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Original Article

Galectin-3 mediates the effect of PDGF on pulmonary arterial hypertension

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Abstract: Aims: The present study aimed to perform in vitro experiments to investigate whether Galectin-3 (Gal-3) mediates the effect of platelet-derived growth factor (PDGF) on pulmonary arterial smooth muscle cells (PASMC) proliferation, apoptosis and migration, and to reveal the mechanism of how Gal-3 functions in the pathogenesis of pulmonary arterial hypertension (PAH). Methods: Pulmonary arterial smooth muscle cells (PASMC) were treated with various concentration of PDGF for indicated times, and the expression of Gal-3 was analyzed by western blotting. Gal-3 siRNA was transfected into the PASMC to knock down endogenous Gal-3. MTT assay was performed to examine cell proliferation. Transwell-migration assay was used to determine cell migration ability. Cell apoptosis rate was determined by flow cytometric analysis. Results: The result showed that the expression of Gal-3 protein was induced by PDGF in a dose- and a time-dependent manner. PDGF contributes to the progression of PAH by inducing cell proliferation and migration, as well as inhibiting cell apoptosis of PASMC. However, these effects of PDGF on PASMC were attenuated by Gal-3 knockdown. Conclusion: The present study provided potential evidence about the role of Gal-3 in the pathophysiological mechanisms of PAH. This study firstly demonstrated that Gal-3 could be induced by PDGF in PASMC, and mediates the effect of PDGF on PASMC proliferation, apoptosis and migration, thus contributing to the pathogenesis of PAH.

Keywords: Galectin-3, pulmonary arterial hypertension, platelet-derived growth factor

Introduction

Pulmonary arterial hypertension (PAH) is a fatal and progressive disease which is clinically characterized by a sustained elevation in pulmonary artery pressure [1, 2]. Various pathological conditions, such as collagen vascular disease, chronic exposure to hypoxia, intracardiac left-to-right shunting and portal hypertension, cause the induction of PAH [3]. Although the medical management of PAH has improved in the past years, patients with PAH still have a greatly increased risk of death, and the median survival of patients with idiopathic/familial PAH is only 7 years [4].

Galectins are a family of soluble β-galactoside-binding lectins, and Galectin-3 (Gal-3) is a member of this lectin family. Gal-3 participates in numerous cellular functions including growth, apoptosis, metastasis and adhesion [5, 6]. Serum galectin-3 levels were higher in systemic sclerosis patients with elevated right ventricular systolic pressure (RVSP) than in those without this symptom [7]. It has been reported by Fenster et al that serum Gal-3 level was significantly increased in the patients with PAH compared with the controls [8]. However, the mechanism of how Gal-3 functions in the pathogenesis of PAH remains unknown.

Platelet-derived growth factor (PDGF) is an important mitogen which acts as a stimulator not only for its own expression [9], but also for the synthesis of other mitogens, such as vascular endothelial growth factor, heparin-binding epidermal growth factor and endothelin- endothelin-1, in vascular smooth muscle cells [10]. Elevated PDGF level has been implicated in the patients with PAH [11-15].

In the present study, we performed in vitro experiments on pulmonary arterial smooth muscle cells (PASMC) to investigate the effect

of Gal-3 on cell proliferation, apoptosis and migration in the presence of PDGF. This study may provide potential evidence about the role of Gal-3 in the pathophysiological mechanisms of PAH.

Materials and methods

Cell culture

Human pulmonary artery smooth muscle cells (PASMC) were purchased from Lonza (Walkersville, MD, USA). The cells were cultured in Smooth Muscle Cell Medium (SMCM; Lonza) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), and maintained at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every three days. The PDGF was obtained from R&D Systems, Inc. (Minneapolis, MN, USA) and dissolved in sterile 4 mM HCl.

Cell transfection

For cell transfection, 1×10^5 cells were seeded into the 6-well plates. When the cells reached about 80% confluence, cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, siRNA and Lipofectamine 2000 were mixed in serum-free Opti-MEMI (Invitrogen) and incubated at room temperature for 20 min. Cells were incubated with the complexes for 6 h, then the cells were washed and maintained in the fresh medium.

Western blotting

The cells were gently washed with PBS and lysed in RIPA buffer (Beyotime, Shanghai, China). The protein concentration in the cell lysis was determined by the bicinchoninic acid protein assay using a BCA Protein Assay Kit (Beyotime). 25 μg of proteins were separated on 10% SDS-PAGE, and then transferred to nitrocellulose membranes (EMD Millipore Corporation, Billerica, MA, USA) by electroblotting. After incubation overnight at 4°C in a blocking buffer (0.1% Tween 20 in PBS) containing 5% bovine serum albumin, the membranes were incubated with mouse monoclonal antibody to Gal-3 (sc-32790; dilution, 1:400, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonal antibody to β-actin (sc-130301; dilution, 1:1000, Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. The membranes were then washed with TBST and incubated with rabbit anti-mouse horseradish peroxidase-conjugated IgG (sc-358914; dilution, 1:5000, Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The signals were detected using an enhanced chemiluminescence detection system (ECL Western Blotting kit; Pierce Biotechnology, Inc., Rockford, IL, USA). The band density was quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA). β-actin was used as an internal control.

Cell proliferation assay

A cell suspension containing 1×10^4 cells was distributed into 96-well plates. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. After incubation for the appropriate times, 10 μL of methyl thiazol tetrazolium (MTT) solution (0.5 mg/mL; Beyotime) was added to each well, and the cells were incubated at 37°C for 4 h. The supernatants were then removed, and 150 μl of dimethyl sulfoxide was added and the plates were oscillated for 15 min. The optical density was measured by a microplate reader (Thermo Labsystems, Waltham, MA, USA) at a wavelength of 570 nm.

Flow cytometric analysis

The cells were digested into a single cell suspension and dual-stained with Annexin V-FITC and PI using the Annexin V-FITC and propidium iodide (PI) apoptosis kit (KeyGEN Biotech. CO., LTD, Nanjing, Jiangsu, China) according to the manufacturer's instructions. After incubation at room temperature for 15 min in the dark, the cells were assayed with flow cytometry (Beckman Coulter, Inc., Miami, FL, USA).

Transwell migration assay

The 6-well Transwell system (8 μm; Corning, Inc., Corning, NY, USA) was used to perform cell migration assay. 1 mL of Smooth Muscle Cell Medium containing fetal bovine serum was added to the lower chamber. The PASMC were suspended in Smooth Muscle Cell Medium without serum, then 2 mL of cell suspension at the density of 5×10^4/mL was added to the Transwell plates. After incubation at 37°C for 24 h, the non-migrated cells were removed using a cotton swab, the migrated cells were fixed in 95% ethanol for 15 min followed by staining with hematoxylin for 10 min. The numbers of migrated cells were counted under a microscope (Nikon, Tokyo, Japan).

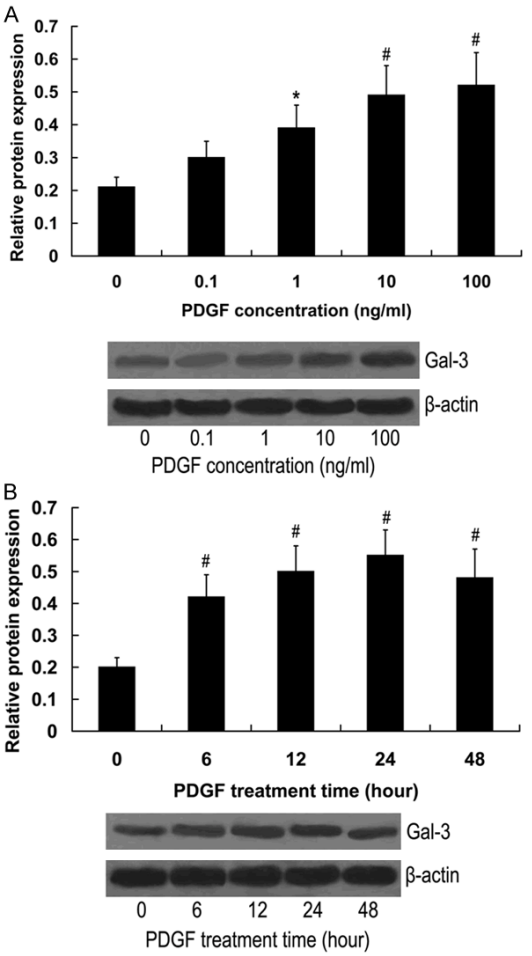

Figure 1. PDGF induced the expression of Gal-3 in PASMC. A. The dose-dependent effect of PDGF on Gal-3 protein expression. $^{*}P<0.05$ and $^{\#}P<0.01$ compared with 0 ng/ml. B. The time-dependent effect of PDGF on Gal-3 protein expression. $^{\#}P<0.01$ compared with 0 h. Gal-3, Galectin-3; PDGF, platelet-derived growth factor.

Statistical analysis

All data were shown as means ± standard deviation. The statistical significance between two groups was determined by Student's *t*-test. A *P*-value of <0.05 was considered to be significant.

Results

PDGF induced the expression of Gal-3 in PASMC

The PASMC were incubated with various concentrations of PDGF for 24 h, subsequently, western blotting analysis was performed to examine the expression of Gal-3 in PASMC. As shown in **Figure 1A**, treatment of PASMC with PDGF induced an increased protein level of Gal-3, which reached a maximum level at 100 ng/ml.

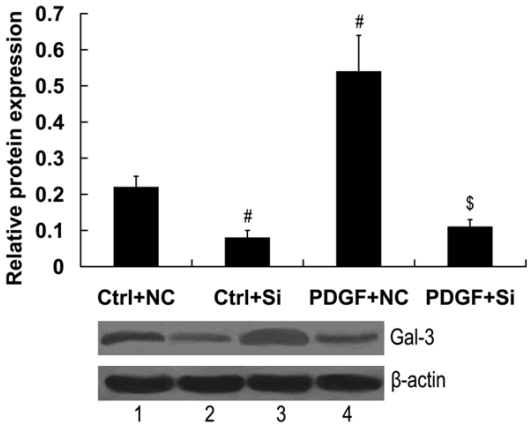

Figure 2. Expression of Gal-3 protein in PASMC following treatment with PDGF and transfection of Gal-3 siRNA. Lane 1, Ctrl + NC; lane 2, Ctrl + Si; lane 3, PDGF + NC; lane 4, PDGF + Si. $^{\#}P<0.01$ compared with Ctrl + NC; $^{\$}P<0.01$ compared with PDGF + NC. Gal-3, Galectin-3; PDGF, platelet-derived growth factor; Ctrl, control; Si, siRNA.

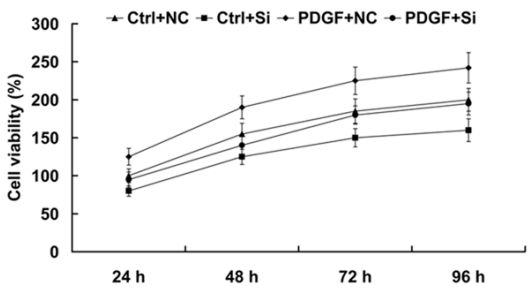

Figure 3. Gal-3 mediates the effect of PDGF on PASMC proliferation. Gal-3, Galectin-3; PDGF, platelet-derived growth factor; Ctrl, control; Si, siRNA.

In addition, the PASMC were incubated with 100 ng/ml PDGF for 6, 12, 24 and 48 h, respectively. The results from western blotting analysis showed that the expression of Gal-3 increased from 6 h after PDGF treatment, and reached maximum level at 24 h. The increasing of Gal-3 expression maintained to 48 h after PDGF treatment (**Figure 1B**).

Gal-3 mediates the effect of PDGF on PASMC proliferation

In order to determine the effect of endogenous Gal-3 on PASMC proliferation, Gal-3 siRNA was

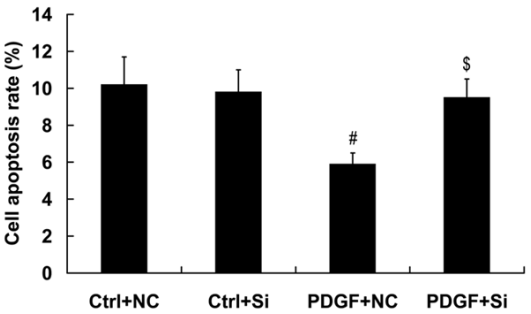

Figure 4. Gal-3 mediates the effect of PDGF on PASMC apoptosis. #*P*<0.01 compared with Ctrl + NC; $*P*<0.01 compared with PDGF + NC. Gal-3, Galectin-3; PDGF, platelet-derived growth factor; Ctrl, control; Si, siRNA.

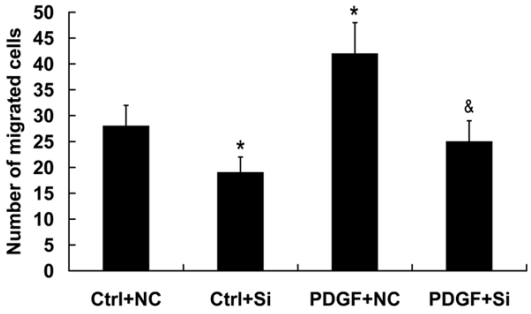

Figure 5. Gal-3 mediates the effect of PDGF on PASMC migration. *P<0.05 compared with Ctrl + NC; &P<0.05 compared with PDGF + NC. Gal-3, Galectin-3; PDGF, platelet-derived growth factor; Ctrl, control; Si, siRNA.

transfected into the PASMC to knock down Gal-3 expression. As demonstrated in **Figure 2**, the expression of Gal-3 protein was significantly decreased in the PDGF-treated or untreated PASMC following Gal-3 siRNA transfection.

MTT assay was performed to examine PASMC proliferation. Cells incubated with 100 ng/ml PDGF for 24 h proliferated at a higher speed compared with the control. The cells transfected with Gal-3 siRNA proliferated at a lower speed compared with the control. In addition, knockdown of endogenous Gal-3 significantly abolished the PDGF-mediated promotion of cell proliferation (**Figure 3**).

Gal-3 mediates the effect of PDGF on PASMC apoptosis

PASMC apoptosis was determined by FCM analysis. As shown in **Figure 4**, compared with the control + NC group, the cell apoptosis rate did not alter in the control + Gal-3 siRNA group, but significantly decreased in the PDGF + NC group. However, Gal-3 knockdown could reverse the effect of PDGF on cell apoptosis. The cell apoptosis rate was significantly increased in the PDGF + Gal-3 siRNA group compared with the PDGF + NC group.

Gal-3 mediates the effect of PDGF on PASMC migration

PASMC migration was determined by Transwell migration assay. It was shown in **Figure 5**, the number of migrated cells was significantly increased in the PDGF + NC group compared with the control + NC group. Furthermore, the number of migrated cells in both the control + Gal-3 siRNA and PDGF + Gal-3 siRNA group was significantly decreased after Gal-3 knockdown.

Discussion

Gal-3 is involved in numerous physiological and pathological processes [16-18]. It controls cell cycle, reacts with many extracellular and/or intracellular proteins, modulates adhesion and affects cellular differentiation [19-22]. Changes in Gal-3 expression have been implicated in the physiopathology of multiple diseases, including liver and kidney fibrosis [23, 24], heart failure [25, 26] and tumors. It has been found in a clinical study that the expression level of Gal-3 in the serum of patients with PAH is higher than that in the control subjects, and Gal-3 levels are associated with multiple indices of right ventricular functional and morphologic changes in PAH [8]. In the present study, we performed in vitro study to further elucidate the role of Gal-3 in PAH.

The mitogenic effect of PDGF on vascular smooth muscle cells is associated with hypertrophy, hyperplasia, migration and vascular remodeling [10, 11, 13, 14, 27]. It has been demonstrated that patients and animal with PAH showed high levels of PDGF in the blood plasma and lung tissues [11, 12, 14, 28], suggesting a critical role of PDGF in the progression of PAH. In this study, we firstly found that the expression of Gal-3 was induced by PDGF in a dose- and a time-dependent manner. Subsequently, we investigated whether Gal-3 is involved in the effect of PDGF on PAH.

PAH is pathologically characterized by pulmonary vascular remodeling [29, 30]. The abnor-

mal proliferation, apoptosis and migration of PASMC contribute to pulmonary vascular remodeling [13, 31, 32]. In the present study, we found that PDGF contributes to the progression of PAH by inducing cell proliferation and migration, as well as inhibiting cell apoptosis of PASMC. However, these effects of PDGF on PASMC were attenuated by Gal-3 knockdown. These findings demonstrated that Gal-3 at least partially mediates the effect of PDGF on PAH.

In conclusion, this study firstly demonstrated the in vitro role of Gal-3 in PAH. Gal-3 could be induced by PDGF in PASMC, and mediates the effect of PDGF on PASMC proliferation, apoptosis and migration, thus contributing to the pathogenesis of PAH. Gal-3 may represent a novel target for the treatment of PAH.

Disclosure of conflict of interest

None.

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