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Pneumoconiosis Compensation Fund Board (PCFB) Research Funding

Final Report

(1/9/2011- 31/08/2014)

Therapeutic effect of a natural agent from Chinese herb --- resveratrol on an experimental pneumoconiosis rat model

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INTRODUCTION:

Pneumoconiosis is the most severe one in occupational diseases around the world. Persistent inflammation and subsequent fibrosis in the lung constitute two main pathological processes of pneumoconiosis (Thakur SA et al 2009). Whole Lung Lavage (WLL) is the current clinical treatment modality. However, the limitations include low compliance to those asymptomatic pneumoconiosis patients at early inflammatory stage and no intervention effect on progressive fibrosis at late stage. It is desirable to develop alternative therapeutic agent specifically targeting early inflammation and late fibrosis in pneumoconiosis.

Polygonum cuspidatum (虎杖), a Chinese herb traditionally used for treating blood stasis, cough, sputum (Ding, 2006), has been shown a potential in adjuvant management of early inflammation and late fibrosis in SARS patients in mainland China. Resveratrol (trans-3,4,5-trihydroxystilbene), a natural polyphenol, is believed as the major bioactive component of *polygonum cuspidatum* and has been shown to inhibit inflammation in vitro (Donnelly, 2004) and fibrosis formation in blemycin-induced rat pulmonary fibrosis (Akgedik R, 2012). However, the efficacy and underlying mechanisms of this natural agent on early inflammation and late fibrosis in pneumoconiosis has not been systemically clarified. The present study aimed to: 1) examine the effect of resveratrol on silica-induced inflammatory response in rat alveolar macrophages and TGF-β1-induced fibrotic response in human lung fibroblasts in vitro, respectively; 2) evaluate the prevention and treatment effects of resveratrol on early inflammation and later fibrosis events in an experimental pneumoconiosis rat model in vivo; 3) investigate the underlying mechanisms of resveratrol’s anti-inflammation and anti-fibrosis actions in experimental pneumoconiosis in vitro and in vivo, respectively.

METHODS AND MATERIALS:

To achieve aim 1, NR8383 (rat alveolar macrophage) cells were exposed to silica particles to induce inflammatory response, cell survival and cytokines were analyzed. WI-38 (human lung fibroblast) cells were incubated with TGF-β1 to stimulate fibroblast proliferation and differentiation, cell proliferation and collagen deposition was investigated. Both cell models were treated with or without resveratrol in the time- and dose-dependent manner. To achieve aim 2, an experimental pneumoconiosis model was established in rats treated with or without resveratrol. Cytokines in bronchoalveolar lavage fluid (BALF) was examined, histological and immunohistochemical tests were also performed to observe inflammatory cells infiltration and collagen deposition in rat lung tissue. To achieve aim 3, western blot, microarray and qRT-PCR analysis were performed in vitro and in vivo. Details were described below.

- **In vitro study:**

1. Chemicals and reagents:

Resveratrol were purchased from Sigma (#R5010); Culture mediums F12K, EMEM, fetal bovine serum (FBS) were gained from Life technology and ATCC. Silica particles (MIN-U-SIL-5) were purchased from US Silica. The manufacture's stated average diameter of Silicon Dioxide (SiO_2) is 1.6 μm. Recombinant human TGF-β1 was from Millipore (#GF111).

2. Cell cultures:

NR8383 (rat alveolar macrophage) cells were purchased from ATCC (#CRL-2192) and maintained in F12K with 15% FBS. Cells were subcultured every 3-4 days. Experiments were run in 6-well, 24-well Costar tissue culture plates. WI-38 (human lung fibroblast) cells were

obtained from ATCC (#CCL-75) and cultured in EMEM medium with 10% FBS, 100ug.l^{-1}streptomycin and 100U.ml^{-1}penicillin. Cells were seeded to 6-well, 24-well or 96-well culture plates for different assays.

3. Macrophage survival analysis:

Macrophage cell death following silica exposure (0-100 μg/cm^2) for 3-12 hours treated with or without resveratrol (0-100 μM), was analyzed by LDH assay according to manufacturer's protocol.

4. Cytokine analysis in macrophage cultures:

Macrophage cells were cultured for 24 hours followed by silica particle incubation (0-100μg/cm^2) for further 6 hours, treated with or without resveratrol (0-100 μM). Cell culture supernatants were collected and inflammatory cytokines IL-1β and TNF-α protein level were measured by ELISA testing.

5. Fibroblast proliferation analysis:

Human lung fibroblast cells were cultured for 24 hours in serum-containing medium followed by 16h culture in serum-free medium. TGF-β1 (10ng/ml) and resveratrol (0-100μM) were then adminstrated for different timelines. MTT assay was used to evaluate the proliferation ability of fibroblasts.

6. Collagen analysis in fibroblasts:

After stimulated by TGF-β1 (10ng/ml), treated with or without resveratrol (0-100μM), fibroblast cells were subjected to Sirius red/fast green staining to examine and semi-quantify the collagenous and non-collagenous proteins in fibroblasts, respectively.

7. Microarray and qRT-PCR analysis:

Total RNAs were extracted with Trizol from each sample (n=3) that was treated with or without resveratrol in silica-exposed (3h) alveolar macrophages and TGF-β1-stimulated (48h) lung fibroblasts, respectively. Microarray analysis was performed using Agilent 4×44k oligonucleotide arrays to investigate potential target markers and pathways involved in the present experiments. Informative genes from microarray analysis, particularly inflammation- and fibrosis-related markers, were confirmed for its altered transcription by qRT-PCR following routine method.

8. Western blot analysis:

Proteins extracted from cell lysis were quantified by Bradford Assay. Primary antibodies against p65, cleaved caspase-1 (p20) and collagen type I were used in in vitro studies, β-actin were used as endogenous control. Enhanced chemiluminescence (ECL) method was used for detecting the protein levels with ChemiDoc™ MP System and Image Lab™ program (Bio-rad, CA, USA).

- **<u>In vivo study:</u>**

1. Animal model establishment through silica aerosol exposure to rats:

Male Sprague Dawley (SD) rats weighting around 70-80g were housed in a whole body inhalation chamber for silica exposure (15 mg/m^3). Exposures were conducted for 6 hours per day (3h x 2 times) with a 45min-interval for animal feeding, 5 days per week, for a total of 20 or 40 days. The rats were on a 12-h light–dark schedule and were exposed to silica aerosol during the dark cycle to coincide with their most active period (Porter et al, 2001).

2. Animal grouping and treatment:

There were two batches of experiments in this study. In the first batch for prevention study, the rats were randomly exposed to silica only, simultaneously exposed to silica and resveratrol treatment at low (10mg/kg/day) or high (20mg/kg/day) dose for 20 or 40 days. In the second batch for treatment study, the rats were randomly assigned to silica exposure only, resveratrol treatment at low (10mg/kg/day) or high (20mg/kg/day) dose after 20-day or 40-day silica exposure, and the treatment lasted for 2, 4, or 6 weeks, respectively. Age-matched rats without any treatments were served as normal control. The animal number in each group is 7. Resveratrol dosage was based on Sener's study (Sener et al, 2007).

3. Bronchoalveolar Lavage fluid analysis:

Bronchoalveolar lavage fluid (BALF) was obtained by washing the airways three times with 5 ml of saline through a tracheal cannula. Cell suspensions were centrifuged and supernatants were collected for LDH assay to examine lung tissue damage. The remaining cells were re-suspended and cultured at a density of 5×10^5 cells/ml in 24-well plates using EMEM medium supplemented with 10% heat-inactived FBS. After 18-h culture, the cell-free supernatants (BAL cell-conditioned media) were collected, then TNF-α and IL-1β protein levels were measured in BAL cell-conditioned media by ELISA testing.

4. Histological and immunohistochemical analysis of lung tissues:

Paraffin-embedded lung sections (5 μm) were stained routinely with hematoxylin & eosin (H&E) to observe the lesions, inflammatory cells infiltration and fibrosis formation in lung tissue. Tissue sections were also specially stained with Sirius Red to evaluate the collagen deposition in

lung tissue. Immunohistochemical staining was done to detect the expression and distribution of collagen type I and type III proteins in lung tissue.

5. Western blot analysis in rat lung tissue

Proteins extracted from homogenized lung tissues were quantified by Bradford Assay. Collagen type I and type III proteins level were detected following common western blot procedure as described previously.