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Leptin promotes pulmonary fibrosis development by inhibiting autophagy via PI3K/Akt/mTOR pathway

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ABSTRACT

Leptin, a protein-related product of the obesity gene, plays an important role in the pathogenesis of fibrotic diseases including pulmonary fibrosis. As a highly conservative process, autophagy regulates various biological functions. Otherwise, insufficient autophagy has been described in alveolar epithelial cells (AEC) to cope with the progression of pulmonary fibrosis. Hence, this study is to investigate the effects of leptin on fibrosis in TGF-β1 induced epithelial mesenchymal transition (EMT) and the potential roles of autophagy in this processes. Our results showed that the elevated leptin level in serum correlated with the severity of lung fibrosis and leptin significantly promoted the EMT in A549 cells as evidenced by promoting collagen I and α-SMA production. Additionally, treatment with leptin decreased autophagosome formation, inhibited the lipidation of LC3I to LC3II, and up-regulated the expression of p62 via activating PI3K/Akt/mTOR pathway, which is indicative of inhibition of autophagy by leptin. Finally, rapmycin pretreatment reversed the pro-fibrogenic effects of leptin. Taken together, our study suggested that leptin accelerated the EMT of A549 cells through inhibiting autophagy via PI3K/Akt/mTOR pathway.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) without exact cause is characterized by progressive deterioration of dyspnea and ultimately respiratory failure [1,2], resulting in median survival of 2–5 years from diagnosis [3]. Evidence shows that IPF is an aberrant wound healing process, characterized by epithelial cell damage and activation, fibroblast proliferation and abnormal deposit of extracellular matrix (ECM) in lung parenchyma [4].

Autophagy is a process of intracellular degradation dependent on lysosomal pathway in which damaged organelles or excessive proteins are cleared [5]. This process can be influenced by multiple factors such as oxidative stress reaction, cytokines, some medicines and so on [6]. Though autophagy plays an important role in the processes of immunity, infection, senescence and cell differentiation [7–9], it does not always mean a protective role. Proper autophagy maintains cell function and survival through recycling of amino acids and fatty acids produced by degradation of cellular components [10]. But deficient autophagy is involved in various diseases such as cardiovascular, neurodegeneration, fibrotic diseases and so on [11,12]. Recently, the insufficient autophagy has been regarded as one of the important features in the pathogenesis of IPF [13]. Furthermore, impaired autophagy results in fibroblast proliferation and differentiation into myofibroblast, cellular senescence, decreasing degradation of collagen, which is important in the progression of pulmonary fibrosis [13,14].

Leptin, as a protein-related product of the obesity gene, plays crucial role in inflammatory, nutritional metabolism, immunity, and autophagy [15–19], following with pathophysiology of many diseases like systemic lupus erythematosus, breast cancer, glomerulosclerosis and alcoholic cirrhosisand etc [20–24]. Furthermore, leptin has been reported to promote the expression and activity of TGF-β1, resulting in pulmonary fibrosis through inhibiting the activity of peroxisome proliferator–activated receptor-γ (PPAR-γ) in bleomycin induced rat model [24]. However, it has been

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argued that bleomycin-induced pulmonary fibrosis in a mouse system reflects all of mechanisms involved in human IPF.

Herein, we tried to explored relation between leptin and main clinical features in IPF patients and found that the higher level of leptin was correlated with severity of IPF, the process of which was regulated by inhibiting autophagy via activating PI3K/Akt/mTOR pathway.

2. Materials and methods

2.1. Study subjects

Patients in the study were enrolled according to the American Thoracic Society (ATS) and the European Respiratory Society (ERS) Recommendations for IPF diagnosis of 2011 [2].

Blood samples were obtained from 24 Chinese adult patients of IPF who were admitted to Nanjing Drum Tower Hospital. 16 healthy controls from the Center of Physical Examination were included for observation. All subjects provided informed consents to participate in this study and granted permission to use the serum and/or lung tissue. This study was approved by the ethics committee of the hospital. Healthy controls were assessed by medical questionnaire, physical examination, and a thoracic X-ray scan. The clinical data of IPF patients, including high-resolution computed tomography (HRCT), and pulmonary function test were collected upon admission.

2.2. Histological examination

Paraffin-embedded lung sections were obtained from surgical resection from patients with IPF. Control lung tissues were obtained from lung tissue far away from tumor region of lung cancer patients.

2.3. Lung function tests

The lung function measurement was performed according to ATS (American thoracic society) Recommendations [25]. Tests of pulmonary function included forced expiratory volume in 1 s, total lung capacity, forced vital capacity, and lung carbon monoxide diffusion function.

2.4. Lung HRCT scoring

The lung HRCT scan of IPF patients was evaluated independently and randomly without knowledge of patients' clinical manifestation. The doctors assessed the extent of areas of ground-glass attenuation, reticular opacities, honeycombing, nodules, consolidation and emphysema. The HRCT results were graded on a scale of 0–4 on the basis of the overall extent of fibrosis (i.e., the extent of reticulation and honeycombing): 0 = no involvement; 1 = 1%–25% involvement; 2 = 26%–50% involvement; 3 = 51%–75% involvement; and 4 = 76%–100% involvement [26,27].

2.5. Cell culture

The human lung cell line A549 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified chamber of 5% CO_2. The culture medium was replaced every other day.

2.6. Measurement of leptin in serum levels

To determine the level of leptin, serum samples were collected at the first visit. All samples were then assayed at the same time to determine the leptin in serum with an ELISA kit according to the manufacturer's instructions (R&D Systems). Optical densities were measured in a Universal Microplate Spectrophotometer (Bio-Rad) at a wave length of 450 nm and then transformed to values according to their standard curves.

2.7. Immunohistochemistry

Paraffin sections (4 μm) were processed for immunohistochemical analysis of leptin and p62. Antibodies used were mouse *anti*-p62 (1:40, Cell Signaling Technology, Danvers, MA), and rabbit *anti*-leptin (1:40, SantaCruz, California, USA). For semi-quantitative score assessment, 10 fields were randomly chosen at × 400 magnification in each slide and the staining intensity was scored as no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3. The extent of positive cells was scored as 0 = no positive cells, 1 = comprising <25%, 2 = 25–50%, 3 = 50–75% and 4 = 75–100% (the percentage of labeled cells, was determined according to the following equation: % = number of labeled cells/total counted cells × 100). The final score was determined by multiplying the intensity scores with the extent of positivity scores of stained cells, with the minimum score of 0 and a maximum score of 12.

2.8. Transmission electron microscopy

A549 cells were fixed at 4 °C in 2.5% glutaraldehyde in 0.15 M sodium cacodylate (pH 7.4) overnight. After postfixation in osmium tetroxide (1% in cacodylate buffer), contrasted in uranyl acetate (1% in ethanol 70%), the cells were dehydrated in ethanol and embedded in epoxy resin. Then, 100 nm-thick sections were stained with uranyl acetate, and then examined by using a Hitachi-7500 electron microscope.

2.9. Confocal assay

A549 cells were plated on coverglass in 24-well plates and treated with or without indicated agents. The cells were washed twice with PBS, fixed by 4% paraformaldehyde for 1 h, and washed three times with PBS after fixation. The cells were prepared and stained with *anti*-LC3B primary antibody overnight at 4 °C. The sections were washed twice, incubated with fluorochrome-labeled secondary antibodies (1:200) for 30 min, and washed three times with PBS after staining. Images were obtained with a Leica SP2 confocal microscope (Leica Microsystems, Exton, PA) and analyzed with Leica confocal software. The autophagosomes were identified by LC3 dots.

2.10. Western blot

Whole cell protein from A549 cells were extracted with lysis buffer. To analyze levels of specific proteins, supernatants with equal amounts of proteins were mixed with 5 × sodium dodecyl sulfates (SDS) sample buffer, boiled for 5 min at 100 °C and then separated by 10% SDS–polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to PVDF membranes (Millipore) by a wet blotting system. After blocked with 5% skim milk, membranes were incubated with primary antibodies overnight at 4 °C: including antibodys of p62 (Santa Cruz Biotechnology), LC3B, phospho–STAT-3, STAT-3, phospho-Akt, Akt and GAPDH (Cell Signaling Technology). The membranes were then

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