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Effect of bone morphogenic protein-7 on the expression of epithelial–mesenchymal transition markers in silicosis model

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ABSTRACT

This study presented the effect of bone morphogenic protein-7 (BMP-7) inhibiting epithelial–mesenchymal transition (EMT) in silicosis model. In vivo, Wistar rats were exposed to silica by intratracheal instillation. Seven days later rats were treated with BMP-7. Rats were sacrificed at 15 and 30 days after exposure of silica. The results demonstrated vimentin expression was down-regulated; and E-cadherin was up-regulated after intervention with BMP-7. The TGF-β expression and phosphorylation-p38 were lower in BMP-7 treated group than in silica group. In vitro, p38 MAPK/Snail signaling pathway was involved in the occurrence of EMT in A549 cells treated by silica. EMT was inhibited by BMP-7. The data showed BMP-7 inhibited EMT induced by silica associated with inhibition of p38 MAPK/Snail pathway.

1. Introduction

Silicosis characterized by diffuse lung fibrosis was one of the most serious occupational lung diseases. Silicosis usually occurred after many years of exposure to crystalline silica (Rimal et al., 2005). There were no effective therapies to control the process of fibrosis currently. It is very important to explore the molecular mechanism of silicosis to find the effective treatments.

Recent studies had confirmed that lung EMT was one of the important mechanisms of pulmonary fibrosis (Liang et al., 2013; Noguchi et al., 2014; Song et al., 2013; Xi et al., 2014; Y.L. Chen et al., 2013). EMT had emerged as a critical event in fibrosis, wound healing and tumor. It was recognized that, after epithelial injury, epithelial cells gradually lost epithelial phenotype characteristics and acquired mesenchymal phenotype characteristics, such as down-regulation of E-cadherin expression and up-regulation of vimentin, improved abilities of cell migration and invasion, transition into myofibroblast-like cells and the generation of extra-cellular matrix (ECM). It was found that many signal molecules could induce EMT, such as hepatocyte growth factor (HGF) (Chang et al., 2011), epidermal growth factor (EGF) (Xiong et al., 2014) and transforming growth factor beta (TGF-β) (H.H. Chen et al., 2013; Y.L. Chen et al., 2013; Yang et al., 2013). Most signal molecules depended on different environments of cells, however, TGF-β was thought to be essential for EMT of almost all epithelial tissues (Akhurst and Hata, 2012; Bi et al., 2013; Jang et al., 2013; Kim et al., 2013; Rodrigues-Diez et al., 2012; Zheng et al., 2013). Thus in our study, to further confirm whether silica could induce EMT, we observed expression of TGF-β under silica-exposure conditions, given the fact that TGF-β is the key inducer of EMT. In recent years, it was reported that bone morphogenic protein-7 (BMP-7) inhibited EMT via TGF-β (Correa-Costa et al., 2014; Veerasamy et al., 2013; Wang et al., 2010; Y. Xu et al., 2009; M. Zeisberg et al., 2007), and BMP-7 was regarded as one of the anti-fibrosis cytokines of application prospects.

Mitogen-activated protein kinases (MAPKs) could regulate intracellular signal transduction, including cell proliferation, differentiation and apoptosis. They were activated in response to a range of extra-cellular stimuli such as growth factors, cytokines, hormones, oxidants, toxins, and physical stress. It was reported that silica induced the activation of MAPKs (Gehrke et al., 2013; Morishige et al., 2012; Tomaru and Matsuoka, 2011). The p38 MAPK, one of the MAPK members, could be activated by inflammation and stress reaction. More than one study showed p38 MAPK signaling pathway involved in EMT induced by TGF-β in lung epithelial cells (H.H. Chen et al., 2013; Kondo et al., 2012). There was no report for BMP-7 to inhibit EMT induced by silica via p38 MAPK pathway resulting in suppression of pulmonary fibrosis.

Abbreviations: BMP-7, bone morphogenic protein-7; EMT, epithelial–mesenchymal transition; MAPK, mitogen-activated protein kinase; TGF-β, transforming growth factor beta; ECM, extracellular matrix; HGF, hepatocyte growth factor; P-p38, phosphorylation-p38; AM, alveolar macrophages; TNF, tumor necrosis factor; IL-1, interleukin-1; IPP, Image-Pro Plus.

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Therefore, we used silicosis model in rats and treated with BMP-7 to explore the relationships among BMP-7, p38 MAPK pathway and EMT in the development of pulmonary fibrosis.

2. Materials and methods

2.1. Silica

Crystalline silica of which 95% of particle diameter was below 5 μm and silica content was >99% was provided by the Center of Occupational Health and Poisoning Control, Chinese Center for Diseases Control and Prevention. The crystalline silica samples were weighed and suspended in saline to a concentration of 50 mg/ml. Then they were autoclaved to sterilize, and later they were supplied with final concentration of 5000 IU/ml penicillin. The silica samples for cell experiments were suspended in Dulbecco's Modified Eagle Medium (DMEM) to a concentration of 5 mg/ml, and then were briefly shaken to assure dispersion of particles which were later diluted to 25–100 μg/ml before they settled.

2.2. Animals

Adult male Wistar rats (200–240 g, SPF species) were purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The rats were fed with a commercial diet and water. All animal experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the experiment protocol was approved by the Laboratory Animal Care and Use Committee at Capital Medical University.

2.3. Experimental design

A total of 60 adult rats were classified into control group (n = 20), silica group (n = 20) and BMP-7 treated group (n = 20). They were anesthetized with ether and administered with silica suspension 1 ml intratracheally except for control group which received 1 ml of saline instead. From the eighth day after instillation, rats in BMP-7 treated groups were injected with 300 μg/kg recombinant human BMP-7 (QuantoBio Limited Company, Beijing, China) intraperitoneally every other day. Rats in the control group and silica group were given 1 ml of saline intraperitoneally to match the BMP-7 injection schedule. They were sacrificed and the lungs were removed for further examination on day 15 or day 30 after silica instillation.

2.4. Histopathological examination

The left lung was fixed in 10% formalin for 48 h and then embedded in paraffin and cut into 5 μm thick slices. The fixed sections were stained with hematoxylin and eosin (HE) and Masson trichrome staining for evaluation of alveolitis and fibrosis (collagen fibers). A light microscope (Olympus D72, Japan) was used to examine slides at low-power (100×) and high-power (400×) magnification.

2.5. Hydroxyproline assay

The collagen content in lung tissues was estimated by measuring hydroxyproline levels using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer's instructions. The data was expressed as milligrams of hydroxyproline per gram of dry lung weight.

2.6. Western blot analysis

Equal amounts of protein (40 μg) was separated by 12% SDS-PAGE and transferred onto a PVDF membrane (GE Health Care, Atlanta, USA). Nonspecific binding was blocked with 5% milk in TBST at room temperature for 1 h. The membranes were then incubated at 4 °C overnight with the following primary antibodies: E-cadherin, vimentin, phosphorylation-p38 MAPK (P-p38 MAPK), TGF-β, Snail, Slug and GAPDH which were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA), and ZEB-1 and ZEB-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). After three washes with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit (Cell Signaling Technology Inc., Beverly, MA, USA) for 1 h at room temperature and then washed. The membranes were incubated with the substrate and exposed to X-ray film. All western blots were repeated at least 3 times.

2.7. Immunohistochemistry and immunofluorescence

Immunohistochemical and immunofluorescence staining in vivo were performed from paraffin-embedded tissue sections; slides were incubated with E-cadherin (1:100 dilution), vimentin antibodies (1:100 dilution) for 1 h, respectively, and followed by development with DAB Envision System according to the manufacturer's instructions or FITC/Alexa Fluor-conjugated secondary antibody examined with Confocal Laser Scanning Microscopy for analysis. Antigens were retrieved by heating the sections in citrate buffer before staining.

Immunofluorescence staining in vitro was as follows: A549 cells were grown on chamber slides and serum were removed overnight before exposing to silica for 72 h. Cells were then fixed with pre-cooled 4% paraformaldehyde for 20 min and blocked with 5% BSA for 45 min at room temperature, then incubated with antibody against E-cadherin (1:100 dilution), vimentin (1:100 dilution) and P-p38 MAPK (1:100 dilution) at 4 °C overnight. Slides were washed three times with PBS and incubated with FITC/Alexa Fluor-conjugated secondary antibody (1:1000 dilutions) for 45 min at 37 °C. Then, the cells were counter stained with DAPI for 10 min and were examined with Confocal Laser Scanning Microscopy for analysis.

2.8. Cell culture and treatment

RAW264.7 (Xiang Ya School of Medicine, Central South University, Hunan, China) mouse peritoneal macrophages were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) which contained 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (purchased from KeyGEN, Nanjing, China) at 37 °C with humidified 5% CO_2 atmosphere. When RAW264.7 cells were grown to approximately 70% confluence, culture medium were replaced by free-serum medium for 24 h. Then the cells were treated with different concentration of silica (0, 12.5, 25, 50, 100 and 200 μg/ml) for 24 h. The supernatant was harvested and filtered through 0.22 μm microporous membrane for future experiments. Following silica exposure, TGF-β concentration was measured in supernatant with the use of an ELISA kit (Shanghai ExCell Biology Inc., Shanghai, China) following manufacturer's instruction.

A549 (Xiang Ya School of Medicine, Central South University, Hunan, China), a cell line of human type II alveolar epithelial carcinoma, was grown in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. For silica treated group, cells were pretreated with the former RAW264.7 supernatant treated by different concentrations of silica for 72 h. For SB203580 treated group (Sigma, St. Louis, MO, USA), cells were pretreated with different concentrations of SB203580, a chemical inhibitor of the p38 MAPK pathway, for 1 h, and then they were exposed to 100 μg/ml of silica supernatant for 72 h. For BMP-7 treated group, cells were treated with 100 μg/ml silica supernatant and different concentrations of recombinant human BMP-7 (R&D systems, USA) (0, 100, 200, 400, 800, 1600 ng/ml) for 72 h.

2.9. Data analysis

All experiments were independently replicated at least three times, unless otherwise stated. The data were expressed as mean ± SD.