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Neogambogic acid prevents silica-induced fibrosis via inhibition of high-mobility group box 1 and MCP-1-induced protein 1

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ABSTRACT

Background: Silicosis is a systemic disease caused by inhaling silicon dioxide (SiO_2); early stages are characterized by alveolar inflammation, and later stages are characterized by progressive lung fibrosis. Mounting evidence indicates that high-mobility group box 1 (HMGB1) is involved in pulmonary fibrosis. Whether neogambogic acid (NGA) inhibits macrophage and fibroblast activation induced by SiO_2 by targeting HMGB1 remains unclear.

Methods and results: Experiments using cultured mouse macrophages (RAW264.7 cells) demonstrated that SiO_2 treatment induces the expression of HMGB1 in a time- and dose-dependent manner via mitogen-activated protein kinases (MAPKs) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway; in turn, this expression causes macrophage apoptosis and fibroblast activation. Pretreating macrophages with NGA inhibited the HMGB1 expression induced by SiO_2 and attenuated both macrophage apoptosis and fibroblast activation. Moreover, NGA directly inhibited MCP-1-induced protein 1 (MCPIP1) expression, as well as markers of fibroblast activation and migration induced by SiO_2. Furthermore, the effects of NGA on macrophages and fibroblasts were confirmed in vivo by exposing mice to SiO_2.

Conclusion: NGA can prevent SiO_2-induced macrophage activation and apoptosis via HMGB1 inhibition and SiO_2-induced fibrosis via the MCPIP1 pathway. Targeting HMGB1 and MCPIP1 with NGA could provide insights into the potential development of a therapeutic approach for alleviating the inflammation and fibrosis induced by SiO_2.

1. Introduction

Silicosis is caused by the inhalation of silicon dioxide (SiO_2) and is one of the most serious occupational diseases worldwide. The pathogenic characteristics of silicosis include chronic inflammation and late pulmonary fibrosis (Leung et al., 2012). Mounting evidence suggests that alveolar macrophages (AMs) and pulmonary fibroblasts (PFBs) play critical roles in silicosis (Liu et al., 2016; Liu et al., 2015; Wang et al., 2015). After being exposed to SiO_2, AMs become activated and release inflammatory mediators, such as reactive oxygen species, reactive nitrogen, chemokines, cytokines and growth factors (Fujimura, 2000; Mossman and Churg, 1998). Activated AMs cannot clear silica but are instead induced to undergo apoptosis, which is a mechanism underlying the development of silicosis (Borges et al., 2001; Gu et al., 2013; Lim et al., 1999).

High-mobility group box 1 protein (HMGB1), a nuclear protein expressed during inflammation, cell differentiation and cell migration, is involved in the pathologic mechanism of pulmonary fibrosis-related diseases, such as asthma, chronic obstructive pulmonary disease (COPD) (Ferhani et al., 2010), cystic fibrosis airway disease (Rowe et al., 2008), and idiopathic pulmonary fibrosis (Hamada et al., 2008). Mounting evidence suggests that the effect of HMGB1 mainly occurs via advanced glycation end-product receptors and toll-like receptors (Liu et al., 2011; Tang et al., 2008; Tang et al., 2005). During inflammation, HMGB1 is released into the extracellular space as a cytokine, which stimulates the release of proinflammatory cytokines, such as tumor necrosis factor, interleukin (IL)-1α, IL-6, and IL-8, from monocytes

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(Andersson et al., 2000), macrophages (Kokkola et al., 2005) and neutrophils (Park et al., 2003). In addition, HMGB1 stimulates endothelial cells to upregulate adhesion molecules (Fiuza et al., 2003) and causes dendritic cell maturation (Messmer et al., 2004). Downstream signaling mediated by HMGB1 interaction with these receptors includes mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB), thereby facilitating cellular responses, including cell migration and the release of proinflammatory cytokines (Yang et al., 2012). Lee et al. (2015) demonstrated that HMGB1 induced human lung myofibroblast differentiation and enhanced migration by the activation of matrix metalloproteinase (MMP)-9. However, whether HMGB1 is involved in the fibrosis induced by SiO_2 remains unclear.

Gambogic acid (GA) is a traditional medicine derived from *Garcinia hanburyi*. Various recent studies have reported that GA is a potent anticancer agent with multiple cellular targets in vivo and in vitro (Ishaq et al., 2014; Yu et al., 2012), as well as anti-inflammatory activities against endotoxic shock (Geng et al., 2013). GA has been approved for clinical trials by the Chinese Food and Drug Administration (Yang and Chen, 2013); it was entered into phase I testing in 2004 and is now being tested in phase II clinical trials (Pandey et al., 2007; Wang et al., 2009). However, the side effect of bleeding and the brief half-life period observed in clinical experiments have limited its application in patients. As reported in recent literature, neogambogic acid (NGA) is a GA isomer including an additional hydroxyl group (Lu et al., 1984). Chen reported the enhancement of radiotherapy in breast cancer cells through the use of cerium oxide (CeO_2) nanoparticles modified with NGA (Chen et al., 2015). However, few studies reported in the literature have investigated the effects of NGA in silicosis or the relevant mechanisms.

In this study, we hypothesized that NGA prevents SiO_2-induced AM activation and apoptosis via HMGB1, which, in turn, contributes to PFB proliferation and migration. We demonstrate that NGA targeting HMGB1 and MCP-1-induced protein 1 (MCPIP1) alleviates the inflammation and fibrosis induced by SiO_2.

2. Methods and materials

2.1. Animals

Male C57 mice between 4 and 8 weeks of age were obtained from Nanjing Medical University Laboratories (Nanjing, China). All animals were 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 the ARRIVE guidelines, and the animal protocols were approved by the Institutional Animal Care and Use Committee of Southeast University.

2.2. Reagents

SiO_2 was obtained from Sigma® (S5631, 1–5 μm), selected by sedimentation according to Stokes' law, acid hydrolyzed, and baked overnight (200 °C, 16 h) to inactivate endotoxin contamination. The SiO_2 dosage (50 μg/cm^2) used in this study was based on the previous dose-response experiments and studies of other laboratories (Brown et al., 2007; Fazzi et al., 2014; Hao et al., 2013; Liu et al., 2015). Fetal bovine serum (FBS), normal goat serum (NGS), Dulbecco's modified Eagle's medium (DMEM; #1200-046), and 10× MEM (11430-030) were purchased from Life Technologies™. Amphotericin B (BP2645) and GlutaMax™ Supplement (35050-061) were obtained from Gibco®, and Pen-Strep (15140-122) was obtained from Fisher Scientific. PureCol® type I bovine collagen (3 mg/ml) was obtained from Advanced BioMatrix. Antibodies against HMGB1 (ab18256) and p65 (ab16502) were obtained from Abcam®, Inc. Antibodies against p53 (SC6243), PUMA (SC374223), MCPIP1 (SC136750), F4/80 (SC26642) and β-actin (SC8432) were obtained from Santa Cruz Biotechnology®, Inc. Bax (2772S), Bcl-xL (2764S), Akt (9272S), p-Akt (9271S), ERK (9107S), p-ERK (9101S), JNK (9258S), p-JNK (9251S), p38 (9212S), p-p38 (9211S), caspase-3 (9662S), cleaved caspase-3 (9661S), histone (9715S), p-p65 (3033S), and α-SMA (14968S) were obtained from Cell Signaling®, Inc. The collagen I (BS1530) and collagen III (BS1531) antibodies were obtained from BioWorld®.

2.3. Cell culture

RAW264.7 and L929 cells were purchased from ScienCell and cultured according to the standard protocols; the cells were maintained in T75 flasks in DMEM containing 10% FBS. Cells were stored at passages 3–7 (P3–7) in liquid nitrogen. When the cells reached between P10 and P15, a new vial of P3–7 cells was thawed, seeded, and passaged upon confluence for use in each experiment.

2.4. Lentiviral transduction of L929 cells with green fluorescent protein

L929 cells were transduced with LV-RFP lentivirus (Hanbio, Inc., Shanghai, CN), as previously described (Chao et al., 2014). In brief, P3–4 L929 cells were cultured in a 24-well plate (1×10^4 cells/well) in DMEM containing 10% FBS for 48 h. The medium was replaced with 1 ml of fresh medium containing 8 μg/ml polybrene. Then, 100 μl of lentivirus solution (10^7 IU/ml) was added to each well, and the cells were then incubated at 37 °C and 5% CO_2 for 24 h. After this incubation, the treatment medium was replaced with fresh DMEM containing 10% FBS, and the cells were cultured at 37 °C and 5% CO_2 until the cells reached >50% confluence. The transduced cells were selected using puromycin in the following manner. In brief, the medium was replaced with DMEM containing 10 μg/ml puromycin and 10% FBS, and the cells were cultured at 37 °C and 5% CO_2 for 24 h. Then, the cells were washed twice with fresh DMEM containing 10% FBS. Pure transduced L929 cultures were expanded and/or stored in liquid nitrogen as previously described (Carlson et al., 2004).

2.5. MTT assay

Cell viability was measured via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. In brief, the cells were collected and seeded in 96-well plates. Different seeding densities were employed at the beginning of the experiments. Following incubation for different periods of time (3–24 h), 20 μl of MTT dissolved in Hank's balanced salt solution was added to each well at a final concentration of 5 μg/ml, and the plates were incubated in 5% CO_2 for 1–4 h. Finally, the medium was aspirated from each well, and 200 μl of dimethyl sulfoxide was added to dissolve the formazan crystals. Then, the absorbance of each well was obtained using a plate reader at the reference wavelength of 570 nm. Each experiment was repeated at least three times.

2.6. In vitro scratch assay

Cell migration ability was evaluated using an in vitro scratch assay. In brief, 1×10^5 L929 cells were seeded in 24-well tissue culture plates and cultured in growth medium for 24 h, at which point the L929 cells were approximately 70–80% confluent. Using a sterile 200-μl pipette tip, a straight line was carefully scratched in the monolayer across the center of the well in a single direction while maintaining the tip perpendicular to the plate bottom. Similarly, a second straight line was scratched perpendicular to the first line to create a cross-shaped cellular gap in 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.