Cell-Based Gene Transfer of Vascular Endothelial Growth Factor Attenuates Monocrotaline-Induced Pulmonary Hypertension

Andrew I.M. Campbell, MD; Yidan Zhao, MD, PhD; Reena Sandhu, PhD; Duncan J. Stewart, MD

Background—Pulmonary arterial hypertension is characterized by increased pulmonary vascular resistance secondary to a decrease in the caliber and number of pulmonary vascular channels. We hypothesized that the targeted overexpression of an angiogenic factor within the lung would potentially minimize the development and progression of pulmonary arterial hypertension by preventing the loss of existing vessels or by inducing the development of new blood vessels within the lung.

Methods and Results—We used a cell-based method of gene transfer to the pulmonary microvasculature by delivering syngeneic smooth muscle cells overexpressing vascular endothelial growth factor (VEGF)-A to inbred Fisher 344 rats in which pulmonary hypertension was induced with the pulmonary endothelial toxin monocrotaline. Four weeks after simultaneous endothelial injury and cell-based gene transfer, right ventricular (RV) hypertension and RV and vascular hypertrophy were significantly decreased in the VEGF-treated animals. Four weeks after gene transfer, the plasmid VEGF transcript was still detectable in the pulmonary tissue of animals injected with VEGF-transfected cells, demonstrating survival of the transfected cells and persistent transgene expression. In addition, delay of cell-based gene transfer until after the development of pulmonary hypertension also resulted in a significant decrease in the progression of RV hypertension and hypertrophy.

Conclusions—These results indicate that cell-based VEGF gene transfer is an effective method of preventing the development and progression of pulmonary hypertension in the monocrotaline model and suggest a potential therapeutic role for angiogenic factors in the therapy of this devastating disease. (Circulation. 2001;104:2242-2248.)

Key Words: angiogenesis ■ gene therapy ■ endothelium-derived factors ■ hypertension, pulmonary ■ remodeling

Primary arterial hypertension is a progressive disease of unknown pathogenesis with few satisfactory long-term treatment options. The majority of patients with primary pulmonary hypertension (PPH) show little or no acute vasoconstrictor response, and their prognosis remains grave, with a mean survival of ≈3 years from the time of diagnosis. The introduction of intravenous prostacyclin analogues has represented a significant advance in the treatment of this disease; the long-term benefits have not been established, however, and the treatment itself is associated with significant morbidity. Endothelin receptor antagonists have been found to have favorable hemodynamic effects in patients with severe congestive heart failure and associated PH, although the experience in patients with primary arterial hypertension is limited. Similarly, the administration of NO has met with some clinical success but difficulties associated with long-term inhalational delivery and concerns over its safety and efficacy have limited widespread application.

Recently, efforts to identify the potential factors underlying PPH have focused on the involvement of growth factors. Upregulation of vascular endothelial growth factor (VEGF) has been described in association with plexiform lesions, possibly representing an incomplete attempt at revascularization distal to arteriolar occlusion. In the monocrotaline (MCT) model of PH, however, an overall decrease in pulmonary VEGF expression has been reported in concert with a dramatic decrease in pulmonary vessel number and a significant increase in vessel wall thickness. Therefore, we hypothesized that the overexpression of VEGF within the pulmonary microvasculature would reduce the development of PH in the MCT model of disease. To deliver VEGF to the lung, we exploited the natural filtering properties of the pulmonary microvasculature by using a novel cell-based pulmonary gene transfer approach.

We now report that cell-based gene transfer of VEGF reduced the development of MCT-induced PH in the rat and decreased the hypertrophic response seen in the right ventricle (RV) and pulmonary vasculature in this model. These results suggest that the delivery of vascular endothelial...
growth factor is an effective method of preventing PH and underscores the importance of VEGF in the maintenance of pulmonary vascular homeostasis.

Methods

Cell-Based Pulmonary Vascular Delivery and Localization

All animal experiments were performed in accordance with the Canadian Council on Animal Care guidelines. Fisher 344 rats (Charles River Co, St Constant, Quebec) were obtained at 21 days of age and euthanized. The main pulmonary arteries were excised and smooth muscle cells cultured. Cells between the fifth and ninth passages were labeled with the viable fluorochrome chloromethyl rhodamine (CMTR, Molecular Probes Inc) and were then injected into the internal jugular vein of recipient Fisher 344 rats. Animals were allowed to survive for 1 minute, 30 minutes, 15 hours, and 24 hours (n=3 per group) and were then euthanized. Lung sections were cut, washed with PBS, fixed in acetone-methanol (1:1) for 10 minutes, and incubated for 1 hour with a rabbit-derived polyclonal anti–von Willebrand factor antibody (Sigma Chemical Co) diluted 1:50 in a PBS solution containing 5% horse serum, 5% FCS, and 0.1% Triton X-100. The sections were incubated for 1 hour with goat anti-rabbit FITC-conjugated IgG (Vector Laboratories) diluted 1:100 in the above buffer. Negative control slides were incubated with the same solutions with the primary antibody omitted. The sections were mounted on glass slides with Vectashield (Vector Laboratories) and examined with a scanning confocal microscope (MRC-600, BioRad). The number of cells present within each lung section and their localization with regard to the endothelial border were assessed.

MCT Experiments

The full-length coding sequence of VEGF165 was generated by reverse transcription–polymerase chain reaction (RT-PCR) using total RNA extracted from human aortic smooth muscle cells and the following sequence-specific primers: sense, 5'-TGGGGCTCTGGGAACCATGTA-3'; antisense, 5'-CTCTGAGGAGACTGTC-3'. This generated a 649-bp fragment that was sequenced and cloned into the expression vector pcDNA 3.1 (Invitrogen) at the EcoRI restriction site, and correct orientation was determined by use of a differential digest. The insert-deficient vector (pCDNA 3.1) was used as a control for the MCT experiments. Smooth muscle cells were transfected by use of Superfect (Qiagen Inc) with either pcDNA 3.1 or pVEGF and were then trypsinized and divided into aliquots of 500,000 cells. Six- to 8-week-old Fisher 344 rats were injected with saline to establish normal hemodynamic and morphometric parameters of 500,000 cells. Six- to 8-week-old Fisher 344 rats were injected with 80 mg/kg MCT SC (Aldrich Chemical Co) either alone (n=9) or together with 500,000 pVEGF-transfected (n=11) or pcDNA 3.1-transfected (n=10) cells delivered via a catheter in the external jugular vein. At 28 days after injection, the animals were reanesthetized, and RV systolic pressure (RVSP) and systemic arterial pressure (SAP) were recorded with a Millar microtip catheter inserted into the RV ascending aorta. Before the catheter was placed into the aorta, 0.5 mL of arterial blood was drawn into a heparinized syringe and inserted into the RV ascending aorta. Before the catheter was placed into the RV ascending aorta. The upstream primers was located within the T7 priming site of the pcDNA 3.1 vector, and the downstream primer was located within exon 4 of the coding region of VEGF, thus selectively amplifying the 480-bp fragment. A 380-bp fragment from the pcDNA 3.1 vector was amplified with the following primers for the constitutively expressed gene GAPDH: sense, 5'-CTCTAAGGCTGTGGGCAAGGTCAT-3'; antisense, 5'-GAGATCCACCCCTGTTGCTGTA-3'. This reaction was carried out for 25 cycles with an annealing temperature of 58°C. In all cases, 10 μL of a 50-μL reaction were run on a 1% agarose gels.

Morphometric Analysis

Paraformaldehyde-fixed rat lungs were paraffin-embedded en face. Sections 5 μm thick were cut and stained with the elastin–van Gieson’s technique. A blinded observer measured all vessels with a perceptively media within each cross section under ×40 magnification using the C-Imaging morphometric software (Compix Inc). The morphometric area of each vessel was determined, and an average was obtained for each vessel size from 0 to 30 and 30 to 60 μm in external diameter for each animal. The averages from each size were compared between the VEGF, pcDNA 3.1, and MCT-alone groups. Similar measurements were made in the experiments in which VEGF gene transfer was given 2 weeks after MCT (ie, delayed gene transfer), except that the images were collected with a Cool SNAP high-resolution CCD camera (Roper Scientific) and analyzed with Scion Image (Scion Corp) morphometric software. In addition, in the delayed gene transfer group, only the VEGF and MCT-alone groups were compared.

Caspase-3 Immunostaining

Lungs were harvested from rats treated with MCT alone or together with VEGF-transfected cells at 1, 2, 3, and 4 weeks. Formaldehyde-fixed sections were cut and mounted, and immunohistochemistry was performed with an antibody for active caspase-3 (Promega).

Statistical Analysis

Data are presented as mean±SD unless otherwise stated. Differences in the number of fluorescently labeled cells over time were assessed by ANOVA, with a post hoc analysis using the Bonferroni test. All pressures, weights, arterial blood gas results, RV/LV ratios, and morphometric data were initially analyzed to determine whether the assumptions for parametric testing (normal distributions and equal variances) had been met. Because these assumptions were met for the pressure, weight, arterial blood gas, and RV/LV ratio data, differences were assessed by ANOVA, with a post hoc analysis using the Bonferroni test. For the morphometric data, the assumption of normal distribution was not met for the 30- to 60-μm vessel grouping. Therefore, a resampling procedure was used to test for differences between the VEGF, pcDNA 3.1, and MCT groups in both the 0- to 30- and 30- to 60-μm groups using sampling with replacement (bootstrapping). The resampling procedure was repeated 2000 times. In all instances, a value of P<0.05 was accepted to denote statistical significance. For the morphometric data in the
delayed gene transfer experiments, because only 2 groups were being compared, a Student’s t test was used for statistical analysis.

Results

Fluorescent Cell Labeling and In Vivo Detection of Labeled Cells

In vitro studies demonstrated that 100% of cultured rat pulmonary artery smooth muscle cells fluoresced intensely after incubation with the fluorophore CMTMR. Five minutes after intravenous injection, nearly all of the CMTMR-labeled cells (99±1%) were found within small arterioles, with little change up to 30 minutes (93±5%, P=NS) (Figure 1). In contrast, by 15 and 24 hours after injection, the majority of CMTMR-labeled cells could be clearly identified outside the endothelial perimeter (73±5% and 67±16%, respectively, P<0.05 versus 5 minutes). No fluorescent signals were seen in lungs injected with nonlabeled smooth muscle cells.

MCT Experiments

Four weeks after injection of MCT alone, RVSP was increased from 22±4 mm Hg in normal rats to 49±6 mm Hg, consistent with the development of PH (P<0.001, Figure 2A). There was no improvement in animals receiving cells transfected with the control pcDNA 3.1 vector, with the average RVSP remaining at 48±6 mm Hg. In contrast, animals treated with the pVEGF-transfected cells, RVSP was reduced to 32±7 mm Hg (P<0.001 versus MCT alone or null vector). This treatment, however, did not completely restore the RVSP to normal values (P<0.05 versus normal animals).

MCT-treated animals receiving pVEGF-transfected cells exhibited better general appearance and weight gain compared with animals treated with either MCT alone or MCT in combination with the delivery of cells transfected with the null pcDNA 3.1 vector (weight increase: 71±80 versus 3±53 and 4±37 g, respectively, P<0.01). Arterial oxygen tension (Po2) was significantly reduced in the MCT-treated rats compared with control animals (Po2, 53±16 versus 81±9 mm Hg, respectively, P<0.01), and delivery of control vector- or VEGF-transfected cells did not result in further worsening of pulmonary gas exchange (Po2, 65±2 and 64±6 mm Hg, respectively, P=NS versus MCT alone).

Consistent with long-standing and severe PH, the RV/LV ratio was significantly elevated in animals treated with MCT alone (0.34±0.05) or MCT together with pcDNA 3.1-transfected cells (0.33±0.05) compared with normal animals (0.21±0.02, P<0.001) (Figure 2B). In contrast, in the group receiving VEGF-transfected cells, the RV/LV ratio was reduced to values not significantly different from those of the control animals (0.24±0.04, P<0.001 versus pcDNA or MCT, P>0.05 versus control).

Figure 1. A, Detection of CMTMR-labeled smooth muscle cells in lung sections at various times after internal jugular injection: a, 5 minutes; b, 15 minutes; c, 30 minutes; and d, 15 hours. B, Percentage of CMTMR-labeled cells clearly identified outside microvascular lumen at various time points after CMTMR-labeled cell delivery at 5 and 30 minutes and 15 and 24 hours, n=3 for each time point. *P<0.05, **P<0.001 vs 5 minutes.

Figure 2. A, RVSP for normal animals (n=7) and for animals 4 weeks after injection of pulmonary endothelial toxin MCT given either alone (n=9) or together with cell-based gene therapy (GT) with null vector–transfected (pcDNA, n=10) or VEGF-transfected (VEGF, n=11) smooth muscle. B, RV/LV ratio for same groups. *P<0.001, **P<0.001 versus control; **P<0.001 vs MCT and pcDNA3.1.
Figure 3 shows representative sections of lung tissue from normal rats and rats treated with MCT alone or together with cell-based gene transfer of the null or VEGF vectors. In both the MCT-alone and null-transfected animals, exuberant hypertrophy of the media of small muscular arteries and arterioles, as well as increased muscularity of the terminal arterioles, was apparent. As in previous reports using the MCT model, morphometric analysis of the pulmonary tissue sections revealed that MCT significantly increased the medial area of small muscular arteries and arterioles compared with rats that were not treated with the pulmonary endothelial toxin (Figure 4). Medial area in the MCT-alone group was not different from the group receiving MCT together with pcDNA 3.1–transfected cells. Cell-based gene transfer with VEGF, however, significantly reduced the medial area measurement in arterioles and between 30 and 60 μm in diameter, compared with either the pcDNA or MCT groups (P<0.005).

PCR Analysis
At the time of death, rats treated with MCT alone or MCT and cells transfected with the control vector demonstrated only weak endogenous VEGF expression. In contrast, rats that received the VEGF-transfected cells had a significant increase in total VEGF levels (Figure 5). By use of primers designed to amplify exclusively the plasmid-derived human VEGF transcript, an exogenous VEGF transgene could be detected only in those animals that received the VEGF-transfected cells, confirming that the VEGF transgene expression persisted even 28 days after cell-based gene transfer. In the majority of these animals, no VEGF transgene could be detected in tissues other than lung. A weak band was detected occasionally (3 of 13 experiments, <10%), however, in the spleen and liver. The intestine and kidney did not exhibit any evidence of transgene expression in any animal studied.

Delayed VEGF Gene Transfer
RVSP was elevated to 27±1 mm Hg 2 weeks after MCT injection. In animals receiving pcDNA 3.1–transfected cells at the 2-week time point, the pressure was further increased to 57±14 mm Hg at 4 weeks after MCT delivery. In the pVEGF-treated animals, however (Figure 6), the RVSP increased only modestly (+11±9 mm Hg, P<0.05, to 37±7 mm Hg, P<0.01 versus pcDNA 3.1) (Figure 6A). The

Figure 3. Compared with lungs from normal rats (a), there was marked medial hypertrophic and hyperplastic response in medium and small muscular pulmonary arteries 4 weeks after subcutaneous injection of pulmonary endothelial toxin MCT (b), as well as increased muscularization of distal arterioles. MCT-treated animals treated with pcDNA-transfected cells (c) had similar appearance. In contrast, after cell-based gene transfer of VEGF (d), a decrease in medial thickness was observed in medium and small vessels, with better preservation of overall alveolar structure. Bar=50 μm. Solid arrows indicate muscular arterioles; open arrows, bronchioles.

Figure 4. Summary data for medial area of pulmonary arterial vessels, 30 μm (A) and 30 to 60 μm (B) in external diameter. Compared with normal control lungs (n=3), lungs from animals treated with MCT alone (n=12) or MCT with pcDNA (n=9) exhibited marked increase in medial area. Treatment with pVEGF-transfected smooth muscle cells (n=11) substantially reduced medial area in MCT-treated animals, particularly in small arterioles (A). Data are means±SEM. *P<0.001, †P<0.05 vs control; ¶P<0.005 vs MCT or pcDNA.
RV/LV ratio was elevated in the pcDNA group to 0.395 ± 0.063, but after VEGF gene transfer, the ratio was reduced to 0.278 ± 0.036 (P < 0.0005 versus pcDNA 3.1). Again, no difference in aortic blood pressure was noted. If anything, the reduction in medial area was more pronounced after delayed VEGF gene transfer for both the smaller (<30-μm) and larger (30- to 60-μm) arterioles (Figure 6B).

Caspase-3 Immunostaining
Apoptosis of vascular cells was studied at 1, 2, 3, and 4 weeks after MCT treatment alone or together with cell-based VEGF gene transfer (n = 2 to 3 for each group). In lungs from animals given MCT alone, staining for active caspase-3 was observed, particularly localized to endothelial cells in the smaller arterioles, and was greatest at 2 weeks (Figure 7A), whereas smooth muscle cells and pericytes only infrequently exhibited caspase-3 positivity. In animals treated with VEGF together with MCT, there was noticeably less endothelial caspase-3 staining (Figure 7B), suggesting that the improvement in pulmonary hemodynamics and arteriolar remodeling induced by VEGF may be associated with reduced endothelial cell apoptosis.

Discussion
Cell-based gene transfer was associated with a high percentage of cells being retained within the lung immediately after intrajugular injection, and the majority of these cells transmigrated through the endothelial layer within 24 hours. The targeting of VEGF-transfected cells to the precapillary resistance vessel reduced the development of MCT-induced PH and inhibited pulmonary vascular and RV remodeling. We have previously shown that cell-based eNOS gene transfer to the pulmonary microvasculature also attenuated the chronic increase in pulmonary arterial pressures in the MCT model of pulmonary vascular remodeling and PH. This approach may offer significant advantages over other strategies to achieve selective pulmonary gene transfer, such as endotracheal gene delivery, which results in predominantly epithelial overexpression, or catheter-based pulmonary vascular gene transfer, which produces diffuse macrovascular and systemic overexpression.

VEGF in Pulmonary Hypertension
To the best of our knowledge, this is the first report demonstrating that an angiogenic growth factor, such as VEGF, might have therapeutic effects in MCT-induced PH. Increased VEGF expression has been reported in association with plexiform lesions in pulmonary tissue from patients affected by PPH; this was interpreted, however, as suggesting a role for VEGF in the pathogenesis of this disease. Against this view are the results demonstrating potentiation of hypoxic PH with selective blockade of the VEGFR-2 (kdr) receptor. Together with the findings of the present study, these data suggest that VEGF expression may represent an adaptive response to microvascular obstruction in PH and that further overexpression achieved by gene transfer may reduce adverse vascular remodeling.
Alternatively, VEGF, which has also been well characterized as an endothelial cell survival factor, may prevent pulmonary microvascular apoptotic cell loss induced by endothelial injury. The demonstration that VEGF gene transfer reduced caspase-3 activation in pulmonary arteriolar endothelium after MCT treatment provides support for this view.

Finally, the well-known angiogenic effects of this growth factor may have contributed to pulmonary microvascular regeneration in the affected lungs and thereby have decreased overall vascular resistance. The efficacy of delayed VEGF gene transfer in established PH is consistent with this possibility; further experiments are necessary, however, to better define which of these mechanisms are involved in the beneficial effects of “angiogenic” gene transfer in the MCT model of PH. Ultimately, the relevance of these findings for patients with arterial PH will need to be determined by appropriately designed clinical studies.

The recent discovery of the genetic defect underlying some cases of familial and sporadic PPH may provide new insight into the molecular mechanisms of this disease. A high proportion of individuals exhibited heterozygosity for various mutations in the bone morphogenic protein receptor-2, suggesting that haploinsufficiency or dominant negative protein interactions may lead to a partial inhibition of transforming growth factor-β signaling and thereby contribute to the development of the pathological features of PPH. Among other things, this mutation may reduce the angiogenic effects of transforming growth factor-β and lead to an overall loss of pulmonary microvessels and the vascular pruning that is characteristic of arterial PH.

In summary, cell-based gene transfer to the pulmonary microvasculature resulted in selective transgene overexpression for periods of up to 4 weeks. Also, VEGF gene therapy using this approach was effective in inhibiting the development and progression of PH and improved vascular and RV remodeling in the MCT model. Therefore, these results suggest that the delivery of angiogenic factors combined with a cell-based method of gene transfer may provide a novel therapeutic strategy for pulmonary vascular disorders, for which at present there are few long-term treatment options.

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