Mesenchymal stem cells transplantation improves functions of circulating endothelial progenitor cells in a rat model of pulmonary hypertension

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Received October 13, 2015; Accepted January 27, 2016; Epub February 15, 2016; Published February 29, 2016

Abstract: Inflammation and dysfunction of circulating endothelial progenitor cells (EPCs) have been recognized as the possible factors for pulmonary hypertension (PH) progression, the present study determined that mesenchymal stem cells (MSCs) suppress inflammation associated down-regulation of circulating EPCs number and functions, and then exert therapeutic effects on PH. A single subcutaneous injection of monocrotaline (MCT) was used to induce a rat model of PH, and then administrated with MSCs transplantation. The therapeutic effects, including changes in hemodynamics and histology, and the plasma levels of TNF-α, the number and functions of circulating EPCs were evaluated. The results indicated that, the level of TNF-α in plasma was significantly increased in the model group compared with it in the control group. Moreover, the circulating EPCs number and functions were all significantly decreased, and the number of circulating EPCs was negatively correlated with the level of plasma TNF-α. Administration of MSCs transplantation could decreased the plasma level of TNF-α, and up-regulated the circulating EPCs number and functions, and then improved the pulmonary hemodynamic abnormality and vascular reconstruction effectively. Furthermore, the negative correlation between the circulating EPCs number and the plasma level of TNF-α could also be observed in the MSCs transplantation group. Thus, the present study suggested that transplantation of MSCs could attenuate MCT induced PH by improving pulmonary vascular repair probably via inhibiting inflammation mediated down-regulation of circulating EPCs.

Keywords: Pulmonary hypertension, mesenchymal stem cell, endothelial progenitor cell, tumor necrosis factor

Introduction

Pulmonary hypertension (PH) is characterized as progressive increase of pulmonary arterial pressure and vascular remodeling. Persistent high pulmonary arterial pressure would increase the resistance of pulmonary circulation system, and eventually results in right ventricular hypertrophy and dilatation, following the occurrence of heart failure. At present, targeted medicines (bosentan, Sildenafil, and etc.) were widely applied in clinic, and have already been as a conventional therapeutic protocol for PH. However, even though application of these medicines could relieve the clinical symptoms of PH partly, but long term prognosis of this disorder is still not so satisfying. So, it is urgent to exploring a more effective protocol for the treatment of PH.

In recent years, along with the advancement in the researches on mechanism of PH, the dysfunction of endothelial progenitor cells (EPCs) has been recognized as one of the possible factors for PH progression. As the precursor of vascular endothelial cells, EPCs play a pivotal role in maintaining the integrity and normal function of the vascular endothelium [1-3]. Previous studies have shown the reduced number and activity of circulating EPCs in PH patients or animal model [4, 5], and the progression of PH could be effectively prevented by up-regulating the number and function of EPCs. Furthermore, inflammation may be a crucial factor in the phenomenon of EPCs dysfunction [6, 7], inflammatory infiltration was found around the remodeled pulmonary vessels in PH patients and animal models [8-11], and produce enormous cytokines such as tumor necrosis factor.
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(TNF)-α, interleukin (IL)-6 [12]. Even though it is still uncertain the exact effects of inflammation on the mechanism of PH, but at least it has been explicated that high levels of inflammatory cytokines decrease EPCs number and function [7], and down-regulation of EPCs would further inhibit the repair process of the damaged pulmonary artery endothelium [5].

Mesenchymal stem cells (MSCs), as primitive stromal cells, have abilities of self-renewal and multi-lineage differentiation, and attract much more attention especially for its immunosuppressive effect in immune disorders [13, 14]. When co-cultured in vitro with activated CD4+ T cells, MSCs could decrease the production of inflammatory cytokines evidently by secreting PGE2 and immuno-regulatory cytokines [15]. Previous studies have been confirmed that transplantation of MSCs could improve PH in animal model [16, 17]. However, studies involving the number and function of EPCs following the transplantation of MSCs in PH are lacking, and it is still unknown to what extent the therapeutic effects of MSCs in PH are accounted by its immune-regulation ability and the effects on EPCs.

In the present study, a single subcutaneous injection of monocrotaline (MCT) was used to induce a rat model of PH, and then administrated with MSCs transplantation. The therapeutic effects, plasma levels of TNF-α, number and functions of circulating EPCs were evaluated, so that to better understands the therapeutic mechanisms of MSCs on PH.

Materials and methods

MSCs isolate and culture

The present study was approved by the Institutional Review Board of Capital Medical University (Beijing, China), and written informed consent was obtained from all donors. MSCs were isolated from human umbilical cord according to the previous study [18] and cultured in DMEM/F-12 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, GE Healthcare Life Sciences, Logan, UT, USA) and 100 U/ml penicillin-streptomycin. At passage 8-10, MSCs were identified by analyzing the ability of osteogenic and adipogenic differentiation, and the cell-surface markers, including CD29, CD31, CD34, CD44, CD90 and CD105 [19].

Experiment animals

All animal studies and protocols were approved by the Institutional Animal Care and Use Committee of Capital Medical University. Sprague-Dawley (SD) rats (male, 8 weeks age), were purchased and housed in specific pathogen-free units of the Division of Laboratory Animals at Capital Medical University (Beijing, China). A total of 24 rats were divided into 3 groups randomly: the PH model group, MSCs transplantation group and control groups, 8 rats in each group. Rats were induced PH by a single subcutaneous injection of MCT (60 mg/kg; Sigma, St. Louis, MO, USA) [20], while administration of PBS as controls. In the MSCs transplantation group, rats were given a single 10⁶ MSCs transplantation 5 days after injection of MCT. PBS was administrated in the PH model group and control group. For transplantation, MSCs at passage 8-10 were detached by using 0.25% trypsin (Gibco Life Technologies) and 0.53 mM EDTA (Sigma-Aldrich). After washing twice with PBS, MSCs (10⁶ cells) were resuspended in 1 ml PBS, and injected slowly into the rats via the caudal vein.

Examination of hemodynamics

At day 21, the right ventricular systolic pressure (RVSP) and mean aortic pressure (MAoP) of rats in each group were assessed according to our previous study [20]. Briefly, the rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). A polyethylene catheter was inserted into the right external jugular vein, and reached the right ventricle (RV), and another was inserted into the right carotid artery, and reached the ascending aorta. RVSP and MAoP were recorded by using a polygraph (Nihon Kohden Corporation, Tokyo, Japan). Subsequently, blood samples were obtained from the external jugular vein, and respectively used to assess the number and function of circulating EPCs and the levels of plasma TNF-α. After completing these procedures, the rats were sacrificed by decapitation, and lung tissues were harvested and fixed in 10% paraformaldehyde (Sigma-Aldrich) for histological examination.

Numbers of EPCs in peripheral blood

The numbers of EPCs in peripheral blood were evaluated by using flow cytometry (BD FACS Calibur Flow Cytometer; BD Biosciences, San Jose, CA, USA) according to our previous study.
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Briefly, 100 μl peripheral blood was sequential incubated for 20 min with 5 μl mouse monoclonal antibody against rat vascular endothelial growth factor receptor (VEGFR)-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and 2 μl fluorescein isothiocyanate (FITC)-conjugated goat monoclonal antibody against mouse immunoglobulin G. After red blood cell lysis, the cells were incubated with 5 μl allophycocyanin (APC)-conjugated mouse monoclonal antibody against rat CD45 (BD Biosciences, Franklin Lakes, NJ, USA) and 5 μl phycoerythrin (PE)-conjugated mouse monoclonal antibody against rat CD34 (BD Biosciences) for 20 min. The samples were washed 2 times with PBS and then evaluated the numbers of cells negative for CD45, but positive for CD34 and VEGFR-2, which termed as circulating EPCs [21].

TNF-α plasma levels

The plasma levels of TNF-α were examined by using an ELISA kit (PeproTech, Inc., Rocky Hill, NJ, USA), and performed according to the manufacturer’s instructions.

Histological examination

Serial 5-μm paraffin sections of lung tissues were stained with hematoxylin and eosin, and then observed on light microscopy (×400). The medial wall thickness of the pulmonary arteriole is expressed as: Wall thickness (WT, %)= [(medial thickness x2)/external diameter] x100 [20].

EPCs cultured in vitro

Peripheral blood was obtained from the external jugular vein of rats, and mononuclear cells were isolated by using Ficoll density gradient centrifugation. After washed twice with PBS, the cells were resuspended with endothelial cell growth medium-2 (EGM-2; Lonza Group AG, Basel, Switzerland) at the density of 1x10⁷/ml, and seeded in 24-well culture plates pre-coated with fibronectin (Sigma). Following 7 days of culture, cells were sequential incubated with 10 μg/ml Dil-labeled acetylated low-density lipoprotein (acLDL; Molecular Probes®, Invitrogen Life Technologies, Carlsbad, CA, USA) and FITC-conjugated lectin from Ulex europeus agglutinin-1 (FITC-UEA-1; Sigma), and examined by using laser scanning confocal microscopy. Differentiating EPCs were identified as double positive for fluorescence staining [7].

Function of EPCs in vitro

EPCs functions assessment, including proliferation, adhesion and migration, were performed according to our previous study [20]. To assess the proliferation ability of EPCs, 10⁵ cells were cultured in 96-well plates for 24 hours, and then 10 μl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS, Promega, USA) were added for 4 hours of culture. The optical density (OD) of supernatant at 490 nm was examined by microplate reader. To assess the adhesion ability of EPCs, 5x10⁴ cells suspended in EGM-2 were incubated for 30 min in 24-well plates pre-coated with fibronectin. Washed 3 times with PBS, the attached cells were counted in high power field (HPF, ×400). To assess the migratory ability of EPCs, 5x10⁵ EPCs suspended in 100 μl EGM-2 with 0.5% FBS and no cytokines were seeded in a modified Boyden chamber (8-μm pore size, corning system), and placed in a 24-well culture plate containing 600 μl EGM-2. After 24 hours of incubation, the chamber was taken out, and cells on the lower membrane were fixed with 4% paraformaldehyde. After stained with 0.1% crystal violet, the cells were counted in HPF (×400).

Statistical analysis

Data are presented as the mean ± standard deviation, and analyzed by using SPSS statistical software (version 13.0; SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to test the differences, and Pearson’s correlation was used to test the correlations. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of MSCs

Human umbilical cord-derived MSCs exhibited a typical fibroblast shape after 3 passagers of culture in vitro (Figure 1A), and demonstrated osteogenic differentiation ability (Figure 1B) and adipogenic differentiation ability (Figure 1C) when cultured in specific conditioned medium. Analysis of cell surface markers indicated MSCs were positive for CD29, CD44, CD90 and
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CD105, negative for CD31 and CD34 (Figure 1D).

**MSCs transplantation improves PH**

Twenty-one days after injection of MCT, the hemodynamic examination indicated that the MAoP was decreased in the model group compared with it in the control group (27.90±3.12 mmHg vs. 45.81±2.63 mmHg; P<0.01) (Figure 2A), while the RVSP increased significantly (112.78±16.77 mmHg vs. 93.39±7.85 mmHg; P<0.01) (Figure 2B). Histological examination shown that, the tunicae media of the pulmonary muscular arterioles were hypertrophic evidently in the model group (Figure 2E) compared with it in the control group (Figure 2D), and the WT increased significantly (30.44±2.23% in the model group vs. 14.63±1.45% in the control group; P<0.01) (Figure 2C).

By administration of MSCs, there were significantly improved in the MAoP (29.01±1.67 mmHg; P<0.05; Figure 2A) and the RVSP (96.78±9.56 mmHg; P<0.01; Figure 2B) compared with those in the model group. While the
Medial hypertrophy of pulmonary muscular arterioles was also attenuated (Figure 2F), and the WT decreased (18.76±1.09%) compared with it in the model group (P<0.01) (Figure 2C).

**MSCs decrease plasma TNF-α level**

Twenty-one days after injection with MCT, plasma TNF-α level was detected. The results indicated that, the plasma concentration of TNF-α was significantly increased in the model group compared with it in the control group (860.90±71.50 pg/ml vs. 132.85±22.51 pg/ml; P<0.01). By administration of MSCs, the plasma concentration of TNF-α was decreased (242.16±25.68 pg/ml) compared with it in the model group (P<0.01) (Figure 3A).
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MSCs up-regulate circulating EPCs number

Twenty-one days after injection with MCT, the number of circulating EPCs, termed as negative for CD45, but positive for CD34 and VEGFR-2, was assessed by using flow cytometry. The results indicated that the number of EPCs in the model group was significantly decreased compared with it in the control group (0.016±0.007 versus 0.034±0.012%, P<0.01) (Figure 3B). By administration of MSCs, the number of EPCs was notably up-regulated (0.029±0.011%) compared with it in the model group (P<0.05) (Figure 3B). Furthermore, the number of circulating EPCs in the model group and MSCs transplantation group were all negatively correlated with the plasma concentration of TNF-α (Figure 3C, 3D).

EPC growth and identification in vitro

Peripheral blood mononuclear cells cultured in EGM-2 for 7 days, abundant spindle-like cells adhered to the bottom of the culture plate were observed (Figure 4A). The cells have the abilities of binding with FITC-UEA-1 (Figure 4B) and phagocytosis of DiI-acLDL (Figure 4C) simultaneously (Figure 4D), and were recognized as the EPCs undergoing differentiation.

MSCs administration improve circulating EPCs function

The functional indices of EPCs derived from the model group, including proliferation, adhesion and migration, were all down-regulated compared with those of EPCs derived from the control group. (Proliferation ability: OD at 490 nm was 0.49±0.07 in the model group versus 0.68±0.07 in the control group, P<0.01; adhesion ability: 6.75±1.67 cells/HPF in the model group versus 12.38±2.26 cells/HPF in the control group, P<0.01; migratory ability: 6.88±1.55 cells/HPF in the model group versus 13.38±2.07 cells/HPF in the control group, P<0.01).
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Figure 4. Circulating EPCs functions in vitro. (A-D) Identification of EPCs: (A) EPCs derived from peripheral blood mononuclear cells of rats showed typical spindle-like morphology after seven days of culture (magnification, ×100). (B) EPCs were immunopositive for fluorescein isothiocyanate-conjugated lectin from Ulex europaeus agglutinin-1 (FITC-UEA-1) and (C) Dil-labeled acetylated low-density lipoprotein (Dil-acLDL) simultaneously (D). (E-G) Circulating EPCs functions in vitro: (E) Proliferation, (F) adhesion and (G) migration (n=8, *P<0.01).
By administration of MSCs, the functional indices of EPCs were all up-regulated (proliferation, OD 0.62±0.06; adhesion, 12.13±1.81 cells/HPF; migration, 12.75±2.66 cells/HPF) compared with those of EPCs derived from the model group (P<0.01).

Discussion

By utilizing a MCT induced rat model of PH, we have demonstrated that, the level of TNF-α in plasma was significantly increased in the model group compared with it in the control group. Moreover, the circulating EPCs number and functions were all significantly decreased, and the number of circulating EPCs was negatively correlated with the level of plasma TNF-α. Administration of MSCs transplantation could decreased the plasma level of TNF-α, and up-regulated the circulating EPCs number and functions, and then improved the pulmonary hemodynamic abnormality and vascular reconstruction effectively. Furthermore, the negative correlation between the circulating EPCs number and the plasma level of TNF-α could also be observed in the MSCs transplantation group. These results indicate, transplantation of MSCs could attenuate MCT induced PH by improving pulmonary vascular repair via inhibiting inflammation mediated down-regulation of circulating EPCs.

PH is characterized as the high blood pressure in pulmonary circulation, which resulted from the persistent pulmonary arterioles contraction. With the progress of the disease, the medial hypertrophy and stenosis of pulmonary arterioles aggravate gradually, and eventually result in the right ventricular hypertrophy and failure. Despite interference with targeted medicines could improve the quality of life, and prolong the survival times of PH patients to a large extent, but the events of heart attack and sudden death are still frequently occurred. So, how to further improve the curative effects and decrease the mortality in PH patients is crucial in the current clinical researches.

Studies have explicated that the injury of pulmonary arteriolar endothelium play a pivotal role in the pathogenesis of PH [22, 23]. EPCs was believed as the main forces of vascular endothelium repair, and the decreased circulating EPCs number and numbers could be observed in the PH patients and experimental animal models [4, 5]. Furthermore, the up-regulation of circulating EPCs usually accompany with the alleviation of PH [24, 25], which further confirm that the abnormality of EPCs involved in the pathology of PH, and may be as a predict factor for the prognosis of PH. As previous studies have indicated that, inflammation is the crucial factor involved in the EPCs dysfunction in some inflammation related diseases [6, 7]. So, in the present study, by using a rat model of MCT induced PH, we have assessed the levels of TNF-α, which as a representation of inflammatory factors, in the plasma, and then analyzed the relevance between the circulating EPCs number and the plasma level of TNF-α. Twenty-one days after injection of MCT, the occurrence of PH was confirmed according to the changes of hemodynamics and histology, and the results indicated that, the concentration of TNF-α in plasma were significantly increased. Simultaneously, down-regulation of circulating EPCs number and functions, and negatively correlated between the circulating EPCs number and the plasma concentration of TNF-α could be observed. Even though we have not elucidate the exact molecular mechanism of how inflammation interfere the EPCs number and functions in PH, but the relative correlation analysis between the levels of plasma TNF-α and the circulating EPCs number confirmed that inflammation could down-regulate the number and functions of EPCs in PH.

MSCs have abilities of multi-differentiation, hematopoietic support, and immunoregulation. High immunosuppressive effect of MSCs makes them as a prospective protocol in immune disorders [13, 14]. Studies have indicated that immunoinflammation was involved in the mechanism of PH [8-11], and our previous study has demonstrated that transplantation of MSCs could decreased the immunoinflammation, and then improved the abnormalities of hemodynamics and histology in MCT induced rat model of PH [26]. For further elucidate the therapeutic mechanism of MSCs in PH, the changes of plasma TNF-α and circulating EPCs, and the correlation between them were assessed in present study. The results indicated that, administration of MSCs in MCT induced rat model of PH could significantly decreased the levels of TNF-α in plasma, and increase the number of circulating EPCs, and up-regulate the functions of circulating EPCs. So that to accelerate the repair process of the
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damaged pulmonary artery endothelium. The negatively correlation between the number of circulating EPCs and the plasma concentration of TNF-α after administration of MSCs further indicated that, the improvement of circulating EPCs number and functions was benefited from the immunosuppressive effects of MSCs. As a result, there was evident improvement in the hemodynamics and histology.

In conclusion, the present study demonstrated that administration of MSCs may prevent the process of MCT induced PH effectively. The therapeutic effects of MSCs were related to the up-regulation of circulating EPCs number and functions via suppressive effect for immunoinflammation, and then accelerate the repair process of the damaged pulmonary artery endothelium. However, the potential side-effects and prospective efficacy of MSCs remain further confirmation.

Acknowledgements

This study was supported by the Clinical study of the capital Beijing Science and Technology Commission (Z161100000516106), and the 2016 Capital Health Development Research Projects (Complement regulatory proteins to guide individual treatment in children with aHUS).

Disclosure of conflict of interest

None.

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