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SiO_2-induced release of sVEGFRs from pulmonary macrophages

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ABSTRACT

Background: The inhalation of silicon dioxide (SiO_2) particles causes silicosis, a stubborn pulmonary disease that is characterized by alveolar inflammation during the early stage. Soluble cytokine receptors (SCRs) play important roles in regulating inflammation by either attenuating or promoting cytokine signaling. However, the role of SCRs in silicosis remains unknown.

Methods and results: Luminex assays revealed increased soluble vascular endothelial growth factor receptor (sVEGFR) family levels in the plasma of silicosis patients. In an enzyme-linked immunosorbent assay (ELISA), cells from the differentiated human monocytic cell line U937 released sVEGFR family proteins after exposure to SiO_2 (50 μg/cm^2). Further Western blot experiments revealed that VEGFR expression was also elevated in U937 cells. In contrast, levels of sVEGFR family members did not change in the supernatants of human umbilical vein endothelial cells (HUVECs) after exposure to SiO_2 (50 μg/cm^2). Interestingly, VEGFR expression in HUVECs decreased after SiO_2 treatment. In a scratch assay, HUVECs exhibited cell migration ability, indicating the acquisition of mesenchymal properties.

Conclusion: Our findings highlight the important role of sVEGFRs in both inflammation and fibrosis induced by SiO_2, suggesting a possible mechanism for the fibrogenic effects observed in pulmonary diseases associated with fibrosis.

1. Introduction

The inhalation of silicon dioxide (SiO_2) particles causes silicosis, a stubborn pulmonary disease that is characterized by alveolar inflammation during the early stage and progressive pulmonary fibrosis during the late stage. The pathogenic mechanisms underlying silicosis remain unknown, and no effective treatment is available.

Considerable evidence suggests that alveolar macrophages (AMs), the first line of defense in the lung, initiate pulmonary dysfunction, which results from chronic inflammation (Keogh and Crystal, 1982; Ward and Hunninghake, 1998). AMs phagocytose silica and release cytokines and chemokines, which stimulate pulmonary fibroblasts (PFBs) to produce collagen, ultimately resulting in pulmonary fibrosis. In addition to cytokines and chemokines, soluble cytokine receptors (SCRs) play important roles in regulating inflammation, either by attenuating or promoting cytokine signaling (Levine, 2004, 2008). A key role of soluble cytokine receptors is preventing excessive inflammatory responses. For example, mutations in the 55-kDa extracellular domain of tumor necrosis factor receptor type 1 (TNFRSF1A, TNFR1) were identified in patients with TNF receptor-associated periodic syndrome (TRAPS). Moreover, a recombinant soluble human TNFR2-Ig fusion protein exerted therapeutic effects in these patients (Hull et al., 2002). SCRs also act as disease biomarkers. For example, elevated circulating levels of soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) are considered a biomarker of inflammation and predict all-cause mortality (Yuan et al., 2013). The mechanism underlying the generation of SCRs includes proteolytic cleavage of cell surface receptors (Blobel, 2005), alternative gene splicing, transcription and translation of cytokine-binding genes (Levine, 2004), and extracellular release of membrane-bound receptors within exosomes (Trajkovic et al., 2008).

In this study, we show that sVEGFRs family members are elevated in silicosis patients. Unlike macrophages, endothelial cells do not release sVEGFRs after SiO_2 exposure; instead, SiO_2 induces the decreased expression of VEGFRs in endothelial cells, which may undergo endothelial-mesenchymal transition (EndMT).

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2. Materials and methods

2.1. Animals

C57BL/6 mice, 6–8 weeks of age, were obtained from Dr. Tao Cheng at Nanjing Medical University Laboratories (Nanjing, China). All animals were male and housed (4 per cage) in a temperature-controlled room (25 °C, 50% relative humidity) on a 12-h light/dark cycle. All animal procedures were performed in strict accordance with ARRIVE guidelines, and animal protocols were approved by the Institutional Animal Care and Use Committee of Southeast University.

2.2. Establishment of a mouse model of silicosis

Animals were anesthetized with pentobarbital sodium via intraperitoneal injection, and their tracheae were surgically exposed. Prepared SiO_2 suspension (0.2 g/kg in 50 mg/ml saline) was instilled intratracheally. Control animals were given the same volume of sterile saline, as previously described (Liu et al., 2017). Plasma samples were collected after the administration of SiO_2 or saline for 7 days.

2.3. Reagents

SiO_2 was obtained from Sigma® (S5631), and 80% of the particles were less than 5 μm in diameter. The particles were selected via sedimentation according to Stokes' law, followed by acid hydrolysis and baking overnight (200 °C for 16 h). The silica samples used for the cell experiments were sterilized by autoclave and then suspended in sterile normal saline (NS) at a concentration of 5 mg/ml. Fetal bovine serum (FBS), normal goat serum and Dulbecco's modified Eagle's medium (DMEM; #1200-046) were purchased from Life Technologies™, and PenStrep (15140-122) was obtained from Fisher Scientific.

2.4. Luminex for cytokine detection

Cytokines were detected with the MILLIPLEX MAP Human Soluble Cytokine Receptor Panel (HSCR-32K-PMX14, Millipore®, USA), which included sCD30 (sTNFRSF8), sEGFR, sgp130, sIL-1RI (sCD121a), sIL-1RII (sCD121b), sIL-2Rα (sCD25), sIL-4R (sCD124), sIL-6R (sCD126), sRAGE, sTNFRI (sTNFRSF1A), sTNFRII (sTNFRSF1B), sVEGFR1 (sFlt-1), sVEGFR2 (sFlk-1) and sVEGFR3 (sFlt-4).

2.5. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell® and maintained in T75 flasks in 10% FBS in DMEM. HUVECs were stored in liquid nitrogen following passages 3-7 (P3-7). A vial of P3-7 HUVECs was thawed, plated, and passaged upon confluence for each experiment, and each experiment was performed using HUVECs between P10 and P15.

The human monocytic cell line U937 was purchased from ATCC and maintained in T75 flasks in 10% FBS in RPMI 1640 medium. Before experiments were performed, the cells were cultured at 8×10^5 cells/well in a 24-well plate. Then, 50 nM phorbol myristate acetate (PMA) was used to differentiate U937 cells for 24 h prior to the experiments.

2.6. Western blot analysis

Immunoblotting was performed as previously described (Carlson et al., 2004), with minor modifications. HUVECs were collected from culture dishes, washed with PBS and lysed using a mammalian cell lysis kit (MCL1-1KT, Sigma-Aldrich®) according to the manufacturer's instructions. Membranes were probed using primary antibodies, followed by alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (1:5000). Signals were detected using chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate, Thermo Scientific). Western blots were repeated using cells from three different donors. A single representative immunoblot is shown in each figure. Densitometry analysis was performed using ImageJ software, and the results from all repeated experiments were combined into one plot.

2.7. Enzyme-linked immunosorbent assay (ELISA)

sVEGFR levels were measured by ELISA. ELISA kits were purchased from SenBeiJia® (Nanjing, China) and used following the manufacturer's protocols. Fifty-microliter aliquots of cell culture supernatant or plasma were added to each well of a 96-well plate for measurements. Each sample was tested in triplicate.

2.8. Lentiviral transduction of HUVECs with GFP

HUVECs were transduced using LV-GFP lentiviruses (Hanbio Inc., Shanghai, China) as previously described (Chao et al., 2014). Briefly, P3-4 HUVECs were cultured in 24-well plates at 1×10^4 cells/well in 10% FBS in DMEM for 48 h. The medium was then replaced with 1 ml of fresh medium and 8 μg/ml polybrene. Next, 100 μl of lentivirus solution (107 IU/ml) was added to each well, and the cells were incubated at 37 °C in 5% CO_2 for 24 h. After incubation, the treatment medium was replaced with fresh 10% FBS in DMEM, and the cells were cultured at 37 °C in 5% CO_2 until > 50% confluence was reached. Transduced cells were selected using puromycin. Specifically, the medium was replaced with 10 μg/ml puromycin and 10% FBS in DMEM, and the cells were cultured at 37 °C in 5% CO_2 for 24 h. The cells were then washed twice with fresh 10% FBS in DMEM. Pure, transduced HUVEC cultures were expanded and/or stored in liquid nitrogen as previously described (Carlson et al., 2004).

2.9. In vitro scratch assay

Cell migratory ability was tested using a 2D culture system with an *in vitro* scratch assay. Briefly, 1×10^5 HUVECs were seeded in 24-well tissue culture plates and cultured in growth medium for 24 h, at which time the HUVECs were approximately 70–80% confluent. Using a sterile 200-μl pipette tip maintained in a position perpendicular to the plate bottom, a straight line was carefully scratched in a single direction in the monolayer across the center of the well. A second straight line was scratched perpendicular to the first line to create a cross-shaped gap between cells within each well. Each well was washed twice with 1 ml of fresh growth medium to remove any detached cells. Digital images of the cell gap were captured at different time points, and the gap width was quantitatively evaluated using ImageJ software.

2.10. Statistics

The data are presented as the mean ± SEM. Unpaired numerical data were compared using unpaired *t*-tests (two groups) or an ANOVA (more than two groups), and statistical significance was set at $p < 0.05$.

3. Results

3.1. Changes in soluble cytokine receptor levels in silicosis patients

Soluble cytokine receptors play critical roles in different physiological and pathological settings. Thus, we first measured the levels of soluble cytokine receptors in plasma from healthy donors and silicosis patients (Table 1). As shown in Fig. 1, 5 of the 14 measured soluble cytokine receptors, specifically sIL-2Ra, sTNFRII, sVEGFR1, sVEGFR2 and sVEGFR3, showed significant increases in ten phase II silicosis patients compared with ten healthy donors. None of the analyzed soluble cytokine receptors exhibited a decrease. Interestingly, all three