Anesthesiology 2008; 108:392–401

Autologous Transplantation of Endothelial Progenitor Cells Attenuates Acute Lung Injury in Rabbits

Chen-Fuh Lam, M.D., Ph.D., Yen-Chin Liu, M.D., M.S.,† Jen-Kuo Hsu, M.D.,‡ Pei-An Yeh, M.D.,§ Ting-Ya Su, M.S.,‖ Chien-Chi Huang, B.S.,‖ Ming-Wei Lin, M.S.,# Ping-Ching Wu, M.S.,# Pei-Jung Chang, M.D.,§ Yu-Chuan Tsai, M.D.***

Background: **Acute lung injury (ALI) and end-stage acute respiratory distress syndrome (ARDS) are among the most common causes of death in intensive care units. Activation and damage of pulmonary endothelium is the hallmark of ALI/ARDS. Recent studies have demonstrated the importance of circulating endothelial progenitor cells (EPCs) in maintaining normal endothelial function as well as endothelial repairing after vascular injury. Here, the authors present the first study demonstrating the therapeutic potential of EPCs in a rabbit model of ALI/ARDS.**

Methods: **Circulating EPCs were obtained from rabbits using Ficoll centrifugation. One week after culturing, ALI was induced in rabbits by oleic acid (75 mg/kg, intravenous), and autologous EPCs were transplanted intravenously. Vasomotor function of isolated pulmonary artery and degrees of lung injury were assessed 2 days later.**

Results: **Endothelial dysfunction in the pulmonary artery was significantly attenuated in rabbits treated with EPCs, whereas the endothelium-independent relaxation responses were not different. Expression of inducible nitric oxide synthase was suppressed in the pulmonary artery of EPC-treated animals. Infiltration of leukocytes in the lung parenchyma was significantly reduced after EPC transplantation. EPCs also decreased water content, hyaline membrane formation, and hemorrhage in lungs.**

Conclusion: **The authors demonstrated that autologous transplantation of EPCs preserves pulmonary endothelial function and maintains the integrity of pulmonary alveolar–capillary barrier. Transplantation of EPCs can be a novel cell-based, endothelium-targeted therapeutic strategy for prevention and treatment of ALI/ARDS.**

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 5A.

This article is accompanied by an Editorial View. Please see: Burnham EL: Circulating progenitors in lung injury: A novel therapy for acute respiratory distress syndrome? ANESTHESIOLOGY 2008; 108:354–6.

* Resident, † Assistant Professor, ‡ Chief Resident, § Clinical Consultant, ‖ Research Assistant, ** Professor and Head, Department of Anesthesiology, National Cheng Kung University College of Medicine and Hospital. # Ph.D. Candidate, Institute of Basic Medical Sciences, National Cheng Kung University College of Medicine.

Received from the Department of Anesthesiology, The National Cheng Kung University Medical College and Hospital, Tainan, Taiwan. Submitted for publication March 23, 2007. Accepted for publication August 22, 2007. Supported by research grant Nos. NCKUH-95-079 (to Dr. Yeh) and NCKUH-95-010 (to Dr. Chang) from the National Cheng Kung University Hospital, Tainan, Taiwan, and research grant No. NSC-94-2314-B-006-046 (to Dr. Tsai) from the National Science Council of Taiwan, Taipei, Taiwan.

Address correspondence to Dr. Tsai: Department of Anesthesiology, National Cheng Kung University, Medical College and Hospital, Tainan, Taiwan. yctsai@mail.ncku.edu.tw.

ACUTE lung injury (ALI) and acute respiratory distress syndrome (ARDS) are commonly developed in patients with sepsis, multiple trauma, massive blood transfusion, burn, and cardiopulmonary bypass.[1] Despite the advances in supportive and pharmacologic treatment, ALI/ARDS remains a serious problem in health care because it carries a high mortality (up to 30–40%) and morbidity.[2] The histologic changes of lung tissues in ALI/ARDS during the acute exudative phase (first 24–48 h) are characterized by infiltration of inflammatory cells and destruction of pulmonary endothelium.[3,4] The injured alveolar–capillary barrier leads to increased pulmonary vascular permeability, pulmonary edema and hypoxemia secondary to increased intrapulmonary shunt fraction, decreased lung compliance, and increased ventilation/perfusion mismatch. Several pharmacologic agents have been examined in the management of ALI/ARDS; unfortunately, none of these have yet met with success.[5]

During the past decade, evidence continues to accumulate on the capability of circulating endothelial progenitor cells (EPCs) in neovascularization and vasculogenesis after their first discovery by Asahara *et al.*[6] in 1997. These circulating endothelial cell progenitors share certain common embryonic cell markers with hematopoietic stem cells, including Flk-1, Tie-2, and CD34.[7] Circulating EPCs are mobilized from bone marrow to peripheral circulation by cytokines, growth factors, and ischemic conditions during endothelial injury and by pharmaceutics such as statins.[8] The endothelial regenerative potential of EPCs has been under intensive investigation in a variety of animal models of vascular injury, including hind limb ischemia,[9] myocardial ischemia,[10] carotid artery injury,[11] and vascular graft survival.[12] Several clinical studies, including randomized controlled trials, also reported some encouraging outcomes in patients with critical limb ischemia and myocardial infarction after administration of EPCs.[13–16] Overall, these studies in diseased animals or patients implicate that circulating EPCs exert important therapeutic function as an endogenous repair mechanism in maintaining integrity of the endothelial monolayer by replacing denuded parts of the injured artery and/or forming new vessels by direct incorporation and paracrine-mediated effects. However, there is currently no published report demonstrating the therapeutic effects of EPCs in animal models or human subjects with ALI/ARDS. Recently, Burnham *et al.*[17] reported an important observational study measuring the circulating EPCs in patients with

ALI. Peripheral blood mononuclear cells were collected from patients with ALI, and colony-forming units of EPCs were counted 7 days after incubation. Their results showed that EPC colony numbers were twofold higher in patients with ALI compared with healthy volunteers. In the 45 patients with ALI, improved survival was associated with a higher EPC colony count, and mortality was significantly increased in ALI patients with EPC colony-forming units below 35. Yamada *et al.*[18] also demonstrated that bone marrow–derived progenitor cells accumulated in the inflammatory site of lung after lipopolysaccharide-induced ALI and differentiated into pulmonary endothelial cells. Currently, regeneration of pulmonary endothelium has been suggested as one of the potential therapeutic targets for ALI/ARDS.[19] Therefore, the current study tested the effects of EPCs on pulmonary endothelial regeneration, as a novel cell-based, endothelium-targeted therapeutic strategy in a rabbit model of ALI.

Materials and Methods

Isolation and Culturing of EPCs

Peripheral blood was obtained from healthy human volunteers and New Zealand White rabbits *via* the peripheral vein (20 ml) and ear artery (10 ml/kg), respectively. Peripheral blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Plaque Plus (Amersham Biosciences, Buckinghamshire, United Kingdom).[20] Mononuclear cells were then washed and plated on six-well plates coated with human fibronectin (Sigma-Aldrich Corp., St. Louis, MO), supplemented with endothelial growth medium 2 (Cambrex Corp., Charles City, IA). At day 7 of culture, the adherent cells (known as early EPCs) were harvested by trypsinization for analysis or transplantation.[11] All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee and were approved by the Institutional Review Board (The National Cheng Kung University, Tainan, Taiwan).

Characterization of EPCs

The early EPCs isolated from rabbits were characterized as previously described.[11,20] Briefly, cells were incubated subsequently with acetyl-LDL (10 μg/ml; Molecular Probes, Carlsbad, CA) and isolectin (5 μg/ml; Molecular Probes). The staining of acetyl-LDL and isolectin in cultured EPCs was detected under fluorescence confocal microscopy at absorption wavelengths of 555 and 495 nm, respectively. In a separated experiment, isolated rabbit mononuclear cells were cultured on four-well glass slides coated with human fibronectin. On day 7, the attached EPCs were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Amresco, Inc., Solon, OH) in phosphate buffer solution. The cells were incubated with primary antibodies against Flk-1 (a specific endothelial cell marker; Santa Cruz Biotechnology, Santa Cruz, CA) and processed for indirect immunofluorescence staining. The expression of Flk-1 was analyzed by confocal microscopy.

Induction of HO-1 and MnSOD in Cultured Human EPCs

Because mature rabbit endothelial cells are not available in our laboratory, mature endothelial cells, namely human umbilical vein endothelial cells, were used to compare with the day-7 EPCs. The protein expression of heme oxygenase 1 (HO-1) and manganese superoxide dismutase (MnSOD) in human EPCs was compared with that in human umbilical vein endothelial cells. Human umbilical vein endothelial cells and human EPCs were incubated with different concentrations of hemin (0–50 μM; Sigma-Aldrich Corp.) in endothelial basic medium 2 for 2 h at 37°C. Solutions were washed three times with phosphate buffer solution. Cells were supplemented with endothelial basic medium and cultured for another 12 h. Expression of HO-1 and MnSOD was determined by Western blotting.

Rabbit Model of ALI and Transplantation of EPCs

Rabbits (2.5–3 kg) were anesthetized with intramuscular injection of ketamine (35 mg/kg), and oleic acid (75 mg/kg; Sigma-Aldrich Corp.) was infused over 20 min *via* the right ear vein. Pulmonary endothelial damage occurs within hours after oleic acid infusion; interstitial/intraalveolar edema and lung hemorrhage with vascular congestion develop by 24 h.[21] Autologous EPCs (approximately 10^5 cells in a volume of 200 μl saline) or placebo (200 μl saline) were transplanted *via* the ear vein of each rabbit 30 min after infusion of oleic acid. Immediately before transplantation, a fluorescent cell tracker, CM-DiI (Molecular Probes, Carlsbad, CA), was used to label EPCs to track their homing in the pulmonary vasculature.[11] Sham-operated animals received either placebo or autologous EPC transplantation without injection of oleic acid. Rabbits were allowed to recover spontaneously from anesthesia after the procedures. Forty-eight hours later, rabbits were killed by intravenous administration of pentobarbital (250 mg/kg). Right and left main pulmonary arteries were isolated, and lung tissues were excised.

Vasomotor Reactivity

Pulmonary artery rings (approximately 2 mm long) were mounted in organ chambers containing 25 ml Krebs solution (118.6 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.1 mM $NaHCO_3$, 10.1 mM glucose, 0.026 mM EDTA) at 37°C (94% O_2–6% CO_2). Changes in force were recorded continuously using an isometric force-displacement transducer (Grass FT03; Grass Instrument, West Warwick, RI). Each ring was gradually stretched to 2.5 g. After a 45-min equilibration period, the rings were contracted by addition of KCl (40 mM) or in-

creasing concentrations of phenylephrine (10^{-9} to 10^{-5} M; Sigma-Aldrich Corp.). Concentration–response curves were obtained by cumulative addition of acetylcholine (10^{-9} to 10^{-5} M; Sigma-Aldrich Corp.), and a nitric oxide donor (DEA-NONOate, 10^{-9} to 10^{-5} M; Sigma-Aldrich Corp.) during contraction to a median effective concentration (EC_{50}) of phenylephrine. Papaverine (3×10^{-4} M; Sigma-Aldrich Corp.) was used to induce complete relaxations of the vessels. All experiments were performed in intact vessels without mechanical removal of pulmonary endothelium.

Western Blot Analysis

Soluble protein extracts (30–50 μg) isolated from pulmonary artery were loaded into polyacrylamide gels (9–12%) and transferred onto nitrocellulose membranes. Anti–inducible nitric oxide synthase (iNOS; BD Biosciences, San Jose, CA), anti–HO-1 (Santa Cruz Biotechnology), and anti-MnSOD (BD Biosciences) antibodies were used. After washing, the membranes were incubated with horseradish peroxidase–linked secondary antibodies, and bands were visualized using enhanced chemiluminescence (Amersham Pharmacia, Buckinghamshire, United Kingdom). Protein levels were quantified by scanning densitometry (Scion Image, Scion Corp., Frederick, MD).

Measurement of Lung Dry-to-wet Ratio

The intermediate lobe of right lung was excised and weighed immediately. Lung tissues were dried in an oven at 80°C for 12 h and reweighed. The lung wet-to-dry ratios were obtained by dividing the mass of the initial specimen by the mass of the dried specimen.

Histologic Examination of Pulmonary Artery and Lung Tissues

Frozen isolated pulmonary artery and lung tissues were sectioned and examined under fluorescence microscopy for the detection of DM-DiI–labeled cells. Other lung tissues were immersed in 10% buffered formal saline and fixed for at least 24 h. Biopsies were processed through increasing grades of alcohol and embedded in paraffin wax. Hematoxylin and eosin–stained lung sections were examined under a light microscope and photographed. An investigator blinded to the treatment was assigned to measure areas of hyaline membrane formation and hemorrhage in the lung sections using the TwinCAD software (TCAM Development, Taipei, Taiwan). Infiltration of polymorphonuclear cells (PMNs) in the lung tissue was also measured using the PMN ratio, as previously described.[22] Briefly, numbers of PMNs and non-PMNs were counted from 40 randomly selected fields in the five domains of each tissue slide under light microscopy (400×). Each observed area was defined with a graticule fitted in the eyepiece (an effective calibrated area of 2,830 μm^2). A ratio of PMNs to non-PMNs was calculated for each tissue slide. According to our previous report, normal human lung tissues yield PMN ratios of 1.3–2.3%, whereas sections obtained from pneumonia or lung abscess yield higher PMN ratios of 8.3–21.8%.[22]

Statistical Analysis

Results are presented as mean ± SEM. Data were compared by an unpaired *t* test or analysis of variance, as appropriate. Statistical significance was accepted at a level of $P < 0.05$.

Results

Characterization of Phenotypes of EPCs

Cultured day-7 rabbit EPCs exhibited phenotyping of endothelial cells, including incorporation of acetyl-LDL and isolectin and expression of Flk-1 (figs. 1A–E). Formation of monolayer colonies with "cobblestone" appearance was also observed 2 weeks after culturing in endothelial basic medium 2 (fig. 1F).[11]

Expression of HO-1 and MnSOD in Isolated EPCs

To determine the endogenous antioxidant capacity in EPCs, we studied the expression of HO-1 and MnSOD in these premature endothelial cells isolated from human peripheral blood after incubation with hemin for 2 h. Compared with human umbilical vein endothelial cells, human EPCs expressed higher levels of MnSOD in unstimulated conditions, and expression of HO-1 was also more significantly up-regulated in a dose-dependent manner after exposure to hemin (fig. 2).

Detection of Transplanted EPCs in the Pulmonary Circulation

Fluorescence-labeled EPCs were detected on the lumens of intralobular pulmonary arterioles, indicating the homing of transplanted EPCs on the injured pulmonary endothelium (fig. 3).

Assessment of Vascular Function of Pulmonary Artery

Vascular function of pulmonary artery was assessed by isometric tension recordings. Contraction responses of isolated pulmonary artery to KCl and phenylephrine were significantly enhanced in rabbits treated with EPCs, but EC_{50}s of phenylephrine were not different (table 1). Transplantation of EPCs attenuated the impaired endothelium-dependent relaxations of pulmonary artery to acetylcholine in rabbits with ALI (fig. 4A). High concentration of acetylcholine (10^{-5} M) induced significantly greater relaxations in the EPC group compared with controls (81.9 ± 4.8% *vs.* 45.1 ± 10.2%, respectively; $P < 0.001$, n = 6–8). In fact, high concentrations of acetylcholine (10^{-7} to 10^{-5} M) induced contractile responses in the control group (fig. 4A). However, the

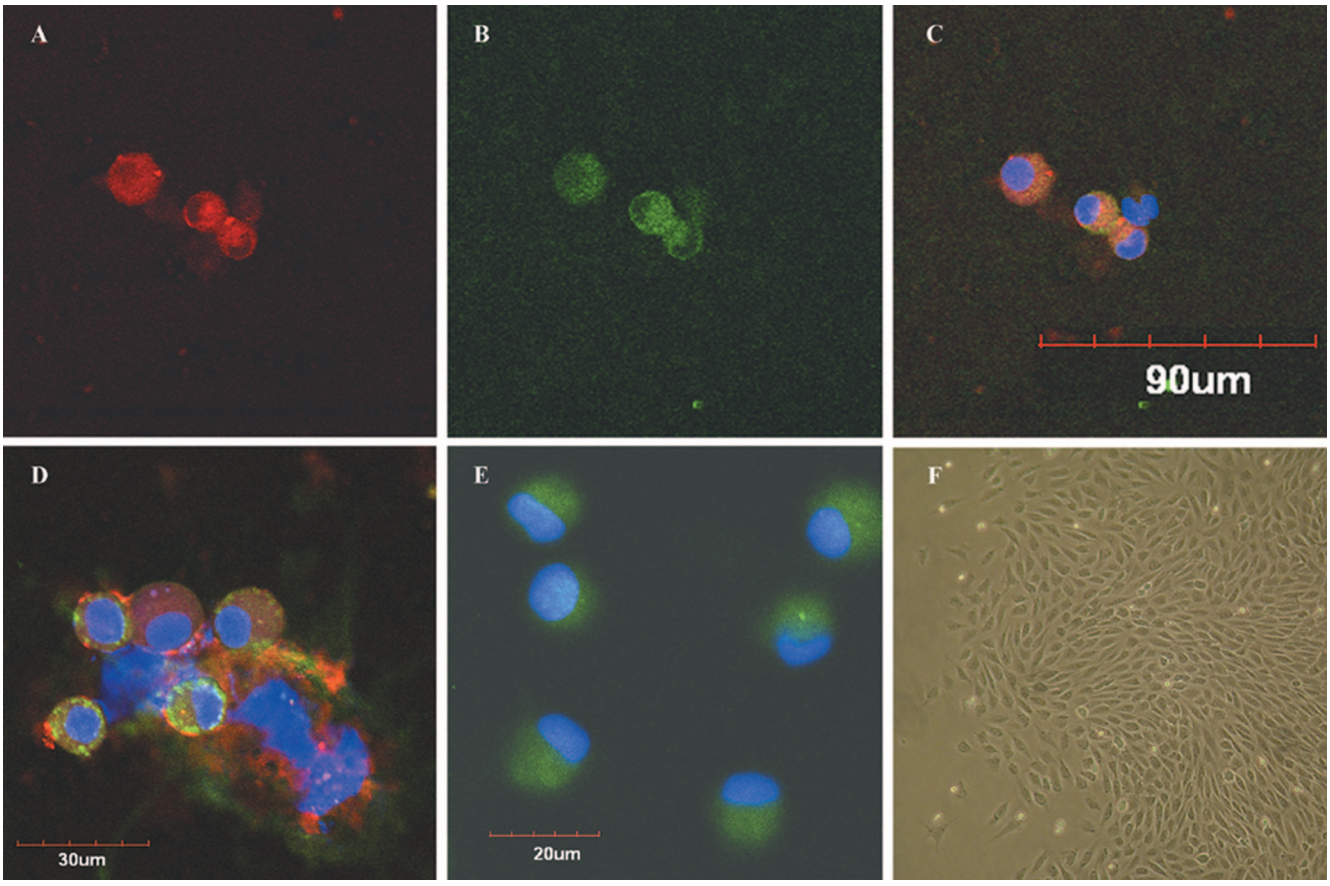

Fig. 1. Characteristics of rabbit circulating endothelial progenitor cells. Mononuclear cells were isolated from peripheral blood of rabbits using the Ficoll centrifugation and cultured in supplementation with endothelial growth medium 2. These endothelial progenitors exhibited phenotyping of endothelial cells with incorporation of acetyl LDL (*A*, *red fluorescence*) and isolectin (*B*, *green fluorescence*). *C* and *D* show overlaying of acetyl LDL and isolectin in the cultured endothelial progenitor cells. Expression of Flk-1 was also detected on endothelial progenitor cells (*E*, *green fluorescence*). *A–E* were photographed under fluorescence confocal microscopy, and nuclei were counterstained in *blue fluorescence*. Colonies of outgrowth endothelial progenitor cells in the typical monolayer "cobblestone" appearance were obtained 2–3 weeks after plating (*F*).

median concentrations of acetylcholine for inducing 50% relaxations (EC_{50R}) of precontracted pulmonary artery in the control and EPC-treated groups were similar ($4.76 \pm 1.55 \times 10^{-8}$ M *vs.* $5.23 \pm 1.55 \times 10^{-8}$ M, respectively; $P = 0.88$, n = 6–8). On the other hand, endothelium-independent relaxations were similar between the two treatment groups (fig. 4B), suggesting that function of vascular smooth muscle was not affected by transplantation of EPCs. Because of superimposition of the concentration–response curves between sham-treated animals receiving placebo or EPC transplantation, results of the latter were not shown.

Expression of iNOS in Pulmonary Artery

Protein expression of iNOS was up-regulated in the pulmonary artery of rabbits with oleic acid–induced ALI (fig. 5). Compared with controls, autologous transplantation of EPCs significantly reduced expression of iNOS in pulmonary artery (fig. 5).

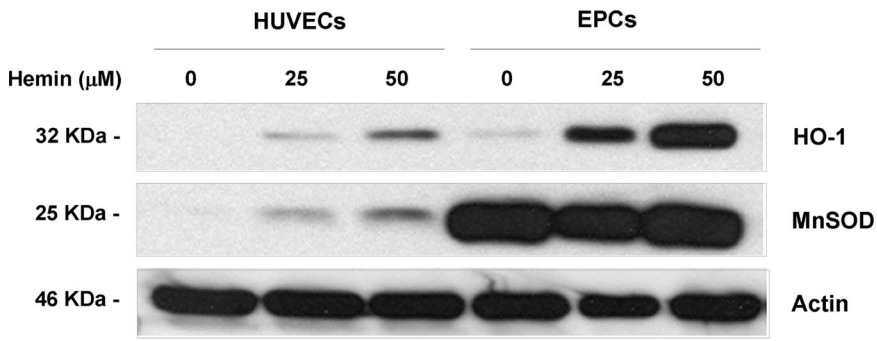

Fig. 2. Representative Western blots of concentration-dependent increased protein expression of heme oxygenase 1 (HO-1) and manganese superoxide dismutase (MnSOD) in human umbilical vein endothelial cells (HUVECs) and human endothelial progenitor cells (EPCs) 12 h after exposure to hemin (0–50 μM). EPCs were isolated from human blood and analyzed 7 days after plating in endothelial growth medium 2. Experiments were performed in blood samples obtained from three independent healthy human volunteers (EPCs) and two different sources of HUVECs.

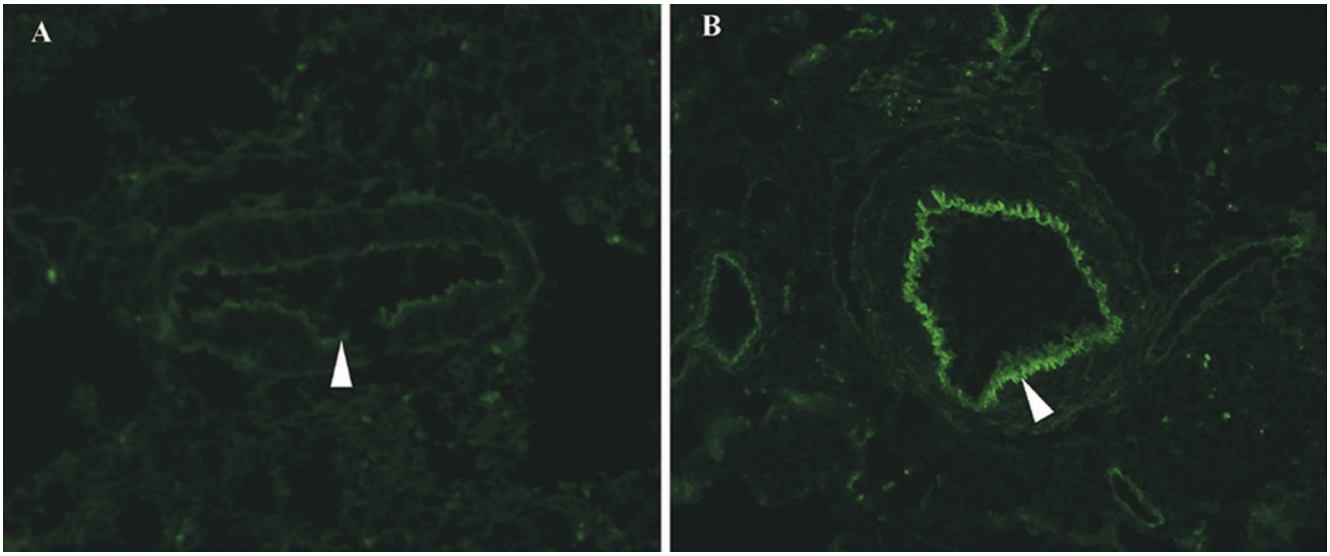

Fig. 3. Detection of fluorescent CM-DiI–labeled cells in the endothelium of intralobular pulmonary arterioles of rabbits under fluorescence microscopy. Fluorescent density was absent in the pulmonary arterioles of placebo-treated animals (*A*), where fluorescent-labeled cells were identified in the lung of animals that received autologous transplantation of endothelial progenitor cells (*B*). *Arrows* indicate the intimal layer of pulmonary arterioles. Histologic sections are shown in 200× magnification. Experiments were performed in the lung biopsies of 3–5 rabbits for each group.

Determination of Degrees of Lung Injury

Disruption of alveolar–capillary barrier was assessed by measuring water and fluid content in the lungs. The lung wet-to-dry ratio was significantly reduced in rabbits received autologous transplantation of EPCs (fig. 6). Degrees of lung parenchymal damage were determined under light microscopy. Formation of hyaline membrane and hemorrhage were dramatically reduced in the lung tissue of rabbits treated with EPCs (fig. 7). Because infiltration of PMNs is the characteristic histologic changes during the acute exudative phase (first 24–48 h) of ALI/ARDS, we therefore examined the counts of PMNs in the lungs. Totals of 1,430 ± 68 and 1,410 ± 62 cells were counted from each lung section under light microscopy in the control and EPC-treated groups, respectively ($P = 0.7$, n = 3 animals in each group). A significantly higher PMN ratio was detected in lung tissue of the placebo-treated animals (fig. 8).

Discussion

We present the first study demonstrating that transplantation of circulating EPCs attenuates lung injury and pulmonary artery endothelial dysfunction in rabbits with oleic acid–induced ALI. Transplantation of EPCs potentiates the relaxation response to acetylcholine in pulmonary arteries and reduces lung water content, suggesting that the therapeutic benefits of EPCs in ALI are most likely derived from their effect on reendothelization of the damaged pulmonary artery wall and alveolar–capillary membrane.

Acute lung injury and ARDS are characterized by diffuse pulmonary infiltration, increased pulmonary capillary permeability, and severe hypoxemia. The initial physiopathologic changes are damage of endothelial surfaces and disruption of the alveolar–capillary barrier function, and flooding alveolar spaces with fluid.[23] Hence, several pulmonary endothelium–targeted therapeutics, such as surfactants, pulmonary vasodilators, an-

Table 1. Contractions to KCl and Phenylephrine in Isolated Pulmonary Artery

		10^{-9}–10^{-5} M Phenylephrine			
Contraction to 40 mM KCl, g		Control		EPC	
Control	EPC	C_{max}, g	$EC_{50} \times 10^{-7}$ M	C_{max}, g	$EC_{50} \times 10^{-7}$ M
3.6 ± 0.4*	5.3 ± 0.6	3.5 ± 0.2*	10 ± 3.3	4.9 ± 0.9	9.9 ± 2.6

Animals in the endothelial progenitor cell (EPC)–treated group received autologous transplantation of EPC after induction of acute lung injury. Contractions (force in grams) to KCl (40 mM) and phenylephrine (10_{-5} M) were 5.3 ± 0.4 and 5.1 ± 0.2 g, respectively, in the isolated pulmonary artery of sham-treated animals (rabbits without oleic acid–induced acute lung injury). n = 6–8 animals in each group. Data were analyzed by unpaired *t* test and are presented as mean ± SEM.

* $P < 0.05$ compared with EPC group.

C_{max} = maximal contraction; EC_{50} = mean concentrations of phenylephrine that induced 50% of C_{max}.

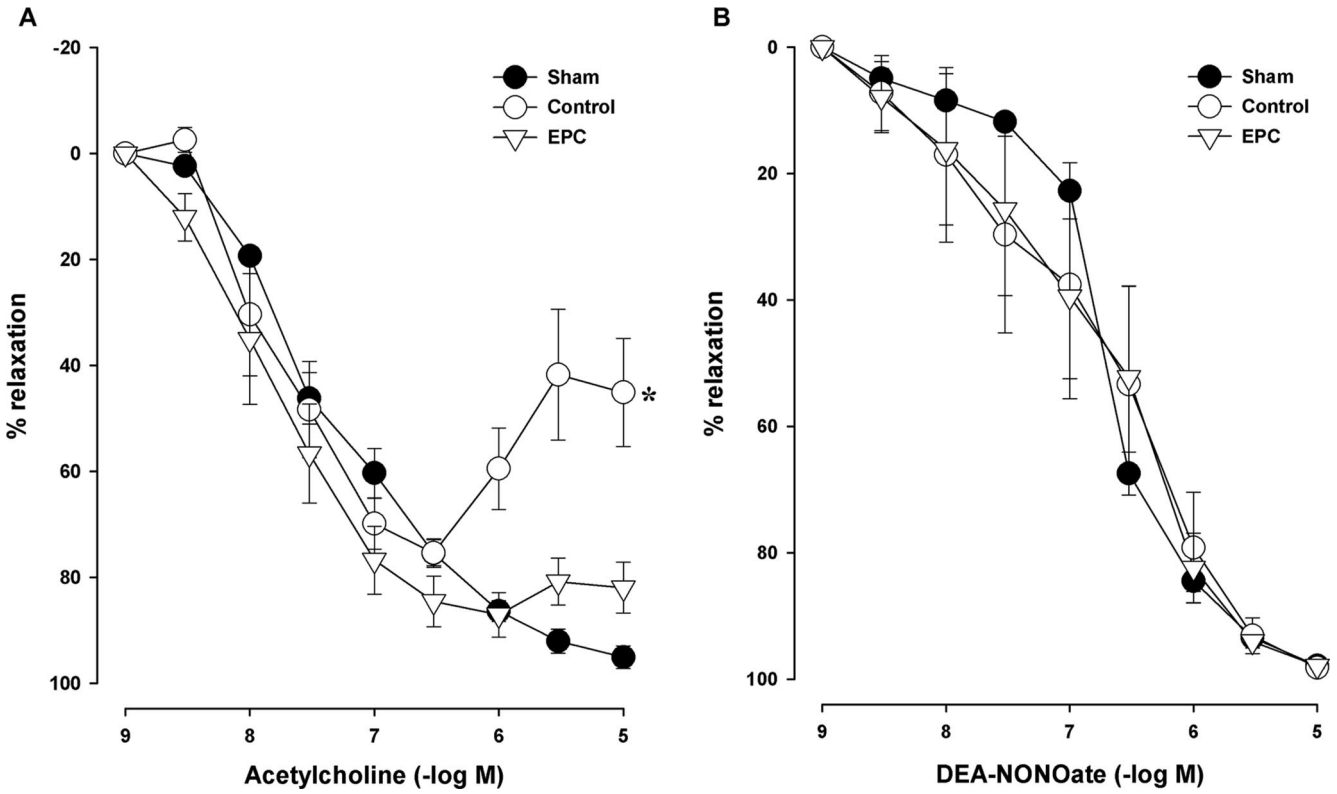

Fig. 4. Measurements of isometric force of pulmonary artery segments in shams and rabbits with oleic acid–induced acute lung injury. Animals with acute lung injury received placebo (control) or autologous transplantation of endothelial progenitor cells (EPCs) 2 days after administration of oleic acid. Sham-treated animals represent rabbits without oleic acid–induced acute lung injury. (*A*) Endothelium-dependent relaxation responses to cumulative addition of acetylcholine. * $P < 0.05$, control *versus* EPCs. (*B*) Relaxation responses to cumulative addition of DEA-NONOate (Sigma-Aldrich Corp., St. Louis, MO), a nitric oxide donor. Relaxations were obtained during contraction to an EC_{50} (the concentration required to achieve 50% of maximum contraction) of phenylephrine. Data were analyzed by analysis of variance and are presented as mean ± SEM. n = 6–8 animals in each group.

tioxidants, antiinflammatory agents, and statins, have been extensively tested in patients with ALI/ARDS, aiming to restore pulmonary endothelial function. However, none of these pharmacologic endothelium-targeted therapeutics reduce mortality of ALI/ARDS. In the current study, we used a novel cell-based, potent endothelial regenerating approach, namely transplantation of EPCs, to test our hypothesis that the high reendothelization capability of EPCs restores damaged pulmonary endothelium and thus provides therapeutic benefits in ALI. One of the foremost concerns for delivering cell-based therapeutics is the survival of target cells in unfavorable pathologic conditions, and homing of these cells in the injured tissues. First, we studied the endogenous antioxidant capacity of EPCs. In comparison with mature endothelial cells, early EPCs isolated from healthy human subjects expressed higher protein levels of MnSOD and HO-1. MnSOD is an abundant mitochondrial enzyme that catalyzes superoxide generated by respiratory chain activity into water and hydrogen peroxide,[24] and HO-1 is potent inducible antioxidant gene that modulates cell growth, inflammation, and apoptosis.[25] High expression of MnSOD in human EPCs was shown by two independent research groups,[20,26] but induction of HO-1 in these premature endothelial cells has not been previously demonstrated. Elevated intrinsic antioxidant enzymatic activity in EPCs may enable them to maintain endothelial regenerative function under conditions of severe oxidative stress, such as ALI/ARDS, and may also attribute to the paracrine cytoprotective effects. On the other hand, we located the transplanted EPCs on the lumen wall of pulmonary arterial system using fluorescence-conjugated cell tracers, indicating that EPCs homed in the pulmonary endothelium and may subsequently reendothelize the injured endothelium after intravenous administration. Nevertheless, we were not able to detect a large amount of DM-DiI–labeled cells from the serial lung sections of rabbits that received EPC transplantation and therefore were not able to provide quantitative data on this measurement. In our opinion, EPCs dispersed in the extremely large surface areas of pulmonary circulation after intravenous transplantation and thus reduced the ability of discovery from thin histologic sections. In addition, low homing efficacy of progenitors in the target organs has been reported after systemic or regional delivery, as a result of washout effect.[27,28] After 24 h, only 2–5% of the transplanted progenitor cells were detected in the bone marrow of recipient animals.[29] Muller-Ehm-

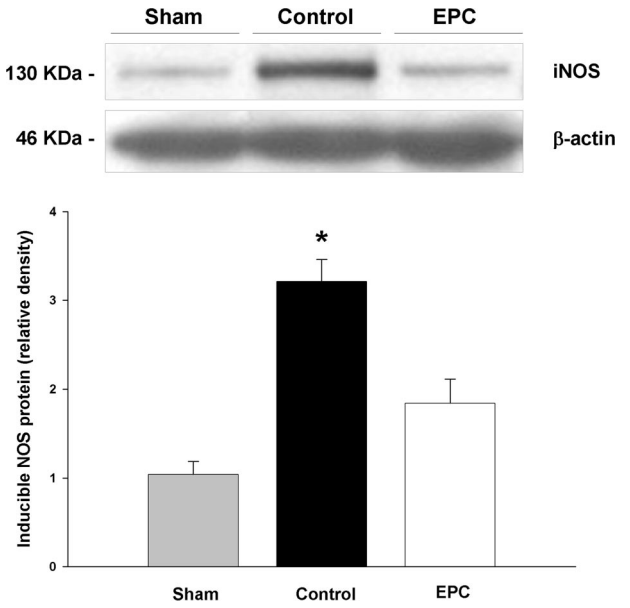

Fig. 5. Western blot analysis for protein expression of isolated pulmonary artery in shams (rabbits without oleic acid–induced acute lung injury) and rabbits with oleic acid–induced acute lung injury. Animals with acute lung injury received placebo (control) or autologous transplantation of endothelial progenitor cells (EPCs) 2 days after administration of oleic acid. Expression of inducible nitric oxide synthase (iNOS) was enhanced after induction of acute lung injury, and transplantation of EPCs significantly suppressed the expression of iNOS in the pulmonary artery. * $P < 0.05$, control *versus* EPCs. n = 4 or 5 animals in each group. Statistical analysis (unpaired *t* test) was performed by comparing the relative density of bands quantified by scanning densitometry (Scion Image Corp., Frederick, MD) between controls and EPC-treated animals. Data are shown as mean ± SEM.

sen *et al.*[30] showed that only 24% of the transplanted cells remained in the recipient heart 1 day after direct ventricular injection. Indeed, our results also support the general concept that apart from direct cell fusion, EPCs may mediate angiogenesis and reendothelization *via* their paracrine effects.

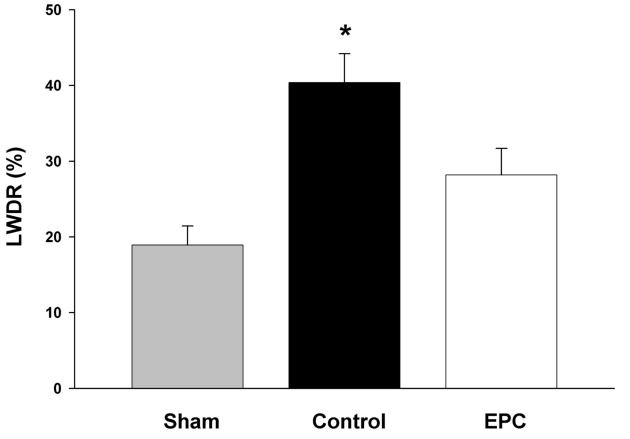

Fig. 6. Lung water content was determined by measuring the lung wet-to-dry ratio (LWDR) in rabbits with and without (sham) acute lung injury. Compared with controls, LWDR was significantly reduced after transplantation of endothelial progenitor cells (EPCs) in animals with oleic acid–induced acute lung injury. * $P = 0.04$, control *versus* EPCs. n = 5 or 6 animals in each group. Data were analyzed by the unpaired *t* test and are shown as mean ± SEM.

Vascular function of the isolated pulmonary artery was assessed by contraction and relaxation responses in organ bath experiments. Vasocontraction was induced by addition of high concentrations of extracellular potassium and phenylephrine. Contraction responses to 40 mM KCl and 10^{-5} M phenylephrine were reduced in the pulmonary artery isolated from control animals. Because morphologic changes in media layer were unlikely, we therefore sought functional alteration in vascular smooth muscle. Consistent with previous reports,[31,32] Western analysis showed that expression of iNOS was significantly up-regulated in the pulmonary artery of animals with ALI, indicating the presence of oxidative stress in the injured pulmonary artery. However, transplantation of EPCs significantly suppressed the protein levels of iNOS in pulmonary artery. Induction of iNOS is commonly suggested to be responsible for diminished vasoconstriction of pulmonary artery due to overproduction of nitric oxide.[33,34] In addition, our result also speculates that transplantation of EPCs attenuates expression of iNOS. However, biologic mechanisms underlying regulation of iNOS by transplantation of EPCs require further investigation. We used different means to assess the function of pulmonary endothelium and the integrity of the alveolar–capillary barrier after placebo or EPC transplantation in rabbits with oleic acid–induced ALI. Endothelial dysfunction of pulmonary artery was confirmed by reduced relaxation response to acetylcholine in rabbits with ALI. Functional analysis of pulmonary artery isolated from rabbits that received EPC transplantation showed that contractile response to KCl and phenylephrine was restored, and endothelium-dependent relaxation response to acetylcholine was significantly potentiated. Instead of causing vasorelaxation, the pulmonary artery tended toward contraction in the control group during cumulative addition of higher concentrations of acetylcholine, suggesting the presence of endothelial injury. Acetylcholine induces vasodilation by releasing endothelium-derived relaxing factor in the presence of intact endothelium, whereas it causes vasoconstriction when the endothelium is removed or damaged.[35,36] Forty-eight hours after transplantation of EPCs, endothelium-dependent relaxation was partly restored (more than 80% of complete relaxation in comparison with 94.8% in sham-treated animals) with the absence of acetylcholine-induced vasocontraction. Together with the normal endothelial-independent relaxation response to nitric oxide donor, we conclude that the vascular regenerative effect of EPC in ALI is endothelium targeted.

The alveolar–capillary membrane is a blood-gas barrier formed by alveolar epithelium, pulmonary capillary endothelium, and lamina densa. This anatomical interface

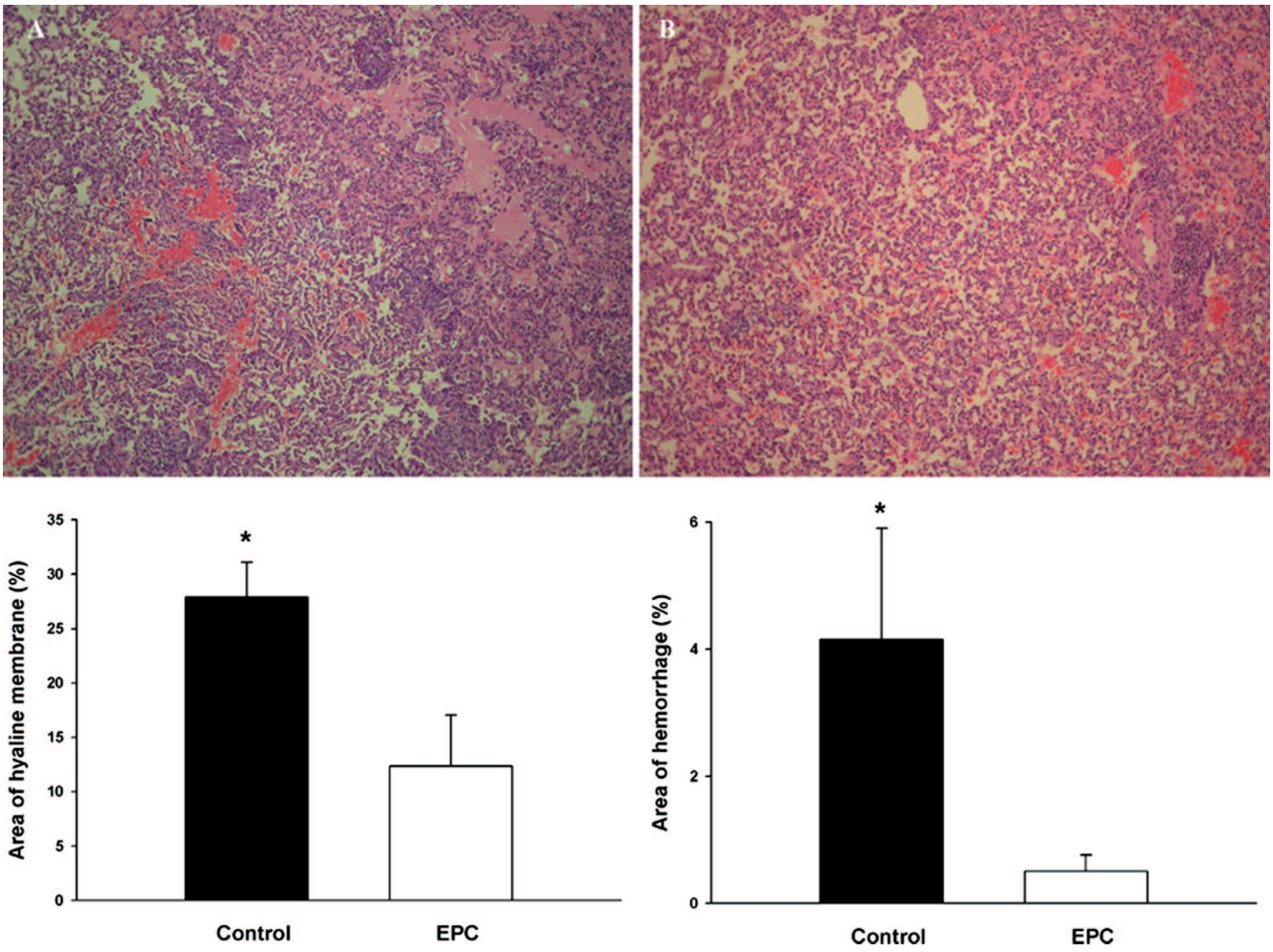

Fig. 7. Degrees of lung injury were assessed by formation of hyaline membrane and hemorrhage in the lung parenchyma of rabbits after transplantation with placebo (control, *A*) or autologous endothelial progenitor cells (EPCs, *B*). Areas of hyaline membrane and hemorrhage were measured using TwinCAD software (TCAM Development, Taipei, Taiwan). Data were analyzed by unpaired *t* test and are presented as mean ± SEM. * $P < 0.05$. n = 4 animals in each group.

allows gas exchange; regulates solute and fluid flux between the alveolar surface, interstitium, and blood; and promotes active fluid clearance from the alveolar lumen to the interstitial space.[37] During the progression of ALI/ARDS, the alveolar–capillary membrane is disrupted by activation of pulmonary endothelium, expression of adhesion molecules, and massive infiltration of PMNs, hence followed by increased protein permeability across the barrier.[5] The characteristic pathologic changes after disruption of alveolar–capillary membrane are noncardiogenic pulmonary edema, hyaline membrane formation, and lung hemorrhage.[38,39] We examined the lung water content in the rabbits with ALI by measuring the lung wet-to-dry ratio. The ratio of wet-to-dry weight was significantly lower in the lungs of animals that received EPC transplantation. Histologic examination also revealed significantly reduced formation of hyaline membrane and area of hemorrhage in the lung sections of EPC-treated animals. Collectively, these data suggest that function of alveolar–capillary membrane was, at least partly, restored by administration of EPCs. In accord with isometric tension analysis of pulmonary artery, these findings reinforce the concept of endothelium-targeting therapeutic potential of EPC transplantation in ALI/ARDS.

Another biologic marker of ALI/ARDS is activation and transmigration of circulating neutrophils, or so-called PMNs, in the lung tissues.[40] In fact, elimination of lung PMNs by cyclophosphamide or antineutrophil antibodies diminished pulmonary edema in hemorrhage- or endotoxin-induced ALI in mice.[41] In the current study, we demonstrated that transplantation of EPCs reduced the numbers of PMN infiltrating the lungs of oleic acid–induced ALI. In addition to endothelial regeneration effect on the pulmonary artery and alveolar–capillary membrane, antiinflammatory effect of EPCs on the infiltration and function of PMNs in the lungs may provide supplementary therapeutic benefit in ALI. However, the actual mechanisms underlying suppression of PMNs after administration of EPCs require further investigation.

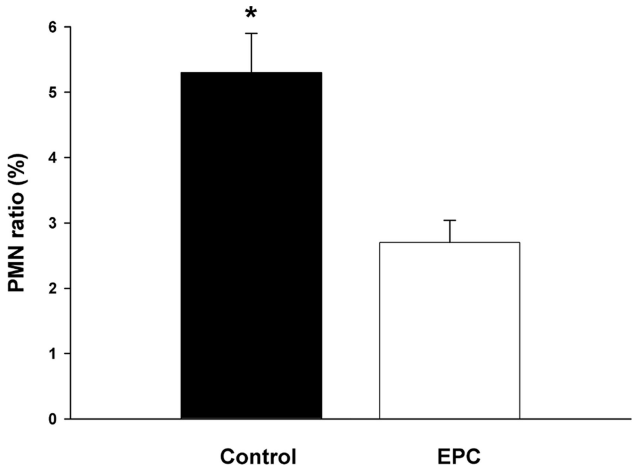

Fig. 8. Degrees of polymorphonuclear cell (PMN) infiltration in lung tissue after acute lung injury were determined using the PMN ratio as previously described.[22] Significantly higher PMN ratio in the lung of control group indicates more inflammatory cell infiltration in the lung tissue secondary to oleic acid administration. A total of approximately 1,500 cells were counted from each lung section under light microscopy (100×). Data were analyzed by unpaired *t* test and are presented as mean ± SEM. * *P* = 0.003. n = 3 animals in each group. EPC = endothelial progenitor cell.

One of the major limitations to generalize these results to clinical practice is that development of ALI/ARDS is almost unpredictable; therefore, autologous transplantation of EPCs may not be clinically feasible. Furthermore, the pathophysiology of ALI/ARDS is a more complicated process than the simple animal model of oleic acid–induced ALI. Further investigation into human subjects is imperative.

In conclusion, we provide the first evidence demonstrating that transplantation of circulating EPCs with high intrinsic antioxidant activity improves endothelial function of the injured pulmonary artery and attenuates damage of alveolar–capillary barrier in ALI/ARDS. Administration of EPCs is a novel cell-based, pulmonary endothelium–targeted therapeutic strategy for ALI/ARDS.

References

1. Bernard GR, Artigas A, Brigham KL, Carlet J, Falke K, Hudson L: The American-European Consensus Conference on ARDS: Definitions, mechanisms, relevant outcomes and clinical trial coordination. Am J Respir Crit Care Med 1994; 149:818–24

2. Frutos-Viva F, Nin N, Esteban A: Epidemiology of acute lung injury and acute respiratory distress syndrome. Curr Opin Crit Care 2004; 10:1–6

3. Mutunga M, Fulton B, Bullock R, Batchelor A, Gascoigne A, Gillespie JI, Baudouin SV: Circulating endothelial cells in patients with septic shock. Am J Respir Crit Care Med 2001; 163:195–200

4. Orfanos SE, Mavrommati I, Korovesi I, Roussos C: Pulmonary endothelium in acute lung injury: From basic science to the critically ill. Intensive Care Med 2004; 30:1702–14

5. Ware LB, Matthay MA: The acute respiratory distress syndrome. N Engl J Med 2000; 342:1334–49

6. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275:964–7

7. Rafii S, Lyden D: Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 2003; 9:702–12

8. Asahara T, Kawamoto A: Endothelial progenitor cells for postnatal vasculogenesis. Am J Physiol Cell Physiol 2004; 287:572–9

9. Urbich C, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S: Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. Nat Med 2003; 9:1370–6

10. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S: Neovascularization of ischemic myocardium by human bone marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001; 7:430–6

11. He T, Smith L, Harrington S, Nath K, Caplice NM, Katusic ZS: Transplantation of circulating endothelial progenitor cells restores endothelial function of denuded rabbit carotid arteries. Stroke 2004; 35:2378–84

12. Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW, Rabken E, Moran AM, Schoen FJ, Atala A, Soker S, Bischoff J, Mayer JE: Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med 2000; 7:1035–40

13. Assmus B, Schachlinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM: Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 2002; 106:3009–17

14. Perin EC, Dohmann HFR, Borojevic R, Silva SA, Sousa AL, Mesquita CT, Rossi MI, Cavalho AC, Dutra HS, Dohmann HJ, Silva GV, Belem L, Vivacqua R, Rangel FO, Esporcatte R, Geng YJ, Vaughn WK, Assad JA, Mesquita ET, Willerson JT: Transendocardial, autologous bone marrow cell transplantation for sever, chronic ischemic heart failure. Circulation 2003; 107:2294–302

15. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schunichen C, Nienaber CA, Freund M, Steinhoff G: Autologous bone marrow transplantation for myocardial regeneration. Lancet 2003; 361:45–6

16. Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, Yu J, Corti R, Mathey DG, Hamm CW, Suselbeck T, Assmus B, Tonn T, Dimmeler S, Zeiher AM: Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 2006; 355:1210–21

17. Burnham EL, Taylor WR, Quyyumi AA, Rojas M, Brigham KL, Moss M: Increased circulating endothelial progenitor cells are associated with survival in acute lung injury. Am J Respir Crit Care Med 2005; 172:854–60

18. Yamada M, Kubo H, Kobayashi S, Ishizawa K, Numasaki M, Ueda S, Suzuki T, Sasaki H: Bone marrow-derived progenitor cells are important for lung repair after lipopolysaccharide-induced lung injury. J Immunol 2004; 172:1266–72

19. Minamino T, Komuro I: Regeneration of the endothelium as a novel therapeutic strategy for acute lung injury. J Clin Invest 2006; 116:2316–9

20. He T, Peterson TE, Holmuhamedov EL, Terzic A, Caplice NM, Oberley LW, Katusic ZS: Human endothelial progenitor cells tolerate oxidative stress due to intrinsically high expression of manganese superoxide dismutase. Arterioscler Thromb Vasc Biol 2004; 24:2021–7

21. Dickey BF, Thrall RS, McCormick JR, Ward PA: Oleic-acid-induced lung injury in the rat: Failure of indomethacin treatment or complement depletion to ablate lung injury. Am J Pathol 1981; 103:376–83

22. Lam CF, Caterina P, Filion P, van Heerden PV, Ilett KF: The ratio of polymorphonuclear leucocytes (PMN) to non-PMN cells: A novel method of assessing acute lung inflammation. Exp Toxicol Pathol 2002; 54:187–91

23. Piantadosi CA, Schwartz DA: The acute respiratory distress syndrome. Ann Intern Med 2004; 141:460–70

24. Kinnula VL, Crapo JD: Superoxide dismutases in the lung and human lung diseases. Am J Respir Crit Care Med 2003; 167:1600–19

25. Slebos DJ, Ryter S, Choi AM: Heme oxygenase-1 and carbon monoxide in pulmonary medicine. Respir Res 2003; 4:7

26. Dernbach E, Urbich C, Brandes RP, Hofmann WK, Zeiher AM, Dimmeler S: Antioxidative stress-associated genes in circulating progenitor cells: Evidence for enhanced resistance against oxidative stress. Blood 2004; 104:3591–7

27. Chute JP: Stem cell homing. Curr Opin Hematol 2006; 13:399–406

28. Oettgen P: Cardiac stem cell therapy: Need for optimization of efficacy and safety monitoring. Circulation 2006; 114:353–8

29. van Hennick P, de Konig A, Ploemacher R: Seeding efficiency of primitive human hematopoietic cells nonobese diabetic/severe combined immune deficiency mice: Implications for stem cell frequency estimates. Blood 1999; 94: 3055–61

30. Muller-Ehmsen J, Whittaker P, Kloner RA, Dow JS, Sakoda T, Long TI, Laird PW, Kedes L: Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. J Mol Cell Cardiol 2002; 34:107–16

31. Sittipunit C, Steinberg KP, Ruzinski JT, Myles C, Zhu S, Goodman RB, Hudson LD, Matalon S, Martin TR: Nitric oxide and nitrotyrosine in the lungs of patients with acute respiratory distress syndrome. Am J Respir Crit Care Med 2001; 163:503–10

32. Kobayashi A, Hashimoto S, Kooguchi K, Kitamura Y, Onodera H, Urata Y, Ashihara T: Expression of inducible nitric oxide synthase and inflammatory cytokines in alveolar macrophages of ARDS following sepsis. Chest 1998; 113:1632–9

33. Griffiths MJ, Liu S, Curzen NP, Messent M, Evans TW: *In vivo* treatment with endotoxin induces nitric oxide synthase in rat main pulmonary artery. Am J Physiol Lung Cell Mol Physiol 1995; 268:509–18

34. Holzmann A, Manktelow C, Taut FJ, Bloch KD, Zapol WM: Inhibition of nitric oxide synthase prevents hyporesponsiveness to inhaled nitric oxide in lungs from endotoxin-challenged rats. ANESTHESIOLOGY 1999; 91:215–21

35. Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, Ganz P: Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med 1986; 315:1046–51

36. Kawano H, Ogawa H: Endothelial function and coronary spastic angina. Intern Med 2005; 44:91–9

37. Guazzi M: Alveolar-capillary membrane dysfunction in heart failure: Evidence of a pathophysiologic role. Chest 2003; 124:1090–102

38. Lamy M, Fallat RJ, Koeniger E, Dietrich HP, Ratliff JL, Eberhart RC, Hill JD: Pathologic features and mechanisms of hypoxemia in adult respiratory distress syndrome. Am Rev Respir Dis 1976; 114:267–87

39. Tomashefski JFJ: Pulmonary pathology of acute respiratory distress syndrome. Clin Chest Med 2000; 21:435–66

40. Reutershan J, Ley K: Acute respiratory distress syndrome: How neutrophils migrate into the lung. Crit Care 2004; 8:453–61

41. Abraham E, Carmody A, Shenkar R, Arcaroli J: Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. Am J Physiol Lung Cell Mol Physiol 2000; 279:1137–45