Influence of Rho Kinase Inhibitor Fasudil on Late Endothelial Progenitor Cells in Peripheral Blood of COPD Patients with Pulmonary Artery Hypertension

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Abstract

The objective of our work was to investigate the influence of Fasudil, a Rho inhibitor on the number and function of the late endothelial progenitor cells in peripheral blood of chronic obstructive pulmonary diseases (COPD) patients with pulmonary artery hypertension. Eighty COPD patients with pulmonary artery hypertension were selected and divided into two groups: the treatment group and the control group, which had 40 patients respectively. The control group received routine treatment, including oxygen uptake, anti-infection and phlegm dissolving. The treatment group received the Fasudil in addition to the routine treatment. The changes on the number and function of the late endothelial progenitor cells in peripheral blood of the patients before and after the treatment were compared between the two groups. The changes on the pulmonary artery pressure were also compared. The number of the late endothelial progenitor cells in peripheral blood of the treatment group increased and the function was enhanced. The pulmonary artery pressure was reduced. The difference before and after the treatment and with the control group was statistically significant ($p<0.05$). The changes on the number and function of the late endothelial progenitor cells in peripheral blood and the pulmonary artery pressure before and after the treatment of the control group were not statistically significant ($p>0.05$). The Rho-kinase inhibitor Fasudil increased the number and enhanced the function of the late endothelial progenitor cells in peripheral blood of COPD patients with pulmonary artery hypertension.

KEY WORDS: Rho Kinase Inhibitor Fasudil, chronic obstructive pulmonary diseases, pulmonary artery hypertension, endothelial progenitor cells, number and function of the late endothelial progenitor cells

INTRODUCTION

Hypoxic pulmonary artery hypertension (PAH) is the core stage of the pathogenesis process of chronic obstructive pulmonary diseases (COPD). The decrease of the pulmonary artery pressure retards the pathogenesis and development of COPD significantly [1]. The search for the ideal method to decrease the pulmonary artery pressure is the key to prevent and cure COPD and cor pulmonate. Studies in recent years demonstrated that the damage and malfunction of endothelium play an important role in the pathogenesis and development of PAH [2]. The endothelial progenitor cells (EPC) participate not only in the formation of human embryonic vessels, but also in the neogenesis of blood vessels after birth and the repairment after endothelium damage [3]. Korean scientists, Hur et al. [4] reported in 2004 the presence of EPC of two kinds of different biological characteristics in adult peripheral blood and named these two kinds of peripheral blood EPC early EPC and late EPC. Following research considered the late EPC, coming from the bone marrow, as the real EPC, which directly takes part in the repairment of vascular endothelium and plays an important role in the neogenesis and repairment of blood vessels [5]. In recent years, the relationship between Rho/Rho-kinase signaling pathway and cardiopulmonary vascular diseases has drawn

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an increasing attention. This research investigated the influence of Fasudil, a Rho kinase inhibitor on the number and function of the late endothelial progenitor cells in peripheral blood of COPD patients with pulmonary artery hypertension by the intervention of Rho kinase inhibitor Fasudil on COPD patients with pulmonary artery hypertension.

MATERIALS AND METHODS

Research Objects

A total of 80 COPD patients with pulmonary artery hypertension hospitalized from July 2010 to April 2012 were selected, in which male patients represented 40 cases and female, 40 cases. The age of the patients ranges from 50 to 75 years old. The average age was 63.5±11.6 years old, conforming to the diagnosis criteria. The patients with diabetes, liver and kidney disease, blood diseases, recent surgery, wounds, inflammation, tumor, cerebrovascular disorder and acute myocardial infarction that could interfere with the EPC were ruled out. The selected patients did not receive statins. The patients were divided randomly into two groups. The treatment group had 40 patients, in which 22 male and 18 female. The control group also had 40 patients, in which 24 male and 16 female. The composition of age and sex of the two groups were not statistically different. All the research objects had been informed of the detailed research process. This experiment had the consent of the patients and was granted by the local ethnics committee.

Research Methods

All the patients of the two groups received such routine treatment as oxygen uptake, anti-infection and phlegm dissolving. The treatment group received intravenous drip of 30 mg of Fasudil hydrochloride injecta added by 100 ml physiological saline. It was applied twice a day and 21 days was a period of treatment.

Measurement of pulmonary artery pressure. The 5500 type color Doppler ultrasound of HP was used. Round chamber view section of the ventricular apex was selected to observe the regurgitation of the tricuspid valve and transprosthetic gradient (ΔP). It was estimated that the systolic pressure of pulmonary artery was ΔP+10mmHg (right atrial pressure).

Separation, culture and identification of late EPC. The peripheral vein blood of the subjects was collected and the mononuclear cells were separated by density gradient centrifugal method. The obtained mononuclear cell suspension was inoculated to a 24 pore cell culture plate covered by human fibronectin. The inoculation density was 2×lo6cells/ml. The plate was cultured in an incubator of CO_2 at 37°C. The fluid was changed for the first time after 4 days. Then, it was changed every 2 days until the 21st day of culture. The cells were washed with PBS for further analysis. The adherent cells were collected to measure the positivity of cell phenotypes CD34 and KDR with a flow cytometry and the absorption of DiLDL combined with FITC-UEA-I (double positivity) by the laser scanning confocal microscope.

Late EPC count. The obtained late EPC were counted under 200 times inverted microscope and the average was obtained. The cellular morphology of early and late EPC was observed respectively.

Measurement of the proliferation ability of late EPC. The adherent cells in the primary culture were digested by 0.25% trypsinase and prepared to be single cell suspension with culture fluid. The cell suspension concentration was adjusted to 3*105/mL. It was inoculated to a 96 pore plate with the density of 104 for every pore. After 48h of culture, its reproduction ability was measured by MTT method.

Measurement of adherence ability

The adherent cells were digested by 0.25% trypsinase and suspended in 500μL culture fluid for counting. Then the equal number of EPC was spread on a culture plate covered by HFN and cultured for 30 min at 37°C. Then, the adherent cells were counted under 200 time microscope.

Measurement of migration ability

The adherent cells were collected and counted as above. 25μL culture fluid and vascular endothelial growth factor (VEGF, 50 μg/L) were added to the lower chamber of the improved Boyden chambers. 2*104 EPC suspended in 50 μL culture fluid was added to the upper chamber. When cultured for 24 h, the unmigrated cells at the surface of the filter membrane were removed and fixated by methane and then stained by Giemsa. 3 random fields of microscope (x 400) were selected to count the cells migrated to the bottom.

Statistical analysis

Statistical analysis was conducted by SPSS13. 0 statistical software. The data was demonstrated by x ±s. The comparison between mean values was conducted with analysis of variance and the comparison between rates was conducted with $\chi 2$ test. Using $P<0.05$ as the variance was considered as statistically significant.

RESULTS

Basic Information

Comparison of age, sex, blood glucose, blood pressure, total cholesterol and low density lipoprotein (LDL) between the two groups.

TABLE 1. Comparison of the basic clinical data between the two groups

Clinical information	Treatment group	Control group
Age (years old)	61.3±5.5	63.7±7.6
Sex (male/female ratio)	22:18	24:16
Smoking (case)	16 (40%)	18 (45%)
Hypertension (case)	4 (10%)	6 (15%)
Blood glucose (mmol/l)	5.27±1.17	4.83±1.03
Total cholesterol (mmol/l)	4.71±1.45	4.15±1.47
Low density lipoprotein (mmol/l)	2.93±0.94	2.75±0.90

The variance of age, sex, blood glucose, blood pressure, total cholesterol and low density lipoprotein betwen the two groups was not statistically significant.(Table 1)

Late EPC Identification

The late EPC cultured in isolation was measured for the positivity of cell phenotypes of CD_{34} and KDR by a flow cytometry (Figure 1) and the absorption of DiLDL combining with FITC-UEA-I (double positivity) (Figure 2).

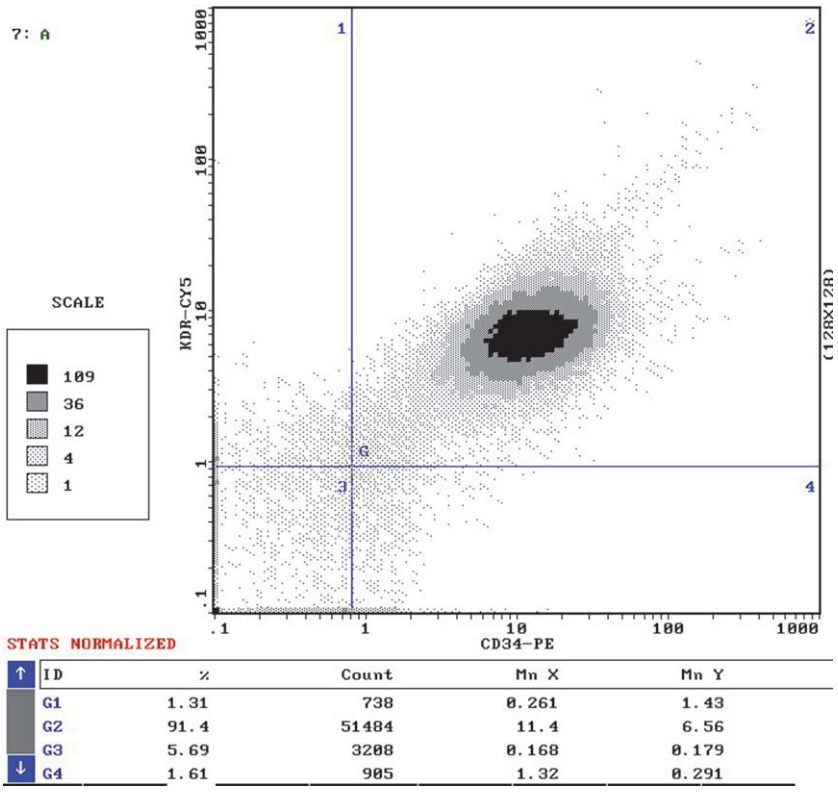

FIGURE 1. The late EPC cultured in isolation was measured for the positivity of cell phenotypes of CD34 and KDR by a flow cytometry.

Comparison of systolic pressure of pulmonary artery before and after treatment of two groups.

In fasudil treatment group, the pulmonary arterial pressures were significantly decreased, the right ventricular outflow tract diameter was reduced and the ejection fraction was significantly improved. They were significantly different from those before treatment and from those in the control group (P<0.05). (Table 2)

Changes on number and function of late EPC in perpheral blood before and after treatment of the two groups.

In fasudil treatment group, the number of late EPC in peripheral blood was increased; the migration, adhesion and proliferation capacities were also increased, which were significantly different from those before treatment and from those in the control group (P<0.05). (Table 3)

TABLE 2. Comparison of the pulmonary arterial systolic pressures before and after treatment between the two groups

		Systolic pressure of pulmonary artery (mmHg)	Internal diameter of outflow tract of right ventricle (mm)	Ray fraction (%)
Treatment group n=40	before treatment	49.00±4.64	33.10±3.53	52.10±4.31
	after treatment	25.00±3.26①	27.50±1.26①	65.00±2.25①
Control group n=40	before treatment	47.00±3.62	32.00±3.15	53.00±3.18
	after treatment	45.15±3.70②	30.28±1.37②	52.00±3.14②

① Variance is statistically different with that before treatment, $p<0.05$; ② Variance is statistically different with the treatment group, $p<0.05$.

TABLE 3. Comparison of the changes in the number and function of late EPC in the peripheral blood before and after treatment between the two groups

		Number	reproduction ability	migration ability	adherenc ability
Treatment group n=40	before treatment	14.35±6.32	0.15±4.55	9.10±4.81	10.50±1.28
	after treatment	**25.00±3.26①**	**0.29±2.25①**	**17.00±3.27①**	**24.85±2.32①**
Control group n=40	before treatment	14.58±3.51	0.15±2.44	9.05±1.27	10.28±2.37
	after treatment	**16.15±2.74②**	**0.14±3.29②**	**10.00±2.19②**	**10.58±2.56②**

① Variance is statistically different with that before treatment, $p<0.05$; ② Variance is statistically different with the treatment group, $p<0.05$.

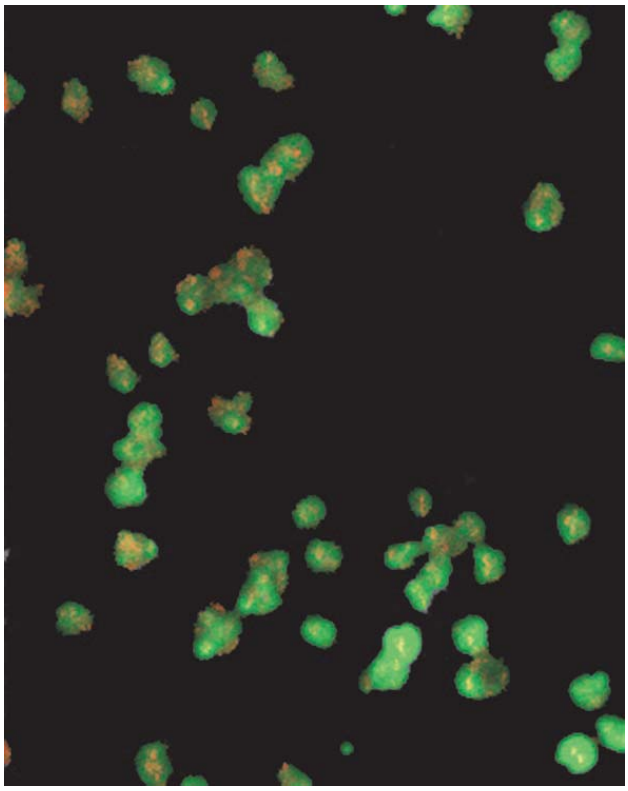

FIGURE 2. The absorption of DiLDL combining with FITC-UEA-I (double positivity)

DISCUSSION

The reformation of pulmonary vessels is the main factor for the pathogenesis of COPD with pulmonary artery hypertension. It is generally believed that systematic endothelial damage is the starting phase of pulmonary artery hypertension. The damage and / or malfunction of pulmonary endothelial cells might cause endogenous unbalance between pulmonary vasodilator and vasoconstrictor. Thus, the relaxation vascular active substance decreases and the contracting vascular active substance increases [6]. In addition, the damaged endothelial cells secrete multiple cell factors, such as fibroblast growth factor, platelet derived growth factor, etc, to promote the hyperplasia and hypertrophy of vascular smooth muscle cells. In a pulmonary artery hypertension case, the damage of vascular endothelial cells is one of the factors in the pathogenic processes. If this process is inhibited, the development of pulmonary arter hypertension could be possibly inhibited. EPC is reproductive and could be differentiated to be vascular endothelial cells to accelerate the repairment of damaged vascular endothelium. Studies showed that endogenous erythrocyte stimulating factor was able to promote the EPC mobilization from chronic hypoxia mouse bone marrow to prevent the PAH process and reduce the hypertrophy of the right ventricle and the reconstruction of pulmonary vessels [8]. As EPC has an important role in the maintaining of the complete functions of endothelium, the treatment of PAH by EPC also draws the attention of researchers home and abroad. This research found that the pulmonary artery pressure of the patients in the Fasudil treatment group was reduced significantly. The number and function of the late endothelial progenitor cells in peripheral blood were also enhanced. It is conjectured that the following mechanisms are possible. In recent years, the relationship between Rho/Rho-kinase signaling pathway and cardiopulmonary vascular diseases has drawn an increasing attention. Fasudil is a selective inhibitor of Rho-kinase signalling pathway. It competes for the combination with the ATO combination site in the kinase area of Rho-kinase with ATP, so as to block the activity of Rho-kinase. It could also inhibit multiple proteinases such as myosin light chain proteinase and protein kinase, leading to the inhibition of phosphorylation at the final stage of contraction of smooth muscle. Thus, the vessels are expanded [9]. Fagan et al. [10] found that the selective inhibitors -Y-27632 and Fasudil of Rho/Rho-kinase signaling pathway were able to lower the pulmonary artery pressure and pulmonary vascular resistance in mice with pulmonary artery hypertension and had important adjustment effects on the vascular structure and function [11]. Recent studies proved that Fasudil could inhibit the synthesis and secretion of ET-1 by endothelial cells and promote the synthesis and secretion of NO, to improve the balance between ET-1 and NO. Therefore, the endothelium mediated relaxation vascular effect was enhanced [12]. Therefore, Fasudil could enlarge the vessels by Rho/Rho-kinase signaling pathway to reduce the pulmonary artery pressure; furthermore, it could adjust the endothelial cells function so that the latter decreases the secretion of blood vessel contraction substance and increase relaxation substance. In this way, the pulmonary artery pressure is lowered. With the development of COPD, the peripheral airway obstruction, lung parenchyma damage and abnormality of pulmonary blood vessels reduce the pulmonary gas exchange capacity and causes hyoxemia, even hypercapnia. Long term of chronic hypoxia results in the pulmonary artery hypertension. The main mechanism is hypoxic pulmonary vasoconstriction (HPV), dysfunction of endothelium of pulmonary vessels and reconstruction of pulmonary vessels. The vascular endothelial cells have important role in adjusting human pulmonary circulation. The endothelial cells could release multiple vascular relaxation substances to adjust the vascular reactions. These substances are called endothelium derived relaxation factors (EDRF) [13]. No is one of them. In COPD patients with pulmonary artery hypertension, EDRF mediated vascular contraction is damaged, pulmonary vascular resistance (RPV) increased. These are the results of endothelial damage caused by shear stress of hemodynamics and hypoxia [14]. The endothelial progenitor cells (EPCs) are precursor cells that can be dif-

ferentiated into vascular endothelial cells directly. EPC plays an important role in the neogenesis and maintaining of the completeness of the function of endothelium of blood vessels [15]. Yamada et al. [16] proved that stem cells were necessary for the repairment of pulmonary tissues with the lipopolysaccharide mediated mouse pneumonia model. Nagaya et al. [17] reported that in the monocrotaline mediated pulmonary artery hypertension nude mouse model, xenotransplantation of EPC reduced the pulmonary vascular resistance by 16%. Gene infected EPC could reduce the pulmonary vascular resistance by 35% and its survival rate was higher [17]. These studies confirmed that EPC could enhance the damaged pulmonary artery endothelial repairment and the reconstruction of artery, so as to reduce the pulmonary artery pressure. In this research, Fasudil reduced the pulmonary artery pressure of COPD patients with pulmonary artery hypertension. One of the mechanisms is to increase the number and function of the late EPC in peripheral blood of the patients to reduce the damage of pulmonary vascular endothelial cells and improve the reconstruction of pulmonary vascular structure, so as to obtain the treatment effects.

CONCLUSION

Fasudil as a Rho-kinase inhibitor can increase the number and function of late EPC in the peripheral blood of COPD patients combined with pulmonary hypertension.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

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