Atorvastatin improves endothelial progenitor cell function and reduces pulmonary hypertension in patients with chronic pulmonary heart disease

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OBJECTIVE: To explore the effects of atorvastatin on the migration and adhesion of endothelial progenitor cells (EPCs) and on pulmonary artery pressure (PAP) in patients with chronic pulmonary heart disease.

METHODS: A total of 68 patients with chronic pulmonary heart disease were randomly assigned to either a control group (n=35) or a treatment group (n=33). In addition, 30 healthy volunteers (17 male, 13 female) were enrolled as healthy controls. Atorvastatin (20 mg per day) was administered to the treatment group. The migration and adhesion activities of EPCs in peripheral blood were assessed before and after six months of treatment. PAP was measured using echocardiography before and after the treatment.

RESULTS: EPC number, migration ability and adhesion activity in the peripheral blood of patients in the treatment group were lower than in patients in the healthy control group at baseline (all P<0.05). After six months of atorvastatin therapy, the number of EPCs in the treatment group was greater than in the control group (P<0.05). Migration and adhesion functions of EPCs in the treatment group were greater than in the control group (all P<0.05). The reduction in PAP in the treatment group was greater than in the untreated control group following six months of therapy (P<0.05).

CONCLUSION: Atorvastatin therapy increased the migration and adhesion activities of EPCs in patients with chronic pulmonary heart disease. Atorvastatin treatment was also associated with a reduction in PAP in these patients.

Key Words: Atorvastatin; Chronic pulmonary heart disease; Endothelial progenitor cells; Pulmonary hypertension

METHODS

Patient selection
A total of 68 patients with pulmonary heart disease, diagnosed between September 2008 and December 2009 at Liaocheng People’s Hospital of Taishan, Taishan, China, were enrolled in the present study. Patients were randomly assigned to either a control group (n=35) or a treatment group (n=33). In addition, 30 healthy volunteers (17 male and 13 female) were enrolled as control subjects.

Chronic pulmonary heart disease was diagnosed according to current guidelines (1). Patients were eligible for inclusion if they met the following criteria: 60 to 85 years of age; in the stable phase of chronic pulmonary heart disease, in which the forced expiratory volume in 1 s (FEV₁) was ≤65% of the predicted value, the ratio of FEV₁ to the forced vital capacity was ≤70% and the pulmonary hypertension systolic pressure was >30 mmHg; and New York Heart Association (NYHA) functional class I or II. Patients with any one of the following conditions were excluded from the present study: a history of valvular heart disease, congenital heart disease, trauma, ulcer, retinopathy, surgery in the previous three months, acute inflammation, tumour, chronic liver or kidney disease, and acute myocardial infarction in the previous three months.

The present study was approved by the Human Research Ethics Committee of Liaocheng People’s Hospital, Taishan, China. Informed consent was obtained from all participants.

Intervention
The management strategy for COPD was identical between the control and treatment groups, and included administration of oxygen, bronchodilation and control of infections if present. Atorvastatin (20 mg/day) was administered to the treatment group for six months.
Cell culture and identification of EPCs
Mononuclear cells were isolated from peripheral venous blood using density gradient centrifugation, according to the method described by Vasa et al (10). Mononuclear cells were plated on 24-well culture dishes coated with human fibronectin (Gibco, USA) and maintained in basal medium (Endothelial Basal Medium-2 [EMB-2], Clonetics Corporation, USA) for four days. The liquid medium was changed and cells were cultured for an additional seven days. Adherent cells were detached using EDTA after seven days of culture.

After detachment, adherent cells were incubated with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labelled acetylated low-density lipoprotein (DiLDL) (Invitrogen, USA) for 1 h at 37°C. Cells were fixed in paraformaldehyde, washed in phosphate-buffered saline and incubated for 1 h with lectin-conjugated fluorescein isothiocyanate (FITC)-labelled Ulex europaeus agglutinin (UEA)-1 (Vector Laboratories, USA). EPCs were characterized by double positivity for Sudan black B and FITC-UEA-1. In addition, Sudan black B-positive cells were also characterized by the appearance of multiple thin, flattened cells projecting from a central cluster of rounded cells, were counted by a single-blinded observer after five days of culture.

Assessment of adhesion activity
After digestion and collection of the cultured adherent cells, equal numbers of EPCs were seeded in 24-well culture plates coated with human fibronectin and cultured for 30 min. Adhesion activity was assessed by counting adherent cells after the nonadherent cells were removed by washing with phosphate buffer solution.

Assessment of migration activity
EBM-2 culture solutions were added into the lower chamber of an improved Boyden chamber (Jiangsu Haimen Qilin Medical Instrument Plant, China) and 2×10^6 EPCs were injected into the upper chamber after being suspended in EBM-2 culture medium for 24 h at 37°C following the digestion and collection of the cultured adherent cells. The nonmobile cells above the filtration membrane were scraped, and cells were fixed with methanol and stained with Giemsa stain. The percentage of the number of colony-forming units per well. EPC colonies, which were characterized by the appearance of multiple thin, flattened cells projecting from a central cluster of rounded cells, were counted by a single-blinded observer after five days of culture.

Colonies forming assay
Mononuclear cells (5×10^6) were seeded in a six-well plate with 20 mg/L human fibronectin. After incubation for two days, nonadherent cells (i.e., in the supernatant) were collected by gentle aspiration and counted for each subject's sample. These cells were replated onto new fibronectin-coated plates at a concentration of 1×10^6 cells/well for the final assessment of the number of colony-forming units per well. EPC colonies, which were characterized by the appearance of multiple thin, flattened cells projecting from a central cluster of rounded cells, were counted by a single-blinded observer after five days of culture.

Assessment of migration activity
After digestion and collection of the cultured adherent cells, equal numbers of EPCs were seeded in 24-well culture plates coated with human fibronectin and cultured for 30 min. Adhesion activity was assessed by counting adherent cells after the nonadherent cells were removed by washing with phosphate buffer solution.

Statistical analysis
Continuous variables were presented as mean ± SD, and were compared using two-tailed Student’s t tests or one-way ANOVA for multiple variables. Categorical data were presented as n and %, and compared using the χ² test or Fisher’s exact test. All data were analyzed using SPSS version 11.0 (IBM Corporation, USA). A two-sided P<0.05 was considered to be statistically significant.

RESULTS

General findings
At baseline, there were no statistically significant differences in age, sex, NYHA class, PaO₂, PCO₂ or medications between the treatment group and the control group (Table 1). PAP was similar between the two groups (P>0.05). All patients completed the six-month follow-up.

Characteristics of EPCs
Mononuclear cells transfomed into fusiform endothelial cells after seven days of culture. EPCs possessed the characteristics of endothelial cells and appeared red after uptake of DiLDL, green after binding lectin and yellow after double staining with DiLDL and lectin (Figure 1).

EPC counts
The number of EPCs was similar in the treatment and control groups before therapy (P>0.05), but was lower than in the healthy control group (P<0.05). After six months of therapy, the number of EPCs was increased in both patient groups, but the increase reached statistical significance only in the treatment group (P<0.05). The number of EPCs in the treatment group were higher than in the control group after six months’ therapy (P<0.05) (Table 2).

Migration and adhesion activity
At baseline, the migration and adhesion activities of EPCs were comparable between the two treatment groups (P>0.05) but were lower than in the healthy control group (P<0.05). After six months of therapy, the migration and adhesion activities of EPCs in the treatment and control groups were increased compared with baseline values (P<0.05). However, the migration and adhesion activities of EPCs in the treatment group were higher than in the control group after six months (P<0.05, Table 2).

DISCUSSION
The present study showed that the numbers and migration and adhesion activities of peripheral blood EPCs were decreased in patients with chronic pulmonary artery disease. However, these parameters were improved after six months of atorvastatin therapy. In addition, atorvastatin treatment was associated with a greater
reduction in PAP compared with the untreated control group. These results indicate that atorvastatin may help improve impaired endothelial function and reduce PAP in patients with chronic pulmonary artery disease.

Recent studies have demonstrated that endothelial dysfunction plays an important role in the pathogenesis of PAH in patients with COPD (5). EPCs are precursor cells that can proliferate and differentiate to become vascular endothelial cells, participating in the repair processes after endothelial injury (5,15). After vascular injury, circulating EPCs can return to the injured vessel and differentiate to become vascular endothelial cells, accelerating the repair of the injured vascular endothelium (16). Recent research demonstrated that the number of circulating EPCs in COPD patients was reduced by 30% to 50%, and this reduction was associated with the severity of hypoxemia and airway obstruction (6). Consistent with these results, we found that EPC counts in patients with chronic pulmonary artery disease were lower than in healthy controls. Furthermore, our study showed that adhesion and migration activities of EPCs were reduced in patients with chronic pulmonary artery disease, which is also consistent with other reports in which reduction of adhesion and migration activities was associated with increased PAP in COPD patients (7).

A major finding of our study was that atorvastatin increased the number of EPCs and improved EPC migration and adhesion activities in patients with chronic pulmonary artery disease. Evidence has shown that statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) exhibit a number of vascular protective effects such as vessel dilation, attenuation of smooth muscle cell proliferation, restoration of endothelial functions, angiogenesis and inflammatory reaction control (17-19). These beneficial vascular effects appear to be independent of the lipid-regulating effects of statins (17-19). A previous study also found that atorvastatin and mevastatin promote EPC proliferation and suppress the aging of circulating EPCs (20). In patients with acute myocardial infarction, the numbers of circulating EPCs in the peripheral blood positively correlated with statin treatment (21). However, most studies have focused on coronary artery disease, heart failure or patients with multiple cardiovascular risk factors (22,23). In the present study, we demonstrated that statins may also facilitate the repair of endothelial function in chronic pulmonary artery disease.

We also found that PAP was decreased after six months of atorvastatin therapy in patients with chronic pulmonary artery disease, although the exact mechanisms remain unclear. In a rat model of severe pulmonary hypertension, statins induced apoptosis in proliferative lesions in the pulmonary arteries (17). In a study involving a rat model of pulmonary hypertension, simvastatin suppressed the expression of inflammatory factors in the lungs by limiting the secretion of monokine-6 and interleukin-8 by inflammatory cells, ultimately reducing pulmonary hypertension (24). Whether attenuation of inflammation is one of the mechanisms leading to the reduction in PAP in our patients remains to be determined.

### Figure 1
Endothelial progenitor cells appear red after the uptake of diocadexyl-tetramethylindocarbocyanine-labelled acetylated low-density lipoprotein (A), green after lectin binding (B), and yellow after double staining (C). Images were produced using inverted fluorescence microscopy (original magnification × 200).

### Table 2
Comparison of EPC and PAP before and post-treatment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Treatment</th>
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<th>Healthy control</th>
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<td>Baseline</td>
<td>Post-treatment</td>
<td>Baseline</td>
<td>Post-treatment</td>
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<td>EPC counts</td>
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<td>14.6±4.3*</td>
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<td></td>
<td>(number of CFUs)</td>
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<td></td>
<td>25.7±5.3</td>
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<td>EPC migration</td>
<td>10.4±3.2*</td>
<td>15.4±3.8†</td>
<td>11.3±3.7*</td>
<td>23.9±4.8</td>
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<td>EPC adhesion</td>
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<td>16.9±4.5†</td>
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</tr>
<tr>
<td>PAP, mmHg</td>
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<td>45.4±6.8†</td>
<td>51.7±7.9*</td>
<td>49.1±7.3</td>
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</table>

Data presented as mean ± SD. *P<0.01 versus healthy control group; †P<0.05 versus baseline values in the same group. CFU Colony-forming unit; EPC Endothelial progenitor cell; PAP Pulmonary arterial pressure.

### Conclusions
EPC counts, migration and adhesion activities were decreased in patients with chronic pulmonary heart disease. Atorvastatin therapy elevated the number of EPCs and improved the migration and adhesion activities of the circulating EPCs in these patients. Furthermore, atorvastatin therapy for six months resulted in a reduction in PAP. These results suggest that statins may help to restore endothelial function and reduce PAP in patients with chronic pulmonary heart disease.
REFERENCES


