Fluor 594 conjugate) were purchased from Cell Signaling (Danvers, MA, USA). Anti-fibronectin and anti-caspase-3 antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-mouse IgG was purchased from Zhong Shan Golden Bridge Biotechnology Co., Ltd. (Beijing, Beijing, China), and goat anti-rabbit IgG was obtained from Millipore (Billerica, MA, USA). SB203580, 3-MA and rapamycin were purchased from Selleck (Shanghai, Shanghai, China), and A-484954 was acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell lines and culture

The human lung fibroblast cell line MRC-5 was purchased from and genetically characterized by ScienCell (Zhong Qiao Xin Zhou Biotechnology, Shanghai, China). MRC-5 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO_2 and 95% air.

2.3. siRNA transfection

siRNA duplexes targeting eEF2K was prepared by RiboBio (Guangzhou, Guangdong, China). Non-silencing scrambled (non-targeting) siRNA was used as a control. siRNA transfection was performed according to the manufacturer's recommended protocol. Cells in the exponential phase of growth were plated in six-well plates at 1×10^5 cells per well, grown for 24 h to 30–40% confluence, transfected with siRNA for 48 h, and then identified by western blotting analysis.

2.4. Cell treatments

MRC-5 cells were transfected with siRNA-eEF2K or nontargeting siRNA for 48 h and then treated with 10 ng/ml TGF-β1 for 24 h. Additionally, MRC-5 cells were cultured in the presence of the autophagy activator rapamycin (1 mM, pre-treated for 4 h), the autophagy inhibitor 3-MA (2.5 mM, pre-treated for 4 h) or the p38 MAPK inhibitor SB203580 (8 μM, pre-treated for 2 h). Additionally, eEF2K was inhibited in the cells using the eEF2K inhibitor A-484954 (10 μM, pre-treated for 2 h) for subsequent experiments.

2.5. Western blot analysis

Following the treatments, the cells were washed twice in ice-cold PBS and scraped off the tissue culture dishes. The cells were collected in microcentrifuge tubes and centrifuged at $1000 \times g$ for 5 min. The cell pellets were lysed at 4 °C. Thirty micrograms of protein were resolved by 10–15% SDS-PAGE and transferred to an Immuno-Blot PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membrane was incubated with the appropriate antibodies. The Pierce ECL western blotting substrate kit (Pierce, Grand Island, NY, USA) was used to detect the protein signals on the immunoblots using the ECL system (Syngene, Cambridge, England, UK).

2.6. Proliferation assay

A total of 5×10^3 MRC-5 cells were cultured in 96-well plates with or without the indicated agents. For the assessment of cell proliferation, the cells were incubated with 20 μl of Cell Counting Kit (CCK) reagent (Beyotime Biotechnology, Shanghai, China) at 37 °C for 1 h, and cell proliferation was measured based on the fluorescence at 450 nm using a 96-well plate reader (BioTek, Winooski, VT, US).

3. TUNEL assay

A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed as follows. A total of 1×10^4 cells were plated onto cover glass-bottom dishes and treated with or without the indicated agents. The cells were fixed in 4% paraformaldehyde and treated with TUNEL buffer containing fluorescein-12-dUTP for 1 h at 37 °C in the dark. The slides were visualized at 200× magnification using a fluorescence microscope (Leica, Buffalo Grove, IL, USA).

3.1. Immunofluorescence

Immunofluorescence staining was performed by plating 1×10^4 cells onto cover glass-bottom dishes and treating the cells with or without the indicated agents. The cells on the dishes were washed three times and then fixed with 4% paraformaldehyde. The cells were then prepared and stained with rabbit polyclonal primary antibodies for LC3B (1:500) overnight at 4 °C. The slides were then washed three times, incubated with FITC-conjugated anti-rabbit secondary antibody (1:40) in the dark for 1 h at 37 °C, washed three times and counter-stained with DAPI for 5 min at room temperature. Following staining, the slides were covered with coverslips and visualized under a fluorescence microscope (Leica, Buffalo Grove, IL, USA).

3.2. Statistical analysis

The data are shown as the means ± SEMs. Statistical evaluations were performed by one-way ANOVA followed by Bonferroni's test. Values of $p < 0.05$ were considered to indicate statistical significance.

4. Results

4.1. eEF2K inhibition aggravates the effects of TGF-β1 on fibroblast-to-myofibroblast differentiation in MRC-5 cells

eEF2K is an unusual protein kinase that regulates the elongation stage of protein synthesis by phosphorylating and inhibiting its only known substrate, eEF2. When phosphorylated on Thr 56 through its amino acid sequence, eEF2 cannot engage with ribosomes and is essentially inactive in translation [20]. eEF2K activity was inhibited in TGF-β1-treated MRC-5 cells, as demonstrated by decreased phosphorylation of eEF2 at Thr 56 ($p < 0.05$; Fig. 1B) and increased cell proliferation compared with the control group ($p < 0.05$; Fig. 1C). In addition, the protein expression levels of α-SMA and fibronectin were higher than those in the control group ($p < 0.05$; Fig. 1B), indicating the differentiation of lung fibroblasts to myofibroblasts ($p < 0.05$; Fig. 1B). We then knocked down eEF2K using siRNA-eEF2K ($p < 0.05$;

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