Rescue of Monocrotaline-Induced Pulmonary Arterial Hypertension Using Bone Marrow–Derived Endothelial-Like Progenitor Cells

Efficacy of Combined Cell and eNOS Gene Therapy in Established Disease

Yidan D. Zhao, David W. Courtman, Yupu Deng, Lakshmi Kugathasan, Qiuwang Zhang, Duncan J. Stewart

Abstract—Pulmonary arterial hypertension (PAH) is characterized by a progressive increase in pulmonary vascular resistance caused by narrowing and loss of pulmonary microvasculature, which in its late stages becomes refractory to traditional therapies. We hypothesized that bone marrow–derived endothelial progenitor cells (EPCs), which normally function to repair and regenerate blood vessels, would restore pulmonary hemodynamics and increase microvascular perfusion in the rat monocrotaline (MCT) model of PAH. Mononuclear cells were isolated from the bone marrow of syngeneic Fisher-344 rats by Ficoll gradient centrifugation and cultured for 7 to 10 days in endothelial growth medium. Fluorescently labeled endothelial-like progenitor cells (ELPCs) engrafted at the level of the distal pulmonary arterioles and incorporated into the endothelial lining in the MCT-injured lung. The administration of ELPCs 3 days after MCT nearly completely prevented the increase in right ventricular systolic pressure seen at 3 weeks with MCT alone (31.5±0.95 versus 48±3 mm Hg, respectively; *P*<0.001), whereas injection of skin fibroblasts had no protective effect (50.9±5.4 mm Hg). Delayed administration of progenitor cells 3 weeks after MCT prevented the further progression of PAH 2 weeks later (ie, 5 weeks after MCT), whereas only animals receiving ELPCs transduced with human endothelial NO-synthase (eNOS) exhibited significant reversal of established disease at day 35 (31±2 mm Hg, *P*<0.005) compared with day 21 (50±3 mm Hg). Fluorescent microangiography revealed widespread occlusion of pulmonary precapillary arterioles 3 weeks after MCT, whereas arteriolar-capillary continuity and microvascular architecture was preserved with the administration of syngeneic ELPCs. Moreover, the delivery of ELPCs to rats with established PAH resulted in marked improvement in survival, which was greatest in the group receiving eNOS-transduced cells. We conclude that bone marrow–derived ELPCs can engraft and repair the MCT-damaged lung, restoring microvasculature structure and function. Therefore, the regeneration of lung vascular endothelium by injection of progenitor cells may represent a novel treatment paradigm for patients with PAH. (Circ Res. 2005;96:442-450.)

Key Words: progenitor cells • pulmonary hypertension • endothelium • endothelial nitric oxide synthase

Pulmonary arterial hypertension (PAH) is a devastating disease that in its most severe form, idiopathic PAH, leads to progressive debilitation and death, often within 2 to 3 years after its initial diagnosis.1 Despite significant advances in the therapy of PAH during the last decade, the prognosis remains poor. Although the genetic basis for some patients with familial PAH has been elucidated,2-4 how these mutations are causally linked to the development of PAH remains unclear.5,6 Evidence from experimental models as well as lung specimens from patients with PAH underlines the importance of microvascular occlusion in the pathogenesis of this disease,7 especially in its advanced stages; however, the precise mechanisms remain uncertain. Recently, it has been suggested that environmental stress in a genetically predisposed host may trigger endothelial cell apoptosis,5,9 which could lead to arteriolar occlusion either directly, possibly by initiating microvascular degeneration,10 or indirectly by promoting the emergence of hyperproliferative, apoptosis-resistant endothelial clones.11,12 Regardless of the mechanism of vascular occlusion, regeneration of lung microvasculature may be a novel and effective therapeutic strategy for restoring pulmonary hemodynamics in patients with advanced PAH.

Our group has suggested that, as in systemic arterial beds, it may be possible to induce the regeneration of pulmonary microvessels in experimental models of lung vascular dis-
ease. We have shown that somatic cell–based gene therapy with endothelial NO-synthase (eNOS)13 or various angiogenic factors, including vascular endothelial growth factor (VEGF) and angiopoietin-1,14,15 can reduce monocrotaline (MCT)-induced PAH in prevention models, possibly by protecting against endothelial cell (EC) apoptosis or inducing microvascular angiogenesis. In addition, the administration of fibroblasts (FBs) transduced with eNOS resulted in significant improvement in right ventricular systolic pressure (RVSP) when delivered to rats with established PAH, associated with evidence of regeneration of the lung microcirculation (unpublished observations, 2004), and consistent with the now well-accepted role of eNOS and NO in angiogenesis.16–18 Recently, it has been recognized that circulating bone marrow–derived endothelial progenitor cells (EPCs) play an important role in repair of endothelial injury and participate directly in postnatal vasclogenesis and angiogenesis in systemic vascular beds.19,20 However, whether EPCs are involved in pulmonary endothelial repair and regeneration is not known, although it has recently been reported that circulating progenitor cells may be recruited to the remodeling adventitia in the bovine hypoxia model of PAH.21

Therefore, we studied the effect of bone marrow–derived endothelial-like progenitor cell (ELPC) transplantation in both the prevention and reversal models of PAH after the administration of MCT, which is well known to induce selective pulmonary endothelial injury in the rat. We now report that MCT-induced PAH could be completely prevented by treatment with syngeneic ELPCs. Moreover, delayed administration of ELPCs to animals 3 weeks after MCT injury prevented further progression of PAH, whereas the transplantation of eNOS-transduced ELPCs induced significant reversal of established disease to levels not different from saline-treated control animals. This was associated with evidence of restoration of distal arteriolar continuity and improved perfusion of alveolar capillaries in this model.

Materials and Methods

Cell Isolation and Culture

Skin biopsies were obtained from 21-day-old Fisher-344 rats (Charles River Co, St Constant, Quebec, Canada), and FBs were cultured using an explant technique. Cells were grown in Dulbecco Modified Eagle Media (DMEM) with 10% fetal bovine serum (FBS) and 2% penicillin/streptomycin (50 U/mL penicillin G; 50 μg/mL streptomycin) in a humidified incubator (20% O2, 5% CO2 at 37°C), and used between passages 2 and 9.

Bone marrow (BM) was aspirated from the femurs of 21-day-old syngeneic Fisher-344 rats. Mononuclear cells (MNCs) were isolated by density gradient (Ficoll-Paque, Amersham) centrifugation at 400g for 30 minutes. BM-MNCs were resuspended in differential endotoxins (EBM-2, Cambrex) with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mmol/L-L-glutamine (Invitrogen), plated on gelatin-coated tissue culture flasks and incubated at 37°C with 5% CO2 for 7 to 10 days, to produce endothelial-like progenitor cells (ELPCs).

For immunocytochemistry, differentiated MNCs were subcultured on 4-well chamber slides (BD Bioscience), and fixed in 2% paraformaldehyde for 10 minutes. Cells were incubated overnight at 4°C with the following primary antibodies: rabbit anti-human Flk-1 (VEGF-R2; Alpha Diagnostic Inc; 1:200); mouse anti-human Tie-2 (Upstate Biotechnology Inc; 1:50), or rabbit anti-human von Willebrand factor (vWF, DAKO; 1:1000). Rabbit anti-mouse or goat anti-rabbit F(ab’2) (Vector; 1:150) conjugated with FITC were used as secondary antibodies, as appropriate. Surface lectin staining was performed using fluorescently labeled UEA-1 Lectin (Sigma) at 10 μg/mL. As well, the ability for live cells to take up fluorescently labeled acetylated-LDL (Di-Ac-LDL; Molecular Probes) was assessed by incubation with Di-ac-LDL (10 μg/mL) for 4 hours at 37°C. ToPro3 (1:1000; Molecular Probes) was used for nuclear counterstaining and images were captured by confocal microscopy (BioRad Radiance).

Transduction

The full-length coding sequence of human eNOS was generated as previously described,13 and ELPCs were transduced with human eNOS cloned into the pVax-1 plasmid vector using electroporation (MaxiCyte) according to a protocol optimized by the manufacturer. The empty (null) pcDNA 3.1 vector was used as a control. After transfection, cells were replated and cultured for 24 hours, trypsinized (0.25% trypsin, 1% EDTA), washed, and resuspended in phosphate buffered saline (PBS), and then divided into aliquots of 500,000 cells/mL for injection. Western blot analysis revealed that electroporation resulted in peak human transgene expression at 72 hours, persisting for more than 1 week (data not shown).

Animal Models of PAH

Two complementary models of MCT-induced PAH were used in this study, as shown in Figure 1, to examine the effect of ELPC delivery on both prevention and reversal. All animal studies were conducted under protocols approved by the animal care committee at St Michael’s Hospital and in accordance with guidelines from the Canadian Council of Animal Care.

Prevention Protocol

Cells were delivered via central venous injection 3 days after MCT, and the animals were euthanized at 21 days. Six-week-old Fisher-344 rats (160 to 180g) were given intraperitoneal (IP) injections of saline (control group, n=13) or 75 mg/kg of MCT (Aldrich Chemical Co). Three days later, MCT-treated animals were assigned to three experimental groups: no cell injection (MCT alone, n=13), or...
1 million ELPCs (n=23) or FBs (n=10). For cell delivery, rats were anesthetized with an IP injection of xylazine (4.6 mg/kg) and ketamine (7 mg/kg), the left cervical area was shaved and cleaned with 70% ethanol, and the external jugular vein was catheterized with a polyethylene cannula flushed with heparinized saline (40 IU/mL). Twenty one days after MCT injection, the rats were reanesthetized, and a 3F Millar microtip catheter was inserted via the right external jugular vein and into the right ventricle to obtain measurements of right ventricular systolic pressure (RVSP; Biopac System, Acknowledge Software). The animals were then euthanized and the hearts and lungs harvested. The ratio of right to left ventricular plus septal weight (RV/LV) was determined as described previously.

In a separate experiment, animals were treated with MCT and randomized at 3 days to receive either no cells (saline, n=13) or ELPCs (n=12) as described above and then followed for longer periods of time to establish the persistence of any therapeutic effect. The animals were monitored daily by experienced animal care personnel in a blinded fashion and euthanized if predetermined criteria of significant morbidity were met (weight loss, hunched posture, poor coat appearance, conjunctival hemorrhage, and labored breathing). RVSP and RV/LV weight ratios were measured at the time of euthanasia as described above.

**Reversal Protocol**

In the reversal model, rats were injected with saline (Control, n=12) or MCT, and 21 days later, baseline RVSP was recorded as earlier to confirm the presence of PAH. Thereafter, polyethylene catheters were inserted into the left external jugular vein and tunneled subcutaneously to the intrascapular region, exiting through a small incision, and sealed to the external environment with a removable plug. All incisions were closed with 3-0 interrupted absorbable sutures. Rats were randomized to receive saline (MCT alone), ELPCs alone, or ELPCs transduced with human eNOS (n=19 to 23/group). Cells were given in three sequential injections of $\text{5} \times 10^5$ over 3 days through the indwelling catheter (total dose $=1.5 \times 10^6$ cells). After the final cell injection, the indwelling catheter was removed, the left external jugular vein was ligated, and animals were euthanized.

![Figure 2](https://via.placeholder.com/150)

**Figure 2.** ELPC phenotype was characterized in vitro (a through d) by assessing Dil acetylated LDL uptake (red, a) and UEA-1 lectin surface staining (green, b), indirect immunofluorescence staining was performed to detect vWF (green, c) and Flk-1 (green, d) expression (blue, ToPro 3 nuclear counterstain in all panels). e through g, Fluorescently labeled (red) ELPCs were injected 3 days after MCT administration, and lungs were perfused with agarose containing fluorescent microspheres (green) just before harvest. At 15 minutes after injection, cells were trapped within distal arterioles (e). At later time points, injected cells were seen to engraft into the endothelial layer of distal precapillary arterioles as confirmed by fluorescent microangiography (green in f). In some areas, complete luminal incorporation was observed (g). h and i, Labeled ELPCs (red) delivered 21 days after MCT could be incorporating in precapillary and larger arterioles (vWF immunostaining in green; calibration bars $=50 \mu m$).
allowed to recover. Fourteen days later (35 days after MCT) RVSP was recorded, the animals were euthanized, and lung and heart tissues were collected for analyses as described.

**Fluorescent Microangiography**

In a subset of animals, a catheter was inserted into the pulmonary artery immediately after euthanasia and the lungs flushed with heparinized PBS at 37°C, immediately followed by perfusion with a warmed (45°C) solution of 1% low melting point agarose (Sigma) containing 0.2 μm yellow-green fluorescent microspheres (505/515 nm peak excitation and emission, Molecular Probes) as described in detail in the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org.

**Engraftment of ELPCs**

In separate experiments, ELPCs were loaded with the vital cytoplasmic fluorescent label, CMTMR (Molecular Probes). Before transplantation, subconfluent cultures of ELPCs were incubated for 40 minutes with 10 μmol/L CMTMR, and 1×10^6 labeled cells were injected into the pulmonary circulation of normal rats at 3 or 21 days after MCT injection via the external jugular vein as described. Lungs or kidneys were harvested at various time points (10 minutes to 3 weeks) after cell delivery and examined by confocal fluorescent microscopy. Quantitation of cell number was performed as previously described.13 In some cases, the lungs were also subjected to fluorescent microangiography and confocal images were captured by optical sectioning as described.

**Arteriolar Muscularization**

The degree of muscularization of small arterioles was assessed in 5 μm lung cryosections immunostained for von Willebrand factor (vWF) and α-smooth muscle actin (α-SM-actin) as described in the online data supplement.

**Statistical Analysis**

Data are presented as mean±SEM. Differences between groups were assessed by analysis of variance (ANOVA), followed by post hoc comparisons using an unpaired t test as appropriate. Differences within groups between the 21- and 35-day time points were assessed using a paired t test. Significance of differences for survival data were determined using the Kaplan-Meier analysis. A value of P<0.05 was considered statistically significant.

**Results**

**In Vitro Characterization of ELPCs**

After 7 to 10 days of culture in endothelial growth medium, BM-MNCs demonstrated a cobblestone appearance typical for endothelial cells and exhibited positivity for a panel of EC markers, including Dil acLDL, UEA-1 lectin staining, and immunostaining for vWF, and Flk-1 varying from 65% to 83% (Figure 2).

**Engraftment of Fluorescently-Labeled ELPCs**

CMTMR-labeled ELPCs were injected into the pulmonary circulation 3 days after administration of MCT. Fifteen minutes after delivery, labeled ELPCs were seen distributed throughout the lung (Figure 2e), nearly exclusively within small precapillary arterioles (insert). Seven days after cell injection, fluorescently-tagged ELPCs were seen surrounding and engrafting distal arterioles (Figure 2f) and on occasions integrating into, and regenerating, the endothelium of larger arterioles (Figure 2g). After the first 3 days, the number of engrafted ELPCs was fairly constant up to 3 weeks (see online Figure WS-2, in the online data supplement). Similar results were obtained when labeled ELPCs were delivered 21 days after MCT (Figure 2h and 2i).

**ELPC Administration in the Prevention Model**

RVSP was significantly increased at day 21 after MCT compared with saline-treated control rats (48±3 versus 26±0.9 mm Hg, P<0.001; Figure 3A). Administration of somatic cells (ie, skin FBs) had no protective effect (RVSP 51±5 mm Hg), whereas the delivery of syngeneic ELPCs nearly completely prevented the rise in pulmonary systolic pressures at 3 weeks after MCT (32±1 mm Hg, P<0.001 versus MCT alone). Similarly, right ventricular hypertrophy as measured by the ratio of RV/LV weight ratio was increased in animals receiving MCT alone (0.36±0.02) or MCT with FBs (0.30±0.01; Figure 3B). In contrast, the delivery of bone marrow–derived ELPCs significantly reduced right ventricular hypertrophy (0.26±0.013, P<0.01 versus MCT) to a level not significantly different from saline-treated control animals (0.23±0.01).
Persistence of Protective Effects of ELPCs

At the time of euthanasia, RVSP was again markedly elevated in animals receiving MCT alone (Figure 4A), whereas with the exception of one animal, ELPC-treated rats exhibited near normal values of pulmonary systolic pressure. Moreover, there was no tendency for RVSP to increase in the ELPC-treated group over more than 60 days (Figure 4B). Nonetheless, despite this dramatic hemodynamic improvement, there was only a modest difference in "survival" between the groups ($P=0.08$), suggesting that the apparent morbidity observed in ELPC-treated animals might be caused by extrapulmonary MCT toxicity. Indeed, necropsy studies in the longest surviving MCT-treated rats revealed markedly enlarged kidneys that exhibited severe histological abnormalities of glomerular and tubular structure consistent with end-stage renal disease in both groups (Figure 4Ca through 4Cd). Unlike the lung, no renal engraftment of ELPCs was seen either immediately or 3 days after cell delivery (Figure 4Ce and 4Cf).

Effect of ELPC Transplantation in the Reversal Model

At 3 weeks after MCT injection, animals assigned to the three treatment groups exhibited similar increases in RVSP compared with saline controls (Figure 5A) and comparable to that of the MCT alone group in the prevention protocol. Two weeks later, RVSP had progressed in the MCT animals treated with saline. The delivery of nontransduced ELPCs prevented the further progression of PAH from day 21 ($43\pm4$ mm Hg) to day 35 ($36\pm4$ mm Hg); however, only animals receiving eNOS-transduced ELPCs demonstrated significant improvement in RVSP at day 35 ($31\pm2$ mm Hg, $P<0.005$; Figure 6A).

Of note, transgene expression was transient and persisted for only $1$ week after electroporation (online Figure WS-2). The ratio of RV to LV and septal weight was significantly increased in the MCT-treated rats receiving only saline, whereas both ELPC-treated groups displayed significant reductions compared with control rats (Figure 5B). Similarly, the effects of MCT on the expression of VEGF and markers of endothelial activation (E- and P-Selectin) were normalized by administration of eNOS-transduced ELPCs (online Figure WS-1).

Effects of ELPCs on Fluorescent Microangiography

In normal lungs, microangiography revealed an even filling of the distal arteriolar bed with a homogeneous pattern of
capillary perfusion (Figure 6Aa). Immunostaining with an antibody directed to α-SM actin showed minimal muscularization of the distal arterioles in normal lungs. In contrast, 3 or 5 weeks after MCT-induced lung injury (Figure 6Ab and Ad, respectively), the distal arteriolar bed showed significant narrowing of distal arterioles with widespread capillary occlusion and evidence of increased distal muscularization. In animals receiving ELPCs 3 days after MCT, there was a marked improvement in the appearance of the lung microvasculature, with preservation of arteriolar continuity and enhanced capillary perfusion (Figure 6Ac). When ELPCs alone were administered 3 weeks after MCT, only modest improvement in capillary perfusion was seen (Figure 6Ad) with persistent distal muscularization. Only eNOS-transduced ELPCs restored a more normal appearance of the lung circulation in the reversal model (Figure 6Af). These observations were confirmed by quantification of microvascular perfusion as shown in Figure 6B (n=6 to 7/group).

Arteriolar Muscularization

In normal lungs, arterioles of <30 μm showed infrequent muscularization with only 14% demonstrating partial muscularization (PM) and no vessels exhibiting full muscularization (FM). In contrast, 35 days after MCT there was a significantly higher proportion of muscularized arterioles (Figure 6C). Treatment with eNOS-transduced progenitor cells reduced arteriolar muscularization, whereas nontransduced ELPCs did not, although there was an increase overall in nonmuscularized vessels in both EPLC-treated groups compared with MCT alone.

Survival Analysis

In one experiment, only one animal in the MCT-saline group survived to the predefined study end point at 35 days after MCT, and therefore, these data were used for survival analysis only (Figure 7). The injection of ELPCs transduced with eNOS nearly completely prevented MCT-induced mortality with all but one animal surviving to end-study (P=0.02 versus MCT alone), whereas delivery of nontransduced ELPCs produced an intermediate survival that was not significantly different from MCT alone. However, when the survival analysis was performed including all 63 animals randomized in the reversal protocol, this trend persisted with the survival benefit in MCT-treated animals receiving nontransduced ELPC now reaching statistical significance (P=0.037 versus MCT alone).

Discussion

We now report that bone marrow–derived ELPCs engrafted the MCT-injured lung and incorporated into the pulmonary microvasculature, resulting in near complete prevention of PAH when delivered into the pulmonary circulation within 3 days of MCT injury. Although nontransduced ELPCs also prevented further increases in RVSP when injected 3 weeks after MCT-induced lung injury, only eNOS-transduced cells resulted in normalization of pulmonary hemodynamics in animals with established PAH, and this effect was associated with a significant survival benefit. Progenitor cell therapy also resulted in marked improvement in the pulmonary microvascular architecture and alveolar capillary perfusion in MCT-treated animals, which could in part be attributed to repair and regeneration of lung microvascular endothelium.

These findings may have important implications both with respect to the mechanisms underlying the development of PAH in this model, and for potential therapeutic strategies for the treatment of this disease. The present data suggest that loss of arteriolar continuity at the precapillary level may be a relatively early and widespread phenomenon, and may contribute directly to the increase in pulmonary vascular resistance by resulting in loss of pulmonary microrcirculation. Fluorescent imaging of the microrcirculation in thick sections revealed widespread discontinuity of the pulmonary arteriolar bed as early as 3 weeks after MCT-induced injury, mainly localized to the level of the precapillary (intraductal) arteriole. Of interest, despite the well-known differences in lung pathology, distal arteriolar occlusions have also been observed in lung tissue from patients with advanced idiopathic pulmonary arterial hypertension. This result may be a consequence of progressive loss of terminal lung vessels, including arterioles and precapillaries, as these vessels are essential for the maintenance of normal capillary perfusion.

We now report that bone marrow–derived ELPCs engrafted the MCT-injured lung and incorporated into the pulmonary microvasculature, resulting in near complete prevention of PAH when delivered into the pulmonary circulation within 3 days of MCT injury. Although nontransduced ELPCs also prevented further increases in RVSP when injected 3 weeks after MCT-induced lung injury, only eNOS-transduced cells resulted in normalization of pulmonary hemodynamics in animals with established PAH, and this effect was associated with a significant survival benefit. Progenitor cell therapy also resulted in marked improvement in the pulmonary microvascular architecture and alveolar capillary perfusion in MCT-treated animals, which could in part be attributed to repair and regeneration of lung microvascular endothelium.

These findings may have important implications both with respect to the mechanisms underlying the development of PAH in this model, and for potential therapeutic strategies for the treatment of this disease. The present data suggest that loss of arteriolar continuity at the precapillary level may be a relatively early and widespread phenomenon, and may contribute directly to the increase in pulmonary vascular resistance by resulting in loss of pulmonary microrcirculation. Fluorescent imaging of the microrcirculation in thick sections revealed widespread discontinuity of the pulmonary arteriolar bed as early as 3 weeks after MCT-induced injury, mainly localized to the level of the precapillary (intraductal) arteriole. Of interest, despite the well-known differences in lung pathology, distal arteriolar occlusions have also been observed in lung tissue from patients with advanced idiopathic pulmonary arterial hypertension. This result may be a consequence of progressive loss of terminal lung vessels, including arterioles and precapillaries, as these vessels are essential for the maintenance of normal capillary perfusion.
PAH,\textsuperscript{7} although the mechanism of arteriolar occlusion in patients with PAH is still a matter of debate.

The observation that administration of ELPCs soon after MCT-induced lung injury protected against the development of PAH favors the concept that endothelial damage plays an important role in the pathogenesis of PAH in this model by contributing to microvascular loss. It is likely that engraftment of ELPCs at the level of the distal arteriolar bed reduced the consequences of MCT damage either by contributing to endothelial repair directly\textsuperscript{19} or indirectly by release of paracrine signals.\textsuperscript{22} This interpretation is supported by fluorescent microangiography showing that the pruning and occlusion of precapillary arterioles induced by MCT was nearly completely obviated by the administration of ELPCs. This effect was dependent on the delivery of “regenerative” cells, because injection of a somatic cell line, ie, skin FBs, had no effect on microvascular structure or function. Of interest, the protective effect of ELPCs appeared to persist as long as it was possible to follow the animals. Indeed, the prevention of MCT-induced vascular damage in the lung unmasked profound renal toxicity, which had been previously recognized in the reports that first characterized the toxicity of MCT.\textsuperscript{23,24} This additional toxic effect of MCT clearly represents a significant limitation for studies of long-term survival after lung-specific therapy of PAH in this model.

The efficacy of nontransduced ELPCs in the prevention of PAH in this study are in contrast with a recent report using Figure 6. Representative confocal projection images of lung sections (A) perfused with fluorescent microspheres (green) suspended in agarose (ie, fluorescent microangiography) and immunostained for α-smooth muscle actin (red). Normal filling of the microvasculature was observed in control rats (a), whereas rats treated with MCT showed a marked loss of microvascular perfusion and widespread precapillary occlusion 21 (b) and 35 (d) days after MCT injection. In the prevention model, animals receiving ELPCs displayed improved microvascular perfusion and preserved continuity of the distal vasculature (c). In the reversal model, eNOS-transduced ELPCs dramatically improved the appearance of the pulmonary microvasculature (f), whereas progenitor cells alone resulted in more modest increases in perfusion and little noticeable reduction in arteriolar muscularization (e, calibration bars=100 μm). Summary data for pulmonary microvascular perfusion (B; 6 to 7 animal/group) for animals treated in the prevention (open bars) and reversal (closed bars) protocols. Proportion of small pulmonary arterioles (C; <30 μm) that are nonmuscularized (NM), partially (PM), or fully muscularized (FM) in the various treatment groups of the reversal protocol (9 to 13 animals per group).

\(^*P<0.05\) vs control; \(^†P<0.05\) vs MCT-saline
human umbilical cord progenitor cells injected into immuno-
deficient rats,25 in which significant prevention of MCT-
induced PAH was seen only with adrenomedullin-transfected
cells, with little or no benefit using ELPCs alone. This
discrepancy may be related to differences in the source of
progenitor cells (ie, cord blood versus bone marrow) or to
species-specific factors that may limit the regenerative effects
of human cells in the rat model.26 Nonetheless, the Nagaya
report confirms a number of previous reports from our group
showing the efficacy of cell-based gene therapy in the
"prevention" MCT model using a variety of somatic cell
types and therapeutic transgenes14,15 including eNOS.13

However, prevention of PAH is not a clinically relevant
therapeutic target because patients only present once this
disease is already in its advanced stages. In the setting of
established PAH, although ELPCs alone reduced progression
of MCT-induced PAH, they did not produce significant
improvement in pulmonary systolic pressures over the sub-
sequent 2-week interval. In contrast, the delivery of ELPCs
transduced with eNOS resulted in near normalization of right
ventricular systolic pressures, which was associated with
marked improvement of microvascular architecture and alve-
olar capillary perfusion. The added benefit of eNOS gene
transfer is consistent with a growing body of literature that
supports an important role for endothelium-derived NO in
angiogenesis and vascular regeneration, both acting as a
downstream mediator for a variety of classical angiogenic
growth factors,16,17,27 as well as by inducing an angiogenic
profile of gene expression.28,29 Of interest, our group has
identified a previously unsuspected role for eNOS in the
development of the fetal lung vasculature and in the matura-
tion of the airway epithelial cells.29 Specifically, eNOS
appears to be essential for the establishment of arteriolar-
capillary continuity in the mid to late gestational fetal lung
circulation, and eNOS-deficient pups exhibit pathological
features that are nearly identical to alveolar capillary dyspla-
sia,30–32 a pernicious form of persistent pulmonary hyperten-
sion of the newborn that is nearly always fatal. Moreover, we
have demonstrated that cell-based gene transfer of eNOS
resulted in partial reversal of MCT-induced PAH even using
a somatic (FB) cell line (manuscript in preparation), although

the magnitude of benefit was less that that seen with eNOS-
transduced ELPCs. Together, these data point to a critical role
for eNOS in control of vascular growth and the development
of a competent pulmonary circulation both during fetal
development as well as in arteriolar regeneration in postnatal
MCT-injured lung.

The present results provide further support for the view
that microvascular degeneration at the critical precapillary
level may play an important role in the development of PAH
by leading to the exclusion of large portions of the pulmonary
microvasculature from the pulmonary circulation. Moreover,
they show for the first time that eNOS-transduced ELPCs can
dramatically improve pulmonary hemodynamics and survival
in animals with established PAH, while restoring the conti-
nuity of the distal arteriolar bed. These data have important
implications for the therapy of this lethal disease and support
the exploration of regenerative cell-based gene strategies for
the treatment of patients with severe refractory PAH for
whom therapeutic options are very limited and the prognosis
is poor.

Acknowledgments

The study was supported by research grants from the Canadian
Institutes for Health Research (MPO-57726) and by Northern Ther-
apuetic Inc. D.J.S. is a member of the Richard Lewar Center of
Excellence. The authors would like to acknowledge the outstanding
technical assistance of Robin N.N. Han, Douglas Ng, Malcolm
Robb, Judy Trogadis, and Danlin Jia.

References

1. Archer S, Rich S. Primary pulmonary hypertension: a vascular biology
and translational research “Work in progress.” Circulation. 2000;102:
2781–2791.

2. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G,
Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodg YE, Knowles JA.
Familial primary pulmonary hypertension (gene PPH1) is caused by
mutations in the bone morphogenetic protein receptor-II gene. Am J Hum

N, Moore KJ, Hodg YE, Knowles JA, Morse JH. BMPR2 germline
mutations in pulmonary hypertension associated with fenfluramine

4. Lane KB, Machado RD, Paicu MW, Thomson JR, Phillips JA, III,
Loyd JE, Nichols WC, Trembath RC. BMPR2 and germline mutations in
BMPR2, encoding a TGF-beta receptor, cause familial primary pul-

5. Morell NW, Yang X, Upton PD, Jourdan KB, Morgan N, Sheares KK,
Trembath RC. Altered growth responses of pulmonary artery smooth
muscle cells from patients with primary pulmonary hypertension to trans-
foming growth factor-b1 and bone morphogenetic proteins. Circulation.

6. Morse JH, Deng Z, Knowles JA. Genetic aspects of pulmonary arterial

Tuder RM. Three-dimensional reconstruction of pulmonary arteries in
plexiform pulmonary hypertension using cell-specific markers: evidence
for a dynamic and heterogeneous process of pulmonary endothelial cell


Waltenberger J, Voelkel NF, Tuder RM. Inhibition of the VEGF receptor
2 combined with chronic hypoxia causes cell death-dependent pulmonary
endothelial cell proliferation and severe pulmonary hypertension. PASEB

Figure 7. Survival to 35 days was analyzed in a separate rever-
sal study. Kaplan-Meier analysis demonstrated that treatment
with eNOS transduced ELPCs 21 days after MCT injection pro-
duced enhanced survival as compared with the MCT alone
group (P=0.02). Although animals receiving ELPCs alone
showed an intermediate response, the improvement was not
statistically significant in this cohort.


Rescue of Monocrotaline-Induced Pulmonary Arterial Hypertension Using Bone Marrow-Derived Endothelial-Like Progenitor Cells: Efficacy of Combined Cell and eNOS Gene Therapy in Established Disease
Yidan D. Zhao, David W. Courtman, Yupu Deng, Lakshmi Kugathasan, Qiuwang Zhang and Duncan J. Stewart

Circ Res. 2005;96:442-450; originally published online February 3, 2005;
doi: 10.1161/01.RES.0000157672.70560.7b
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/96/4/442

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2005/03/02/96.4.442.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Web Supplement

Detailed methods:

**Fluorescent microangiography**: The agarose solution containing fluorescent beads (0.2% total solids) was injected under controlled pressure (between 40-60 mmHg) until agarose could be seen flowing out of an incision in the left atrial appendage. The lungs were then fixed in 4% paraformaldehyde in PBS for 48hrs at 4°C and 150–200µm thick sections were prepared on an oscillating blade microtome (Leica, Richmond Hill, ON). Some sections were immunostained for α-smooth muscle actin (α-SM actin) by 2-hour incubation with a fluorescently conjugated monoclonal antibody (Sigma). Confocal optical sectioning (BioRad Radiance) was used to produce a Z stack of images spanning 150 µm of section depth. Projection images were created from the Z-series to demonstrate vessel architecture throughout the entire thickness of the section. Fluorescence intensity was quantified in 3 separate sections per animal and 3 representative regions per section by a blinded observer, for this analysis a single confocal optical section was taken at a predefined depth (10 µm) using fixed gain and power settings. The resultant image was analyzed for threshold intensity (Image J, NIH 2002) and perfusion index (PI) was calculated as the area ratio of the image surpassing the threshold value. The perfusion index represents the proportion of the optical section filled by vascular perfusion.

**Arteriolar muscularization**: Sections were fixed for 10 minutes with 2% phosphate buffered paraformaldehyde, washed, and sequentially incubated with a von Willebrand Factor (vWF) rabbit polyclonal antibody (DAKO, Mississauga, ON) and smooth muscle
specific alpha actin monoclonal antibody (Sigma, ON). Fluorescently conjugated secondary antibodies directed to mouse (FITC conjugate) or rabbit (rhodamine conjugate) IgG were used to localize primary antibody staining. Omission of the primary antibodies was used as a negative control. Arterioles <30 μm in diameter (identified by vWF staining) were classified as non-muscular (NM), partially muscular (PM - at least one smooth muscle cell, but no continuous media), or fully muscular (FM - SMC around the entire arteriolar circumference). The proportion of arterioles exhibition PM and FM were expressed as a percentage of the total vessel numbers counted from the section.

**Quantitative RT-PCR:** Total RNA was extracted from frozen lung tissues using TRIZOL reagent following manufacturer’s instructions and subjected to reverse transcription as previously described using random-hexamers (0.5 µg) and M-MLV enzyme mix (Invitrogen-Lifetech, Burlington, ON). The expression of genes of interest (see table) was analyzed by real time PCR (ABI 7900HT, Applied Biosystems, Foster City, CA), using SYBR green PCR Core Reagents (ABI, Foster City, CA). Quantification of each transcript was measured against standard curves constructed from the relevant cDNA fragments produced by a two step conventional PCR reaction comprising of an initial denaturation step at 95°C for 5 min and 18 cycles at 95°C (30 sec), 50°C (45 sec), and 72°C (45 sec), followed by a final cycle at 72°C for 2 min. Quantitative PCR was performed in a 30µl reaction using 4µl of complementary DNA. Target genes were amplified using the primers shown in the table. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 40 cycles at 95°C for 15 sec and 62°C
for 1 min. The results of real-time PCR are presented relative to normal lung (fold-
increase or decrease). All measurements were performed in triplicates.

Table 1: Primers for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1</td>
<td>acg agt gtg aat cga aaa ccg aag</td>
</tr>
<tr>
<td>IL-6</td>
<td>agg aga atg agt ctg ctc aga gca c</td>
</tr>
<tr>
<td>E-selectin</td>
<td>gtt agt att cac cct cta ata gat g</td>
</tr>
<tr>
<td>P-selectin</td>
<td>agt ctt cac gaa cgc tgc ata tga c</td>
</tr>
<tr>
<td>TNF-α</td>
<td>cca gga gaa agt cag cct cct</td>
</tr>
<tr>
<td>VEGF\textsuperscript{164}</td>
<td>cat agg aga gat gag ctt cct gc</td>
</tr>
<tr>
<td>peNOS</td>
<td>aag ctt gct agc gtt taa act taa gc</td>
</tr>
</tbody>
</table>

Figure legends:

**Figure WS-1**: Change in transcript expression for VEGF\textsuperscript{164} (A), P-Selectin (B) and E-
Selectin (C) expressed as fold change relative to the expression level in normal lungs. * p
<0.05 vs. MCT; ** p <0.01 vs. MCT. No significant changes were seen for IL-6, TNF-α, or VCAM-1 (data not shown).

**Figure WS-2**: Changes in human eNOS transgene expression (expressed as transcripts
per well) is shown following injection of transduced EPLCs in the prevention protocol of
MCT-induced PAH (A). CMTMR-labeled ELPCs were counted in three sections per lung
(apical, mid and base) and the total number of cells per lung (B) was estimated using the
previously described method\textsuperscript{13}. There was a progressive reduction of about 10-fold over
the first 3 days after EPLC injection in the prevention protocol, which leveled off over
the subsequent 3 weeks. The persistence of labeled cells from 20 minutes to 3 days after
injection was comparable between the prevention and reversal models (C).
Figure WS-1
Figure WS-2