Autologous Transplantation of Peripheral Blood-derived Circulating Endothelial Progenitor Cells Attenuates Endotoxin-induced Acute Lung Injury in Rabbits by Direct Endothelial Repair and Indirect Immunomodulation

Jian-Ping Cao, M.D.,* Xing-Ying He, M.D.,† Hai-Tao Xu, M.S.,‡ Zui Zou, M.D.,‡ Xue-Yin Shi, M.D.§

ABSTRACT

Background: Studies have demonstrated the role of circulating endothelial progenitor cells (EPCs) in maintaining normal endothelial function and in endothelial repairing. This study was aimed to observe the protective effects of autologous transplantation of circulating EPCs against endotoxin-induced acute lung injury in rabbits and to investigate the underlying mechanisms.

Methods: One-hundred-and-fifty rabbits were enrolled. After acute lung injury was induced by endotoxin, autologous circulating EPCs, endothelial cell, or normal saline were transfused intravenously, respectively. PaO2/FiO2 ratios, concentrations of plasma nitric oxide, malondialdehyde, and activity of superoxide dismutase were examined. The lung wet-to-dry weight ratios were counted; polymorphonuclear cell ratios and areas of hyaline membrane formation and hemorrhage were measured. The levels of interleukin-1β, E-selectin, intercellular adhesion molecule-1, interleukin-10, vascular endothelial growth factor protein, and inducible nitric oxide synthase protein were analyzed.

Results: PaO2/FiO2 ratios were significantly increased with EPC transfusion. Infiltration of polymorphonuclear cells, lung wet-to-dry weight ratios, and area of hyaline membrane and hemorrhage in lung tissue were significantly decreased after EPC transplantation. Plasma level of nitric oxide and malondialdehyde were significantly inhibited, and the activity of superoxide dismutase was enhanced in the EPC-treated animals. EPC transplantation significantly increased level of interleukin-10 and vascular endothelial growth factor protein and reduced levels of interleukin-1β, E-selectin, intercellular adhesion molecule-1, and inducible nitric oxide synthase in injury lung tissues.

Conclusions: Autologous transplantation of circulating EPCs can partly restore the pulmonary endothelial function and effectively attenuate endotoxin-induced acute lung injury by direct endothelial repair and indirect immunomodulation of antioxidation and antiinflammation.

What We Already Know about This Topic

• Presently, there is no specific therapy that improves endothelial and/or epithelial damage in endotoxin-injured lungs.

What This Article Tells Us That Is New

• Intravenous administration of endothelial progenitor cells improved endotoxin-injured rabbit lungs by increasing vascular endothelial growth factor expression, decreasing proinflammatory cytokines in the lungs, increasing interleukin-10, and decreasing neutrophil infiltration into the lungs.

10, vascular endothelial growth factor protein, and inducible nitric oxide synthase protein were analyzed.

Results: PaO2/FiO2 ratios were significantly increased with EPC transfusion. Infiltration of polymorphonuclear cells, lung wet-to-dry weight ratios, and area of hyaline membrane and hemorrhage in lung tissue were significantly decreased after EPC transplantation. Plasma level of nitric oxide and malondialdehyde were significantly inhibited, and the activity of superoxide dismutase was enhanced in the EPC-treated animals. EPC transplantation significantly increased level of interleukin-10 and vascular endothelial growth factor protein and reduced levels of interleukin-1β, E-selectin, intercellular adhesion molecule-1, and inducible nitric oxide synthase in injury lung tissues.

Conclusions: Autologous transplantation of circulating EPCs can partly restore the pulmonary endothelial function and effectively attenuate endotoxin-induced acute lung injury by direct endothelial repair and indirect immunomodulation of antioxidation and antiinflammation.

* Associate Professor, Department of Anesthesiology, Changzheng Hospital, The Second Military Medical University, Shanghai, China, and Associate Professor, Department of Anesthesiology, PLA455 Hospital, Shanghai, China. † Attending Doctor, ‡ Associate Professor, § Professor, Department of Anesthesiology, Changzheng Hospital, The Second Military Medical University.

Received from the Department of Anesthesiology, Changzheng Hospital, The Second Military Medical University, Shanghai, China. Submitted for publication December 3, 2010. Accepted for publication February 9, 2012. Supported by National Nature Science Funds (grant no. 30772092), Military Science and Technology Funds (grant no. 08GQ0712), Key Basic Research Projects (grant no. 09JC140500), and Key Medical Research Projects (grant no. 10411951300) from Science and Technology Commission, Shanghai Municipality, China. Drs. Jian-Ping Cao and Xing-Ying He contributed equally to this work.

Address correspondence to Dr. Shi: Department of Anesthesiology, Changzheng Hospital, The Second Military Medical University, 445 Fengyang Road, Shanghai, China. shixueyin1128@yahoo.com.cn. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue.

Copyright © 2012, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins. Anesthesiology 2012; 116:1278–87

Anesthesiology. V 116 • No 6 June 2012

1278
Acutely lung injury (ALI) is characterized by diffuse pulmonary infiltration, increased pulmonary capillary permeability, and severe hypoxemia. The initial pathophysiologic changes include damage of endothelial surfaces, disruption of the alveolar-capillary barrier function, and flooding alveolar spaces with fluid. Until now, several attempts aiming to restore the pulmonary endothelial function, such as surfactants, pulmonary vasodilators, antioxidants, anti-inflammatory agents, and statins, have been tested in patients with ALI. Unfortunately, none of them showed a satisfactory effect. Asahara et al. first reported the role of circulating endothelial progenitor cells (EPCs) in neovascularization and vasculogenesis in 1997. In the past decades, the regenerative potential of EPCs in the injured endothelia, including limb ischemia, myocardial ischemia, carotid artery injury, and vascular graft survival, has been extensively investigated. Furthermore, circulating EPCs have also been indicated to play roles in both ALI and fibrotic lung diseases. Previous study indicated that autologous transplantation of EPCs preserved the pulmonary endothelial function and maintained the integrity of pulmonary alveolar-capillary barrier in oleic acid-induced ALI. Recent study reported that allogeneic transplantation of bone marrow-derived EPCs improved survival and attenuated lipopolysaccharide-induced lung injury in rats. Although the beneficial effect of EPCs has been recognized in the setting of ALI, whether treatment with EPCs can obtain a better prognosis is still uncertain.

The circulating EPCs have been proven to be mobilized from the bone marrow by cytokines and growth factors, and localize at the injury sites, where they differentiate into vascular endothelial cells, consequently promoting neovascularization. Several studies reported that bone marrow-derived mesenchymal stem cells (MSCs), sharing similar properties with EPCs, could modulate immune cell response by adjusting the secretion of tumor necrosis factor-α and interleukin-10 (IL) and promote the proliferation and reepithelialization of lung cells. Furthermore, a recent study reported that MSCs ameliorated the parenchymal and vascular injury of bronchopulmonary dysplasia, which suggested protective mechanisms of MSCs contributed to either a paracrine effect of immunomodulatory factors release or direct tissue repair. However, whether the protective role of EPCs is through the immune regulatory effect has not yet been well studied.

Therefore, we hypothesized that the autologous transplantation of circulating EPCs could effectively attenuate endotoxin-induced ALI in rabbits, and the beneficial effects of EPCs might be because of the immunomodulation role of the inflammatory response. Using endotoxin-induced ALI models in rabbits, we observed the plasma levels of nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), and IL-10 in the injury lung tissues after autologous transplantation of EPCs.

Materials and Methods

Isolation and Culture of Peripheral Blood EPCs

Approval was obtained from the Animal Care and Use Committee of The Second Military Medical University (Shanghai, China). The peripheral blood was obtained from the ear artery of New Zealand White rabbits (10 ml/kg). Peripheral blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Plaque Plus (Amersham Pharmaacia Biotech AB, Uppsala, Sweden). Mononuclear cells were then washed and plated on six-well plates supplemented with EGM-2 MV Single Quots (Lonza Corp., Basel, Switzerland). Mononuclear cells were incubated at 37°C, 5% CO₂, and fed daily with EGM-2. The nonadherent cells were removed after 48 h of culture. At day 7 of culture, the adherent cells (known as early EPCs) were detached with 0.025% trypsinase containing 0.02% EDTA and used for analysis or transplantation.

Characterization of EPCs

The EPCs isolated from rabbits were characterized as previously described. Cells were incubated with Dil-acetyl-low density lipoprotein (LDL) (10 μg/ml; Invitrogen, Carlsbad, CA) and fluorescein isothiocyanate-ulex europaeus agglutinin-1 (UEA-1) (5 μg/ml; Sigma-Aldrich, Saint Louis, MO). The staining of acetyl-LDL and UEA-1 in cultured EPCs was detected under fluorescence confocal microscopy at the absorption wavelengths of 555 and 490 nm, respectively. Dual fluorescent staining positive for both fluorescein isothiocyanate labeled UEA-1 and Dil labeled acetyl-LDL (double-positive cells) were identified as differentiating EPCs. Immunostaining of vascular endothelial growth factor receptor 2 (Santa Cruz Biotechnology, Santa Cruz, CA) and CD133 (Santa Cruz Biotechnology) was performed as previously described.

Lentivirus-eGFP Label EPCs

Enhanced green fluorescent protein (eGFP), as a fluorescent cell tracker, was used to label EPCs to track their homing in the injured lung tissues. Lentivirus was produced in 293T cells (GenePharma, Shanghai, China) by packaging eGFP into human immunodeficiency virus vector (lentiviral vector). The titer recombinant lentiviral vectors with eGFP (Lenti-eGFP) were made. Before transplantation, EPCs were transduced with lentiviral vectors at a multiplicity of infection of 20; EPCs carrying eGFP were harvested.

Rabbit Model of ALI and Transplantation of EPCs

The animals were anesthetized with intravenous 1% pentobarbital sodium (20 mg/kg), and ALI was induced by intravenous administration of endotoxin (E. coli, 5 mg/kg). The animals were then randomly divided into three groups (controls, EPCs, and eGFP-labeled EPCs). Autologous bone marrow-derived EPCs were isolated from the ear artery of rabbits. After 7 days of culture, the adherent cells were harvested and labeled with eGFP using lentiviral vectors before transplantation. The animals were then randomly divided into three groups (controls, EPCs, and eGFP-labeled EPCs). Autologous bone marrow-derived EPCs were isolated from the ear artery of rabbits. After 7 days of culture, the adherent cells were harvested and labeled with eGFP using lentiviral vectors before transplantation. The animals were then randomly divided into three groups (controls, EPCs, and eGFP-labeled EPCs). Autologous bone marrow-derived EPCs were isolated from the ear artery of rabbits. After 7 days of culture, the adherent cells were harvested and labeled with eGFP using lentiviral vectors before transplantation.
venous injection of endotoxin (*Escherichia coli* lipopolysaccharide, 0111:B4, Sigma-Aldrich) 500 μg/kg over 30 min via the ear vein. Sham-operated animals received either placebo or autologous EPC transplantation without injection of endotoxin. Autologous EPCs (EPC group, approximately 10⁶ cells in a volume of 500 μl normal saline), or 500 μl normal saline (control group) were transplanted via the ear vein to each rabbit at 4 h after infusion of endotoxin. As the control cell, mature vascular endothelial rabbit cells (EC group, approximately 10⁶ cells in a volume of 500 μl normal saline) were transplanted at 4 h after infusion of endotoxin. Rabbits were allowed to recover spontaneously from anesthesia. At 48 h after endotoxin injection, all rabbits were killed with intravenous injection of an overdose of 5% pentobarbital sodium (250 mg/kg), and the whole lung tissues were excised for further investigations. Of 154 male New Zealand White rabbits enrolled in the study, three animals were excluded for accidental death and one animal was not included for unsuccessful model of ALI. All procedures were performed in accordance with the guidelines of the National Institutes of Health, Bethesda, Maryland, for the ethical use of laboratory animals.

**Track of EPCs in Lung Tissue**

The expression of eGFP genes was detected using immunohistochemistry or fluorescent microscope. Briefly, rabbits were sacrificed at 48 h after ALI and the lung tissue sections were examined by immunohistochemistry or fluorescent microscopy. The paraffin-embedded lung sections were used for immunostaining, EGFP was detected with an antirabbit eGFP antibody (N-Terminal; Sigma-Aldrich) and goat antirabbit IgG conjugated with horseradish peroxidase, using an ABC Elite kit (Vector Laboratories, Burlingame, CA). The sections with avidin– biotin horseradish peroxidase complexes were color-developed with 3,3-diaminobenzidine, 0111:B4, Sigma-Aldrich) 500 μl normal saline), or 500 μl of saline (control group) were transplanted at 4 h after infusion of endotoxin. As the control cell, mature vascular endothelial rabbit cells (EC group, approximately 10⁶ cells in a volume of 500 μl normal saline) were transplanted at 4 h after infusion of endotoxin.

**Arterial Blood Gases Analysis**

Samples of arterial blood were collected before endotoxin injection and at 4 h, 8 h, 12 h, 24 h, and 48 h after endotoxin injection at various experimental groups. At various times, arterial blood gases (Pao₂/FiO₂ ratio) were determined automatically with a blood-gas analyzer (i-STAT; Abbott Point of Care Inc., Princeton, NJ).

**Plasma Nitric Oxide, Malondialdehyde, and SOD Measurements**

Arterial blood was collected and plasma was isolated at 48 h after endotoxin-induced ALI in rabbits. The plasma levels of nitric oxide and malondialdehyde and the activities of SOD were measured by Griess reaction, thiobarbituric acid, and xanthine oxidase methods, respectively.

**Lung Water Measurement**

The lung wet-to-dry weight ratios (LWDR), an index of microvascular permeability, were determined to assess the severity of lung edema. Rabbits were sacrificed before endotoxin injection and at 4 h, 8 h, 12 h, 24 h, and 48 h after endotoxin injection at various experimental groups. The left lung lobe was excised and weighed immediately. Lung tissues were dried to a constant weight at 60°C for 24 h in an oven and reweighed. The lung wet-to-dry ratio was calculated as LWDR = (weight_wet – weight_dry)/weight_wet · 100%.

**Histopathologic Examination of Lung Tissue**

Lung tissues were immersed in 4% paraformaldehyde and fixed for 24 h. Hematoxylin-eosin stained lung sections were examined under a light microscope and photographed. Areas of hyaline membrane formation and hemorrhage in the lung sections were measured using the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). As previously described, infiltration of PMNs in the lung tissue was also measured using the PMN ratio. Using light microscopy (40-fold magnification), we counted the numbers of PMNs and nonPMN cells from 40 random fields in the five domains of each tissue slide and calculated PMN ratio = (PMN/non-PMN cells) · 100%.

**ELISA Analysis of Cytokines in Lung Tissue**

Lung tissue samples were obtained from rabbits. The levels of IL-10, IL-1β, E-selectin, and ICAM-1 in lung tissue homogenate were measured using an enzyme-linked immunosorbent assay (ELISA kits; R & D Systems Inc., Minneapolis, MN).

**Western Blot**

Soluble protein extract was isolated from lung tissues. Protein concentration was determined by the Bradford assay. Equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto the nitrocellulose membranes. Anti-iNOS antibodies and anti-VEGF antibodies (Santa Cruz Biotechnology) were used. After washing, the membranes were subsequently incubated for 30 min with horseradish peroxidase-linked secondary antibodies (Santa Cruz Biotechnology), and bands were visualized using enhanced chemiluminescence. Densitometric signals were quantified by Quantity One (Bio-Rad, Hercules, CA).

**Statistical Analysis**

All data were presented as mean ± SD. Parametric data were analyzed using one-way ANOVA and variations of different groups were compared with the Tukey post hoc test. The /FiO₂ ratio was analyzed using two-way repeated-measures ANOVA, and follow-up was done with one-way ANOVA and repeated-measures ANOVA. The LWDR were analyzed using two-way ANOVA followed by Bonferroni correction. Significance level was considered α ≈ 0.05.
SPSS version 16.0 statistical software (SPSS, Chicago, IL) was used for the data analysis.

Results

Characterization of EPCs

Seven days after cultivation, EPCs (approximately 2 to 3 × 10^6 cells) were proliferated from 20 ml peripheral blood of each rabbit. Formation of monolayer colonies with “cobblestone” appearance was also observed 2 weeks after culturing in endothelial basic medium 2 (fig. 1A). The EPCs also displayed expression of endothelial markers vascular endothelial growth factor receptor 2 (fig. 1B) and CD133 (fig. 1C). Fluorescence confocal microscopy illustrated that these early EPCs exhibited phenotyping of endothelial cells, including incorporation of DiI-ac-LDL and binding of fluorescein isothiocyanate-UEA-1. Dual fluorescent staining positive for both fluorescein isothiocyanate labeled UEA-1 and DiI-ac-LDL (double-positive cell) were identified as differentiating EPCs (figs. 1D–F).

Detection of Transplanted EPCs

EPCs were cultured (fig. 2A) and transfected with eGFP (figs. 2B, C) on day 7. EGFP-label EPCs were detected by immunohistochemistry (fig. 2D) or fluorescence microscopy (fig. 2E) in injury lung tissues 2 days after the infusion of EPCs, indicating the homing of transplanted EPCs on the injured endothelium. Control nuclei were stained with 4′,6-diamidino-2-phenylindole (fig. 2F).

Improvement of Lung Oxygenation and Reduction of LWDRs

There was a significant reduction in the PaO_2/FiO_2 ratio after endotoxin injection in each group. Compared with the control group and EC group, the PaO_2/FiO_2 ratios were significantly increased at 24 h and 48 h after endotoxin administration in rabbits receiving autologous transplantation of EPCs (fig. 3A). There was a significant increase in the LWDR after endotoxin injection in all groups. Compared with the control group and EC group, the LWDR was significantly reduced at 24 h and 48 h after endotoxin injection in the EPC-treated animals (fig. 3B).

The Plasma Levels of Nitric Oxide and Malonyldialdehyde, and Activities of SOD

Plasma levels of nitric oxide and malonyldialdehyde were significantly increased and the activities of SOD were significantly reduced at 48 h after endotoxin injection in rabbits. Compared with the control group and EC group, the plasma levels of nitric oxide and malonyldialdehyde were significantly inhibited, but the activities of SOD were significantly enhanced in the animals treated with EPCs (table 1).

The Expression of IL-1β, E-selectin, ICAM-1, and IL-10 in Lung Tissue

Two days after endotoxin-induced ALI, the inflammatory cytokine, IL-1β, E-selectin, and ICAM-1 levels were significantly increased in lung tissue. Compared with the control group and EC group, the levels of IL-1β, E-selectin, and
ICAM-1 were significantly inhibited in rabbit lung tissue after autologous transplantation of EPCs. However, the IL-10 level, which was considered as an anti-inflammatory cytokine, was significantly reduced in the control group and EC group, and increased in the EPC-treated group compared with the sham group (table 2).

**Degrees of Acute Lung Injury**

Degrees of rabbit lung parenchymal damage were determined under light microscopy. There were no lesions in the lung tissues of sham-treat rabbits (fig. 4A). In the control group and EPC group, hematoxylin-eosin staining showed typical ALI pathologic traits, such as thickening of alveolar wall, formation of hyaline membrane, hemorrhage, and infiltration of PMNs (fig. 4B, C). Histologic examination revealed that the areas of hyaline membrane formation and hemorrhage were dramatically reduced in lung tissue of rabbits treated with EPCs compared with the control group and EC group (fig. 4D, E). The increased infiltration of PMN in the lung tissue is positively correlated with degree of ALI. A significant higher PMN ratio was detected in the lung tissue of the control group and EC group than the EPC group (fig. 4F).

**The Expression of iNOS and VEGF in Lung Tissue**

The protein expression levels of iNOS and VEGF in rabbit lung were analyzed by Western blot and normalized with β-actin. Protein expression of iNOS and VEGF were up-regulated in lung tissue of rabbits with endotoxin-induced ALI. The expression of iNOS was significantly reduced and the expression of VEGF protein was increased in the autologous transplantation of EPC group compared with normal saline and EC-treated animals (fig. 5, 6).

**Discussion**

In the present study, we found that autologous transplantation of circulating EPCs could improve oxygenation, decrease infiltration of PMNs, and reduce lung edema, formation of hyaline membrane, and area of hemorrhage in EPC-treated animals. Moreover, we were the first to find that VEGF expression in lung tissue was significantly up-regulated after receiving autologous transplantation of EPCs in rabbits with ALI. Our results also indicated EPCs influenced the expressions of inflammatory cytokines and oxidant free radicals during ALI.

ALI and acute respiratory distress syndrome are common causes of morbidity and mortality in the intensive care unit. During the progression of ALI, the alveolar-capillary membrane was disrupted by activation of pulmonary endothelium, expression of adhesion molecules, and massive infiltration of PMNs, resulting in increased protein permeability across the barrier, pulmonary edema, hyaline membrane formation, and lung hemorrhage. Mao et al. reported that allogeneic transplantation of bone marrow-derived EPCs attenuated lipopolysaccharide-induced lung injury in rats. In our study, we transplanted the autologous circulating EPCs into the rabbits with endotoxin-induced ALI. We determined the effect of EPCs on the gas exchange of rabbits with endotoxin-induced ALI. Lung oxygenation function was assessed via blood gas analysis in rabbits with spontaneous respiration. Our results showed PaO2/FiO2 ratio was decreased in rabbits with endotoxin-induced ALI. The PaO2/FiO2 ratios were increased significantly in rabbits receiving
autologous transplantation of EPCs, indicating improved pulmonary oxygenation function. Further, we found the ratio of lung wet-to-dry weight was significantly lower in animals receiving EPC transplantation. Consistent with previous reports, our histologic examinations also revealed significantly reduced formation of hyaline membrane and area of hemorrhage in the lung sections of EPC-treated animals. Therefore, the therapeutic benefits of EPCs could be attributable to reendothelialization of the damaged pulmonary vascular wall and maintenance of alveolar-capillary barrier integrity in rabbits with ALI. Together, these functional and morphologic data indicated that the function and structure of alveolar-capillary membrane was, at least partly, restored by administration of EPCs.

Although it is not clear how the progenitor cells accumulate, differentiate, and participate in lung repair, two possible mechanisms have been reported previously: by intercalating directly with endothelial monolayer or accumulating behind the vascular wall, or by secreting growth factors and cytokines, which then induce neovessel formation by activating resident stem/progenitor cells. In the present study, we located the transplanted EPCs on the lumen wall of pulmonary vascular using immunohistochemistry or fluorescence-conjugated cell tracers, indicating that EPCs homed to the pulmonary endothelium and might subsequently reendothelialize the injured endothelium. Nevertheless, as a result of washout effect, we were not able to detect large amount of eGFP-label EPCs from the injured lung sections of rabbits after EPC transplantation, suggesting other possible mechanisms underlying EPCs’ protective effect. We believe that, after intravenous transplantation, EPCs dispersed in a very large surface areas of pulmonary circulation, making them hard to be detected in the histologic sections. Owing to limitations in the study design, we did not observe ultrastructure of vascular wall with the EPCs, and the duration from EPC infusion to animal death was too short. Therefore, the exact role of EPCs in preserving or restoring pulmonary vascular structure is not clearly understood.

Previous study indicated VEGF could stimulate proliferation, migration, and survival of endothelial cells, which in turn facilitated EPCs’ reendothelialization and recovery of endothelial function. VEGF gene-transferred EPCs enhanced EPCs’ proliferation, adhesion, and incorporation into endothelial cell monolayers. Recent study in vitro observed that MSCs produced and secreted more proangiogenic cytokines such as VEGF, promoting angiogenesis. In the present study, Western blot analysis showed that the VEGF expression was significantly increased after autologous EPC transplantation, indicating that transplanted EPCs could secrete more VEGF to promote proliferation and repair of injured endothelial cells in ALI rabbits. However, because of limitation of the study design, how EPCs exerted the paracrine effect via VEGF still needs to be clarified.

Table 1. The Plasma Level of Nitric Oxide, Malondialdehyde, and the Activity of Superoxide Dismutase

<table>
<thead>
<tr>
<th>Groups</th>
<th>( \text{NO}_2^-/\text{NO}_3^- ) (( \mu \text{M} ))</th>
<th>MDA (nmol)</th>
<th>SOD (NU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>75.4 ± 13.8</td>
<td>2.5 ± 0.5</td>
<td>95.6 ± 11.2</td>
</tr>
<tr>
<td>Control</td>
<td>112.5 ± 14.3*</td>
<td>4.7 ± 0.7*</td>
<td>60.5 ± 7.5*</td>
</tr>
<tr>
<td>EC</td>
<td>115.4 ± 15.4*</td>
<td>4.6 ± 0.5*</td>
<td>61.1 ± 8.7*</td>
</tr>
<tr>
<td>EPC</td>
<td>89.8 ± 12.7†</td>
<td>3.1 ± 0.6†</td>
<td>78.4 ± 9.4†</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD, n = 15. * Significantly different compared with sham group (\( P < 0.05 \)). † Significantly different compared with control group and endothelial cell group (\( P < 0.05 \)).

EC = endothelial cell; EPC = endothelial progenitor cell; MDA = malonyldialdehyde; \( \text{NO}_2^- \) = nitrogen dioxide; \( \text{NO}_3^- \) = nitrates; SOD = superoxide dismutase.

**Fig. 3.** PaO\(_2\)/FiO\(_2\) ratio and lung wet-to-dry weight ratio (LWDR) were determined in rabbits. (A) A significant reduction in PaO\(_2\)/FiO\(_2\) ratio at 4 h after endotoxin injection (\( P < 0.05 \)). Autologous transplantation of endothelial progenitor cells (EPCs) significantly increased PaO\(_2\)/FiO\(_2\) ratios at 24 h and 48 h after endotoxin injection. \# \( P < 0.05 \), EPC versus control and endothelial cells groups; n = 7. Data are presented as mean ± SD. Statistical analysis was first performed by two-way repeated ANOVA. Follow-up one-way ANOVA and repeated-measures ANOVA were used with the Tukey post hoc test for multiple groups. (B) A significant increase of LWDR 4 h after endotoxin injection (\( P < 0.05 \)). The LWDR was significantly reduced at 24 h and 48 h after endotoxin injection in the EPC-treated animals. \# \( P < 0.05 \), EPC versus control and EC; n = 5. Data are presented as mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Bonferroni correction multiple comparison procedure. Control = normal saline; EC = endothelial cells; EPC = endothelial progenitor cells.
Various cytokines and adhesion molecules participated in ALI and acute respiratory distress syndrome, which mobilized signaling cascades leading to inflammatory response.\textsuperscript{26} In the present study, we found that the levels of proinflammatory cytokines, such as IL-1\textsubscript{H9252}, E-selectin, and ICAM-1, were significantly inhibited in lung tissue after transplantation of EPCs, whereas the antiinflammatory cytokine IL-10 level was significantly increased. IL-10 has been suggested to mediate antiinflammatory response through suppression of tumor necrosis factor-\textgreek{a} and other proinflammatory cytokines.\textsuperscript{27} The increased level of IL-10 in the EPC-treated group was consistent with a reduced inflammation observed in the lung tissues. On the other hand, PMNs plays a key role in the development of ALI.\textsuperscript{28} We also confirmed that transplantation of EPCs reduced the numbers of PMNs infiltrating the lung parenchyma of endotoxin-induced ALI. Previous studies reported MSCs have a remarkable ability to modulate the immune system, partly through releasing proinflammatory and antiinflammatory cytokines and lipid mediators, such as prostaglandin E\textsubscript{2}.\textsuperscript{29,30} Intravenously injected MSCs markedly improved mortality from peritoneal sepsis in mice, an effect that was explained by the release of prostaglandin E\textsubscript{2} from the MSCs, which reprogrammed alveolar macrophages to increase production of IL-10.\textsuperscript{31} EPCs and MSCs were derived from bone marrow and had similar properties. However, the immune regulatory role of EPCs has not yet been well studied. In the current study, we also found EPCs may have modulated immune cell response by adjust-

Table 2. The Content of Interleukin-10, Interleukin-1\textbeta, E-selectin, and Intercellular Adhesion Molecule-1 in Lung Tissue Homogenate

<table>
<thead>
<tr>
<th>Groups</th>
<th>IL-1\textbeta (pg/mL)</th>
<th>E-selectin (ng/mL)</th>
<th>ICAM-1 (ng/mL)</th>
<th>IL-10 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>105.7 ± 9.8</td>
<td>0.854 ± 0.145</td>
<td>105.6 ± 8.5</td>
<td>18.5 ± 1.5</td>
</tr>
<tr>
<td>Control</td>
<td>574.5 ± 68.4*</td>
<td>6.852 ± 0.531*</td>
<td>691.4 ± 38.2*</td>
<td>11.4 ± 0.8*</td>
</tr>
<tr>
<td>EC</td>
<td>536.4 ± 60.8*</td>
<td>6.542 ± 0.443*</td>
<td>672.5 ± 41.7*</td>
<td>11.2 ± 0.7*</td>
</tr>
<tr>
<td>EPC</td>
<td>420.7 ± 74.2*†</td>
<td>4.135 ± 0.243*†</td>
<td>446.7 ± 43.5*†</td>
<td>48.8 ± 6.3*†</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD, n = 15. *Significantly different compared with sham group (\(P < 0.05\)). †Significantly different compared with control group and endothelial cell group (\(P < 0.05\)).

EC = endothelial cell; EPC = endothelial progenitor cell; ICAM-1 = intercellular adhesion molecule-1; IL-1\textbeta = interleukin-1\textbeta; IL-10 = interleukin-10.

Fig. 4. Lung injury degrees assessed by formation of hyaline membrane, hemorrhage, and polymorphonuclear cells (PMNs) infiltration. Hematoxylin-eosin staining (200 \(\times\) magnification) displayed infiltration of a large number of PMNs, formation of hyaline membrane, and hemorrhage in transplantation normal saline (control, B) or autologous endothelial progenitor cells (endothelial progenitor cells, C) than in sham animals (A). Compared with the control and endothelial cells group, areas of hyaline membrane (D), hemorrhage (E), and PMN ratio (F) were significantly reduced in endothelial progenitor cells group. Data were analyzed by one-way ANOVA followed by the Tukey post hoc test and are presented as mean ± SD; n = 15. *\(P < 0.05\), endothelial progenitor cells versus control and endothelial progenitor cells groups. Control = normal saline; EC = endothelial cells; EPC = endothelial progenitor cells.
ing proinflammatory cytokines and antiinflammatory cytokine IL-10, and inhibited ALI-induced pulmonary PMN infiltration and inflammatory cascade. In our unpublished study, we indicated that the role of IL-10 after EPC transplantation in endotoxin-induced acute lung injury was exerted as both antiinflammatory and antioxidization stress effect, whereas the direct endothelial repair effect by the EPC was not influenced; even the IL-10 was blocked in mice. In addition to the endothelial-repairing effect on the alveolar-capillary membrane, our results also suggested that antiinflammatory effect of EPCs on the infiltration of PMNs and inhibition of inflammatory cytokines in the lungs might provide supplementary therapeutic benefit in ALI.

Excessive production of reactive oxygen species and reactive nitrogen oxide species, such as superoxide radical, nitric oxide, and so on, have been proven to play crucial roles in the pathogenesis of endotoxin-induced ALI. Among them, the toxicity of nitric oxide in inflammatory conditions was mainly because of secondary oxidizing and nitrating species peroxynitrite (NO$_2^-$/NO$_3^-$). Although it has been established that antioxidant enzymes, namely SOD, are also critical in the defense against oxidative stress, the antioxidant profile of progenitor cells remains poorly understood. Moreover, the level of serum malonyldialdehyde and SOD indirectly reflected production and clearance of the free radical with endotoxin-induced ALI. In the present study, transplantation with EPCs significantly inhibited plasma levels of nitric oxide and malonyldialdehyde and increased the activities of SOD in ALI rabbits. Consistent with the previous report that high enzymatic activity of SOD was a critical mechanism protecting EPCs against oxidative stress, the mechanisms underlying high induction of SOD by EPCs were still not clear. Additionally, the iNOS was activated in macrophages, and its high level indicated an increased inflammatory state. Two studies using iNOS knockout animals and nitric oxide synthase inhibitors showed that increased iNOS played an important role in lipopolysaccharide-induced ALI. Our Western blot analysis showed iNOS protein was significantly up-regulated in the lung of ALI rabbits, indicating the presence of oxidative and inflammatory stresses in the injured lung. Autologous transplantation of EPCs significantly decreased iNOS in lung tissue, suggesting inhibited inflammatory response. Altogether, EPCs might maintain endothelial regenerative function under severe oxidative stress by increased intrinsic antioxidant enzymatic activity.

In conclusion, EPC-based transplantation may provide a novel pulmonary endothelium-targeted therapy for ALI/acute respiratory distress syndrome, by directly repairing the
damaged endothelial, relieving the inflammatory response through regulating secrete of proinflammatory and anti-inflammatory cytokines, elevating intrinsic antioxidant property, and, as a consequence, attenuating acute lung injury. However, the pathophysiology of ALL/acute respiratory distress syndrome is a very complicated process, and the exact mechanisms underlying the immune regulatory role of EPCs need further investigation. One of our limitations is that the occurrence of ALL is often unpredictable, so it is very hard to obtain enough EPCs in advance for autologous transplantation, which hampers its clinical application. Although there is a long way to go before cell-based therapy can be used clinically for acute lung injury, we believe that preclinical study on its mechanisms will help to test its safety and efficacy.

References


