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Ginsenoside Rg1 enhances lymphatic transport of intrapulmonary silica *via* VEGF-C/VEGFR-3 signaling in silicotic rats

Jie Yu, Lijun Mao, Li Guan, Yanlin Zhang, Jinyuan Zhao*

Department of Occupational Disease, Peking University Third Hospital, 49 North Garden Road, Beijing, 100191, China

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ABSTRACT

Ginsenoside Rg1, extracted mainly from Panax ginseng, has been shown to exert strong pro-angiogenic activities in vivo. But it is unclear whether ginsenoside Rg1 could promote lung lymphangiogenesis to improve lymphatic transport of intrapulmonary silica in silicotic rats. Here we investigated the effect of ginsenoside Rg1 on lymphatic transport of silica during experimental silicosis, and found that ginsenoside Rg1 treatment significantly raised the silicon content in tracheobronchial lymph nodes and serum to reduce the silicon level in lung interstitium, meanwhile increased pulmonary lymphatic vessel density by enhancing the protein and mRNA expressions of vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor receptor-3 (VEGFR-3). The stimulative effect of ginsenoside Rg1 on lymphatic transport of silica was actively correlated with its pro-lymphangiogenic identity. And VEGFR-3 inhibitor SAR131675 blocked these above effects of ginsenoside Rg1. These findings suggest that ginsenoside Rg1 exhibits good protective effect against lung burden of silica during experimental silicosis through improving lymphatic transport of intrapulmonary silica, which is potentially associated with the activation of VEGF-C/VEGFR-3 signaling pathway.

Silicosis is a chronic interstitial pulmonary fibrotic disease characterized by alveolitis, silicon proteinosis and progressive pulmonary fibrosis, caused by long-term exposure to silica particles (<5 μm) in occupational and environmental settings [1,2]. Although good improvement in monitoring and controlling system for occupational safety and health make this disorder well preventable, silicosis remains a worldwide health problem, particularly in developing countries [2]. In China, the number of cases has increased rapidly in recent years [3]. As inducing irreversible pulmonary fibrosis, silicosis may strengthen susceptibility to tuberculosis, lung cancer, and pulmonary heart disease. However, there has so far been not an effective therapy for silicotic disease.

The cumulative dose of silica is the most fundamental pathogenic factor of silicosis, associated with crystalline silica content and exposure duration [4–6]. Silica is mainly divided into two types of crystalline and amorphous structures. Animal data suggest that crystalline silica is more fibrogenic than is amorphous silica [7]. And it is found that freshly fractured quartz is more pathogenic than does aged quartz, because freshly fractured quartz may produce more active oxygen species [8]. Accordingly the removal of intrapulmonary silica should be an etiologically ideal therapeutic strategy for silicosis, but remains a problem.

Lymphatic vessels play an important role in the removal of tissue fluid, cells, and macromolecules from the interstitium, and return them to the blood circulation through the lymph [9–11]. Lymphatic vessels are more actively involved in interstitial clearance than blood capillaries in the lung [12,13]. Pathological analysis of autopsy in pneumoconiosis patients has found that tracheobronchial *or* hilar lymph nodes can absorb silica particles from lung tissues [14–16], indicating that pulmonary lymphatic system should play an important role in the extrapulmonary clearance of intrapulmonary silica during silicosis.

Ginsenoside Rg1, which is one of the most active ingredient in more than 30 ginsenosides extracted mainly from panax ginseng, which is proved to have a strong angiogenic activity in the treatment of cardiovascular diseases [17–19]. And its angiogenic effect may be associated with the high expression of VEGF in tissues [18–20]. These reports suggest that ginsenoside Rg1 treatment could promote the formation of newborn vessels to improve microcirculation. But little is known role of ginsenoside Rg1 in lymphatic circulation during silicosis. Therefore, in this study, we aim to explore the effect of ginsenoside Rg1 on lymphatic

* Corresponding author.
E-mail address: zhaojinyuan@sina.com (J. Zhao).

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microcirculation and lymphatic transport of intrapulmonary silica particles in silicotic rats, and clarify its mechanisms.

1. Materials and methods

1.1. Reagents

Ginsenoside Rg1 (chemical structure C42H72O14, molecular weight = 801) was purchased from Beijing Bellancom Chemistry Company. Silica dust (99% particle diameter 0.5–10 μm with 80% of particles having diameters of 1–5 μm) was purchased from Sigma Aldrich. SAR131675 was purchased from Selleck Chemicals.

1.2. Animals

Male Sprague–Dawley (SD) rats, weighting 200–220 g, were purchased from the Experimental Animal Center of Peking University (Peking, China). Rats were housed in an air-conditioned room at room temperature with a 12 h light–dark cycle and abundant access to food and water, and allowed to acclimate upon arrival for a week before the experiment. All procedures performed on rats were approved by the Animal Care and Use Committee of Peking University Third Hospital, China.

1.3. Induction of experimental silicosis

As previously reported [21], a rat model of silicosis was established. A 50 mg/mL standard suspension of silica dust in saline was prepared. Prior to tracheal instillation, this solution was autoclaved and then mixed with penicillin (80,000 U/mL). Rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g i.p.). Under direct observation in virtue of a laryngoscope, 1 mL of this suspension was intratracheally instilled by using a syringe with a plastic tube.

1.4. Groups and treatments

All rats were randomly allocated to four different groups (n = 6 in each group): the sham group (Sham), the vehicle group (Vehicle), the ginsenoside Rg1 group (Rg1) and the ginsenoside Rg1+SAR131675 group (Rg1+SAR131675). According to the previous study [19], we selected 10 mg/kg/day as the experimental dosage of ginsenoside Rg1 in rats. And the experimental scheme was conducted as follows: (1) Sham: saline instillation only (n = 6); (2) Vehicle: silica instillation + distilled water (5 mL/kg/day, n = 6); (3) Rg1: silica instillation + ginsenoside Rg1 (10 mg/kg/day, n = 6). (4) Rg1+SAR131675: silica instillation + ginsenoside Rg1 (10 mg/kg/day) + SAR131675 (50 mg/kg/day). The drug was dosed once daily using gastric gavage for 4 weeks. Rats were sacrificed at 1, 2, 3, and 4 weeks after treatment. After blood collection, animals were euthanized, and the left lung was lavaged immediately with 2 mL PBS twice. The lavage collections were centrifuged at 1409 g for 10 min at 4 °C, and the supernatant collected for enzyme linked immunosorbent assay (ELISA). The right lung was sliced into small pieces and subsequently used for real-time PCR and pathological examination. And the serum, supernatant, tracheobronchial lymph nodes and left lung were procured for inductively coupled plasma-optical emission spectrometer (ICP-OES).

1.5. ELISA

In this study, ELISA was performed as described previously [21]. VEGF-C levels in bronchoalveolar lavage fluid (BALF) and serum were quantified using the VEGF-C assay kit (Cloud-Clone, Houston, TX, USA) according to the manufacturer's instructions. All standards, controls, and samples were measured in duplicate wells. Samples were frozen at the time of collection and stored at −80 °C. Samples were not subjected to freeze–thaw cycles.

1.6. RNA isolation and real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Real time-PCR was performed as described previously [21] with the primers shown as follows: VEGF-C: forward primer 5′-AACTGCTCCTCCAGGTCTTTGC-3′, reverse primer 5′-TGCTGTGCTTCTTGTCTCTGGC-3′, 172 bp; VEGFR-3: forward primer 5′- CTTCCAAGTCTCCTCCTATCAGC-3′, reverse primer 5′-ATTCACATCGGT AACCACCTCA-3′, 227 bp; LYVE-1: forward primer 5′-CTTCCAAATCAGG ACACCCAC-3′, reverse primer 5′- AAGGACCAAGTTGAAACAGCC -3′, 141 bp; β-actin: forward primer 5′-GTTGGCATAGAGGTCTTTACGG -3′, reverse primer 5′-TGCTATGTTGCCCTAGACTTCG-3′, 240 bp. The level of β-actin mRNA in each sample was used as an internal control.

1.7. Immunohistological staining

Lung sections (5 μm) were deparaffinized with xylene and rehydrated with graded ethanol. Antigen retrieval was performed by boiling the sections in low-pH citrate buffer for 2 min. The sections were stained and visualized by ZSGQ-BIO ABC kit (ZSGQ-BIO, Beijing, China). Primary antibodies used in this study were as follows: sheep polyclonal to lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (R&D Systems, Minneapolis, MN, USA) at a 1:200 dilution; rabbit polyclonal to vascular endothelial growth factor-C (VEGF-C) at a 1:50 dilution (Abcam, Cambridge, UK). Specimens were examined with a Leica DM2500 polarizing light microscope.

2. ICP-OES

Tracheobronchial lymph nodes (TBLNs), lung and blood samples were collected to determine the silicon content in rats. The wet samples of tracheobronchial lymph nodes and lung tissues (excluding tracheobronchial structures) were weighed, then digested with nitric acid by heating and then analyzed for silicon content using iCAP6000 ICP-OES (Thermo Scientific, MT, US).

2.1. Statistical analysis

All values were expressed as mean ± standard deviation (S.D.). Data were analyzed using two-way analysis of variance. Correlations between variables were assessed using Pearson correlation analysis. Results were considered statistically significant when $P < 0.05$. All statistical analyses were calculated using SPSS13.0 (IBM, Chicago, IL, USA).

3. Results

3.1. Ginsenoside Rg1 decreases lung burden of silica in silicotic rats

To explore effect of ginsenoside Rg1 treatment on the lung burden of silica during silicosis, we applied ICP-OES to determine the silicon content in lung interstitium. And ICP-OES analysis showed that the silicon content in lung in the Vehicle group increased significantly in a time-dependent manner, which was higher than the Sham group (Fig. 1A). And the silicon content in lung interstitium was decreased significantly after ginsenoside Rg1 treatment, compared with the Vehicle group (Fig. 1A). These data suggested that ginsenoside Rg1 may decrease lung burden of silica during experimental silicosis.