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Effects of dynamic changes in histone acetylation and deacetylase activity on pulmonary fibrosis

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ABSTRACT

Objective: Histone deacetylases (HDACs) play an important role in dysregulation of histone acetylation/deacetylation, which is the main driving force of the progression of pulmonary fibrosis. Here we investigated the changes in histone acetylation/deacetylation, and the contribution of specific class I and class II HDACs in the progression of pulmonary fibrosis.

Methods: Male C57BL/6J mice received a single dose of tracheal administration of bleomycin to establish the pulmonary fibrosis model. The changes in acetylation rate of histone 3 (H3) and histone 4 (H4), and the activity of HDAC2 and HDAC4 in the lung tissue during the progression from alveolitis to pulmonary fibrosis were measured.

Results: The acetylation rate of H3/H4 significantly decreased during alveolitis and the early and middle stages of fibrosis, but restored in the late stage of fibrosis. Correlation analysis showed that H4 deacetylation affected both alveolitis and pulmonary fibrosis. H3 deacetylation only affected alveolitis. HDAC2 activity significantly increased in the middle and late stages of pulmonary fibrosis. There was no significant difference in HDAC4 activity between bleomycin and saline groups. However, HDAC4 activity changed significantly with the progression of the disease in bleomycin group. The changes in HDAC2 and HDAC4 activity were different. HDAC2 had long-lasting effects, while HDAC4 had transient effects. Correlation analysis showed that HDAC2 and HDAC4 activity was positively correlated with alveolitis score and fibrosis score.

Conclusions: The changes in histone acetylation may directly regulate the gene expression of inflammatory cytokines/fibronectin and thus affect the progression of pulmonary fibrosis. The injury-induced histone deacetylation switched into acetylation at the late stage of pulmonary fibrosis, which may be involved in the repair process. HDAC2 is mainly involved in the chronic progression of pulmonary fibrosis, and HDAC4 is mainly involved in early stress response to pulmonary fibrosis.

1. Introduction

The pathogenesis of pulmonary fibrosis has not been fully revealed. Current hypotheses include lesion, inflammation, impaired repair, abnormal cytokine expression and release, hyperactivation of fibroblasts, epithelial-mesenchymal transition (EMT), extracellular matrix accumulation, etc. However, these factors cannot fully explain its pathogenesis. The growth of epigenetic research provides a new concept for the pathogenesis of pulmonary fibrosis.

Epigenetics refers to the regulation of gene expression without altering the DNA sequence [1]. Over the past 10 years, studies in liver, kidney and myocardial fibrosis demonstrate that dysfunction of epigenetic regulation may be an important driving force for the development of fibrosis [2]. In the process of cell activation and differentiation, epigenetic changes, such as histone modification, are often the key to gene transcription. More and more evidence reveals the underlying mechanisms of epigenetic modification in the process of fibrosis and its gene regulation [3].

The balance between histone acetylation and deacetylation is controlled by the antagonistic actions of two types of enzymes: histone acetyltransferase (HAT) and histone deacetylase (HDAC) [4]. HAT acetylate conserved lysine amino acids on histone proteins by transferring an acetyl group from acetyl-CoA to form ε-*N*-acetyl lysine, which binds to the positive charge residues of histone lysine and

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increases hydrophobicity. This results in the formation of a loose chromatin structure that facilitates the transcription of deoxyribonucleic acid (DNA) [5]. During deacetylation HDAC promotes the removal of the acetyl group from acetyllysine and restores positive charge. This results in the formation of dense chromatin structure and blocks gene transcription sites [6].

Changes in chromatin structure are critical to the regulation of gene expression [7]. This theory provides a better explanation for inflammatory gene expression and activation. Fibrosis is usually associated with chronic inflammatory states, persistent inflammation and growth factor expression, fibroblast phenotype alteration and excessive extracellular matrix secretion, leading to collagen fibrillogenesis [8]. In recent years the research of histone modification on organ fibrosis is focused on the role of histone deacetylation, histone deacetylation-regulated signaling pathways, multiple effects of histone deacetylase, and histone deacetylase inhibitors.

Pulmonary fibrosis is a chronic progressive disease. It is still unclear about the changes in histone acetylation and the role of each member of the HDAC family during disease progression. In this study, we used tracheal administration of bleomycin in mice to establish an animal model of simulated pulmonary fibrosis. The degree of inflammation/fibrosis, histone 3 (H3)/histone 4 (H4) acetylation, I/II HDACs activity were dynamically monitored during disease progression. The changes in type I and II HDAC during the progression of pulmonary fibrosis were analyzed to define potential therapeutic targets and precise timing of treatment.

2. Materials and methods

2.1. Experimental animal model establishment

Male C57BL/6J mice, SPF grade, 6–7 weeks of age, were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (license number: SCXK (Beijing) 2012–0001) and housed in Capital Medical University Department of Animals (license number: SCXK (Beijing) 2015–0012). All animal experiments were approved by the Capital Medical University Animal Experiment and Experimental Animal Welfare Committee (ethical number: AEEI-2016-005).

Mice were house in individual ventilated cages (IVC), < 5 mice/cage, 12 h of light, 12 h dark, the temperature of 21–22 °C, relative humidity of 50% -70%. All animals had free access to water and food. Mice were randomly divided into non-intervention, saline and bleomycin groups. The mice were anesthetized with intraperitoneal injection of 10% w/v chloral hydrate (3.5 μL/g body weight). Mice in the bleomycin group received a tracheal administration of bleomycin A2 (manufactured by Nippon Kayaku Co., Ltd.) of 5 mg/kg (2.5 mL/kg) [9]. Mice in saline group were given saline solution (2.5 mL/kg). The non-intervention group did not receive any treatment. Mice in each group were randomly selected and sacrificed at 7, 14 and 21 days post-treatment, and lungs were harvested.

2.2. Histological examination

Fresh lung tissues were slowly perfused with 10% formalin for internal fixation, and then quickly placed in 10% formalin and post-fixed for > 24 h. The lung tissues were embedded in paraffin and sectioned at 4 μm of thickness. Tissues were stained with hematoxylin-eosin staining (HE staining) and Masson trichrome staining, respectively.

(1) Alveolitis score [10]: HE staining sections were evaluated for alveolitis score. All visual fields under 100 × objective were selected and graded. The large trachea and large blood vessels in the field were avoided. The scores from all the visual fields were averaged and defined as the alveolitis score of the sample. The evaluation was performed in a blinded manner.

(2) Fibrosis score [11]: Masson stained sections were evaluated for fibrosis score. All visual fields under 100 × objective were selected. For each field, a 40 × zoom-in image was randomly selected. The large trachea and large blood vessels in the field were avoided. The scores from all the zoom-in images were averaged and defined as the fibrosis score of the sample. The evaluation was performed in a blinded manner.

2.3. Nuclear protein extraction and quantification

Nuclear proteins were extracted from fresh lung tissue using the nuclear extraction kit (EpiQuik ™ OP-0002) according to the manufactures' instructions. Bradford was used to quantify nucleoprotein (Pik Wan Tian Bi).

2.4. HDAC2, HDAC4 activity assay

The HDAC2 and HDAC4 activity was measured by colorimetric method with a two-step procedure according to the manufacturer's instructions (EpiQuik ™ P-4006, P-4042). In the first step, the sample was incubated with the HDAC assay substrate to deacetylate the substrate. In the second step, the activator solution releases p-nitroaniline from the substrate or standard. The absorbance was read at 450 nm. HDAC activity was calculated according to the following formula:

HDAC change% = (intervention groupOD − blank OD) / (non-intervention group OD − blank OD) ∗ 100%.

2.5. Histone acetylation rate

The total protein of H3 (1:1000 (v/v), CST, # 9715) and H4 (1: 500 (v/v), CST, # 979), acetylated H3 (1: 1000 (v/v), CST, # 9649) and acetylated H4 (1: 500 (v/v), CST, # 13534) were measured using Western blot. PCNA (1:1000 (v/v), Bi Yun Tian, AF0261) was used as an internal reference to normalize the target protein expression. The denatured nucleoprotein samples (100 °C, 5 min; 50 μg per lane) were separated using 12% SDS-PAGE. The protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, IPVH00010) by electroporation (Bio-Rad, Power Pac Basic). The membrane was blocked at room temperature with a TBS solution containing 5% skimmed milk for 2 h. The membrane was incubated with primary antibody overnight at 4 °C. After wash, the membrane was incubated with fluorescent goat anti-rabbit antibody (1: 10,000 (v/v), LI-COR, IRDye® 680RD) or fluorescent goat anti-mouse IgG (1:10,000(v/v) LI-COR, IRDye® 800CW) for 2 h at room temperature. The protein bands were analyzed using an Odyssey LI-COR SA two-color infrared laser imaging system. The histone acetylation rate was calculated by acetylated histone/total histone.

2.6. Preparation of lung tissue homogenates and ELISA

5 mL of ice-old PBS (0.02 mol/L, pH 7.0–7.2) was added to 0.5 g fresh lung tissue, and homogenized for 6–8 min on ice. After one freeze-thaw cycle, tissue homogenates were centrifuged at 3000 rpm for 5 min, and the supernatant was collected. The protein levels of TGF-β1, Smad2/3, Smad7 and Snail in lung tissue were detected by ELISA kits (Shanghai BlueGene), according to the manufacture's instruction.

2.7. Statistical analysis

All data were analyzed using SPSS 17.0 software. Data were expressed as mean ± SD. One-way analysis of variance (ANOVA) and Post Hoc Tests, including least significant difference (LSD) and Student-Newman-Keuls (SNK) were used for multiple group comparisons. The correlation between the data was analyzed by binary correlation. All variables were normally distributed. The correlation coefficient was calculated by Pearson formula. $p < 0.05$ was considered statistically