Protective Role of Angiopoietin-1 in Experimental Pulmonary Hypertension

Yidan D. Zhao, Andrew I.M. Campbell, Malcolm Robb, Douglas Ng, Duncan J. Stewart

Abstract—Angiopoietin-1 (Ang-1), a newly discovered ligand of the endothelial-specific tyrosine kinase receptor Tie-2, has been found to promote cell survival, vascular maturation, and stabilization. We hypothesized that Ang-1 gene transfer to the pulmonary microcirculation would improve pulmonary hemodynamics and vascular remodeling in experimental pulmonary hypertension. Rat pulmonary artery smooth muscle cells were transfected with Ang-1 cDNA or null (pFLAG-CMV-1) vector. Syngeneic Fisher 344 rats were treated with monocrotaline (MCT) (75 mg/kg IP) with or without delivery of $5 \times 10^7$ Ang-1–transfected cells into the right jugular vein. After 28 days, plasmid-derived Ang-1 mRNA was consistently and robustly detected by reverse transcriptase–polymerase chain reaction in lungs from all animals receiving Ang-1 gene therapy. Tie-2 receptor expression was markedly downregulated in rats treated with MCT, and this was partially restored by gene therapy with Ang-1. Animals receiving MCT exhibited 77% mortality by 28 days. In contrast, in pAng-1–treated animals, the 28-day mortality was only 14% ($P<0.0001$). In addition, right ventricular systolic pressure was reduced from $52 \pm 1.3$ mm Hg in the MCT-treated group to $38 \pm 1.3$ mm Hg by Ang-1 gene transfer ($P<0.01$), whereas the measurement of right to left ventricular plus septal weight ratio was also reduced from $0.41 \pm 0.03$ to $0.31 \pm 0.01$ ($P<0.05$). Moreover, MCT resulted in increased apoptosis, mainly in the microvasculature, and reduced endothelial NO synthase mRNA expression, both of which were prevented by Ang-1 gene transfer. Thus, cell-based gene transfer with Ang-1 improved survival and pulmonary hemodynamics in experimental pulmonary hypertension by a mechanism involving the inhibition of apoptosis and protection of the pulmonary microvasculature. (Circ Res. 2003; 92:984-991.)

Key Words: gene therapy • angiopoietin-1 • Tie-2 • endothelial nitric oxide synthase • apoptosis

Angiopoietin-1 (Ang-1) is a newly discovered ligand for the endothelial-selective tyrosine kinase receptor, Tie-2. Like vascular endothelial growth factor (VEGF), Ang-1 has been shown to play an essential role in embryonic vasculature development. However, unlike VEGF, Ang-1 seems to act at a later stage of the angiogenic process, promoting the stabilization and maturation of primitive vessels and their organization into stable vascular structures. It has also been recently recognized that Ang-1 is a potent endothelial cell (EC) survival factor, acting via the Akt/phosphatidylinositol-3 kinase pathway to inhibit EC apoptosis in response to cytokines or serum withdrawal. In contrast, Ang-2, another ligand for Tie-2, seems to act as a natural antagonist, blocking the stabilizing effects of Ang-1 and facilitating EC activation in response to VEGF in postnatal angiogenesis. Both Ang-1 and Tie-2 are widely expressed in the adult vasculature, and their interaction may be crucial for the maintenance of vascular homeostasis and normal quiescent state of vascular EC, whereas Ang-2 is expressed only in regions of active angiogenesis and vascular remodeling.

Primary pulmonary hypertension (PPH) is a rare disorder that is characterized by elevated pulmonary vascular resistance leading to right heart failure and death. Although the mechanism underlying PPH is uncertain, considerable evidence now points to abnormalities of EC function as an early feature of this disease. Endothelial dysfunction has been reported in experimental and human PH and is characterized by an imbalance of vasodilator and vasoconstrictor factors, including an altered ratio of thromboxane and prostaglandin, decreased expression of endothelial NO synthase (eNOS), and increased endothelin-1 (ET-1). Therapies designed to counteract EC dysfunction, such as the administration of prostacyclin or antagonists of the endothelin system, have proven to be effective in the treatment of patients with PPH.

Interestingly, the adult lung exhibits one of the highest levels of Tie-2 expression of any organ under physiological conditions. Therefore, we hypothesized that tonic activation of Tie-2 may play an important role in protecting the pulmonary vasculature by preventing EC activation in response to toxic stimuli. Moreover, alterations in expression of components of the angiopoietin system, specifically an increase in Ang-2 or a decrease in Tie-2, may contribute to the...
development of PH. Indeed, decreased levels of pulmonary Tie-2 expression and variable changes in Ang-1 and -2 have been reported in response to short-term exposure to hypoxia; however, the possible relevance of these changes in the development of PH during chronic hypoxia or in response to MCT has not been explored.

Thus, the aims of the present study were to characterize for the first time the changes in Ang/Tie-2 expression in the rat MCT model and to determine the effect of Ang-1 gene overexpression on the development of experimental PH. We now show a profound reduction in pulmonary Tie-2 expression at 4 weeks after MCT, with no change in the levels of Ang-1 or Ang-2 in the lungs. Moreover, cell-based gene transfer of Ang-1 inhibited the downregulation of Tie-2 and markedly improved survival in this model, and these effects were associated with significant reduction in the right ventricular (RV) pressure, EC apoptosis, and RV and pulmonary arterial remodeling.

Materials and Methods

Cell Culture and Transfection

The full-length coding sequence of Ang-1 was a generous gift of Dr. I. Kim (Woosuk University, Chonju, South Korea), and the 1115-bp insert was cloned into the expression vector pFLAG-CMV-1 (Sigma). Pulmonary artery smooth muscle cells (SMCs) were isolated from syngeneic Fisher 344 rats (F-344, Charles River Co, St Constant, Quebec), as described previously. Cells between the fifth and ninth passages were transfected with the null vector, pFLAG-CMV-1 (pFLAG), or the same vector containing the full-length cDNA for human Ang-1 using Superfect (Qiagen, Inc) (see the online data supplement, available at http://www.circresaha.org). Cells were allowed to recover for 12 to 24 hours and then trypsinized and divided into aliquots of 5 x 10^5 cells for delivery into the pulmonary circulation.

Cell Delivery

Six-week-old F-344 rats (160 to 180 g) were anesthetized, and the left external jugular vein was cannulated with PE50 tubing. Aliquots of ~5 x 10^5 transfected cells were injected, and the jugular vein was then ligated, the incision was closed, and the animals were allowed to recover from anesthesia. F-344 rats (160 to 180 g) were randomly assigned to 1 of 4 groups. Normal animals received either saline (n=7), null-transfected cells (n=7), or Ang-1–transfected cells (n=7). The remaining animals were injected intraperitoneally with 75 mg/kg of MCT (Aldrich Chemical Co). The MCT-treated rats then received either no additional treatment (MCT alone, n=18) or 5 x 10^5 cells transfected with Ang-1 (pAng-1, n=29) or the null vector (pFLAG, n=12). All animal experiments were performed in accordance with the Canadian Council on Animal Care guidelines.

Hemodynamic Measurements and Tissue Collection

Twenty-eight days post-MCT or saline injection, the animals were reanesthetized, and a Millar microtip catheter was inserted via the right external jugular vein to record right ventricular systolic pressure (RVSP). The animals were then sacrificed and the hearts excised for measurement of right to left ventricular plus septal weight (RV/LV) ratio. The left lungs were inflated with Tissue-Tek (OCT) and cut into pieces that were either fixed in a 4% paraformaldehyde-0.1% glutaraldehyde solution for paraffin embedding and sectioning or frozen on dry ice for cryostat sectioning. The right lung segments were snap frozen in liquid nitrogen for subsequent RNA extraction and analysis.

Reverse Transcriptase–Polymerase Chain Reaction

From each animal, 5 µg of total RNA was reverse transcribed using the murine leukemia viral reverse transcriptase (RT). An aliquot of the resulting cDNA was amplified by polymerase chain reaction (PCR) using sequence-specific primers for the endogenous Ang-1, Ang-2, Tie-2, or the plasmid-derived exogenous Ang-1 transcript, as detailed in the online data supplement.

Western Analysis

Lung tissues were homogenized in lysis buffer and subjected to electrohoresis in SDS-PAGE gels as described in the online data supplement. The membranes were incubated with primary polyclonal antibody against Tie-2 (1:3000, Santa Cruz Biotechnology, Inc), monoclonal antibody against eNOS (1:2000, Transduction Laboratories), or monoclonal antibody against β-actin (1:4000, Santa Cruz Biotechnology, Inc) and developed as described in detail in the online data supplement.

TIE-2 Receptor Phosphorylation

Tissue samples were homogenized in lysis buffer, as described above. From each tissue lysate, 500 µg of total protein was immunoprecipitated with a Tie-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif). Immunocomplexes were recovered on protein A Sepharose and separated by SDS-PAGE, transferred to the blotting membrane, as described above. The membranes were probed with anti-Tie-2 (Santa Cruz) and then with antiphosphotyrosine (4G10, Upstate Biotechnology Inc; 1:3000) for receptor phosphorylation state. Bands were visualized using the ECL system (Amersham Pharmacia Biotech). Densitometry was performed using a scanning densitometer coupled with Molecular Analyst software (Bio Rad).

Morphometric Analysis

The rat lungs were fixed in 4% paraformaldehyde. Five-micrometer sections were cut and stained using H&E. Vessels with a perceptible media within each cross-section were measured by a blinded observer under ×10 magnification using the Scion imaging system (Scion Image, National Institutes of Health). All vessels that were tangentially cut were excluded. The medial area of small arterioles was determined, and an average was obtained for vessel sizes from 30 to 60 µm and <30 µm in external diameter for each animal. The averages were compared between the Ang-1, MCT only, and normal groups.

Caspase-3 Activity

In a separate series of experiments, 28 additional rats were treated with MCT alone or together with Ang-1–transfected cells, and lung tissue was harvested at 1, 2, 3, and 4 weeks. Lung caspase-3 activity was determined using a colorimetric assay kit (Promega) according to the manufacturer’s instructions, and caspase-3 immunostaining was performed as detailed in the online data supplement.

Immunofluorescent Microscopy

Cells between the fifth and ninth passages were transfected with pFLAG-CMV-1 vector containing the full-length cDNA for human Ang-1 using Superfect (Qiagen, Inc). The cells were allowed to recover for 24 hours and then labeled with the viable fluorophore, chloromethyl trimethyl rhodamine (CMTMR, Molecular Probes Inc) and were then injected into the internal jugular vein of recipient Fisher 344 rats (N=5). Animals were allowed to survive for 48 hours and were then killed. Lung sections (25 µm) were cut, washed with PBS, fixed in acetone-methanol (1:1) for 10 minutes, and then subjected to immunofluorescent confocal microscopy, as described in detail in the online data supplement.

Statistical Analysis

Data are presented as mean±SEM. Differences were assessed by ANOVA (with post hoc comparisons using unpaired t test or Mann-Whitney tests as appropriate). Survival data were analyzed...
Results

Effect of MCT on Angiopoietin and Tie-2 Expression

Ang-1, Ang-2, and Tie-2 mRNA expression was determined by semiquantitative RT-PCR in lung samples from normal and PH rats 28 days after administration of MCT. In normal lungs, all 3 components of the angiopoietin system were readily detected. There was no change in Ang-1 and Ang-2 mRNA, even in the presence of severe PH (Figure 1B). In contrast, Tie-2 mRNA was consistently reduced in the MCT-treated versus control lungs. This decrease in Tie-2 expression was confirmed at the protein level by Western analysis (Figure 1C).

Expression of the Human Angiopoietin Transgene

Using primers designed to selectively amplify only the plasmid-derived Ang-1 transcript, the amount of exogenous Ang-1 being transcribed could be compared in the control, Ang-1, and MCT-treated groups using RT-PCR. The plasmid-derived Ang-1 signal was detected only in animals that had received the Ang-1–transfected cells (Figure 1A), confirming transgene expression 28 days after cell-based gene transfer. Interestingly, Ang-1 gene transfer resulted in a partial normalization of the reduction in Tie-2 expression induced by MCT (Figure 1C).

Effect of Ang-1 Gene Transfer on Survival

Of the 18 animals that received MCT alone or the 12 that received MCT together with null-transfected SMCs, only 7 (23%) survived to end study (Figure 2A). Two of these animals underwent end study on day 27 at the request of the animal facility because they were deemed to be moribund. Of the 29 animals receiving Ang-1–transfected SMCs together with MCT, all but 4 survived to 28 days (86%, P<0.0001 versus MCT and MCT/null). Treatment with MCT resulted in a marked increase in RVSP (B) at 28 days in rats receiving MCT alone or MCT+pFLAG (open bars) compared with normal rats (left bars). In contrast, RVSP was reduced in MCT rats receiving cell-based Ang-1 gene transfer (closed bars). Similarly, the ratio of right ventricular to left ventricle and septum (RV/LV ratio, C) was increased in MCT-only or MCT+pFLAG–treated animals and reduced by Ang-1 gene transfer (*P<0.05; **P<0.01).
Ang-1 only, 23 ± 1.43; null vector, 26 ± 0.67 mm Hg). In the animals receiving MCT alone (52 ± 1.3 mm Hg) or together with null-transfected SMCs (49 ± 1.8 mm Hg), RVSP was similarly increased (P < 0.01 versus control, Figure 2B). In the pAng-1–treated MCT rats surviving to end study, RVSP was significantly reduced (38 ± 1.3 mm Hg, P < 0.01) compared with MCT alone and null-transfected animals.

Body Weight and RV/LV
The initial mean body weight of the MCT-treated and Ang-1–treated animals was similar (180 ± 6 and 183 ± 6 g, respectively). By day 28, the rats receiving MCT alone gained 13 ± 13 g compared with 59 ± 15 g (P < 0.05) for the MCT + Ang-1 rats. RV/LV was 0.26 ± 0.02 in the control rats (Figure 2C), whereas rats receiving MCT either alone or with null vectors developed RV hypertrophy, with an increase in RV/LV to 0.41 ± 0.04 (P < 0.05, MCT versus control). Animals receiving Ang-1 gene transfer together with MCT developed significantly less RV hypertrophy (0.31 ± 0.01, P < 0.05, pAng-1 versus MCT).

Assessment of Lung Morphology
Lungs from rats injected with MCT with or without null-transfected cells exhibited marked medial hypertrophy of the pulmonary arteries in conjunction with increases in cellularity of the alveolar septa and muscularization of the distal arterioles (Figure 3A). In comparison, MCT rats treated with pAng-1–transfected cells demonstrated improvement in the overall appearance of the lung and reduced muscularization of the pulmonary arteries and arterioles. Morphometric analysis revealed that MCT significantly increased the medial area of muscular arterioles compared with normal rats that were not treated with MCT (Figures 3B and 3C). In comparison, cell-based gene transfer with Ang-1 significantly reduced medial thickness in arterioles (30 ± 30 μm (P < 0.001, Figure 3B) and between 30 and 60 μm (P < 0.05, Figure 3C) in diameter compared with MCT control groups.

Caspase-3 Immunostaining and Activity
Caspase-3 immunostaining was used to assess pulmonary cell apoptosis. In the MCT-treated rats, there was abundant vascular endothelial staining for activated caspase-3 at 2 weeks, localized to the endothelium of smaller pulmonary arterioles (Figure 4A). At 4 weeks, other cell types, including smooth muscle and inflammatory cells, exhibited increased caspase-3 activity. In animals treated with Ang-1 together with MCT, less caspase-3 staining was apparent at both 2 and 4 weeks, consistent with reduced vascular cell apoptosis in response to Ang-1 (Figure 4A). In a separate series of experiments, caspase-3 activity was measured in serial lung sections.
tissue lysates (1, 2, 3, and 4 weeks) in the MCT-alone group (n=3 to 5 per each group) and the MCT + Ang-1 group (n=3 to 6 per each group) (Figure 4B). There was an increase in caspase-3 activity in the MCT-alone group, with a peak at 2 weeks (P<0.05). Ang-1 gene transfer prevented the increase in caspase-3 activation in response to MCT treatment. The changes in caspase activity were also correlated with improved pulmonary hemodynamic parameters in these animals. RVSP exhibited a progressive rise over the 4 weeks after MCT alone that was markedly blunted by Ang-1 gene transfer (Figure 4C).

Expression of Ang-1 Transgene In Situ and Effect on Tie-2 Activity
To confirm that genetically engineered cells expressed the Ang-1 protein after engraftment in the pulmonary microvasculature, Ang-1–transfected cells were labeled with CMTMR before their delivery into the pulmonary circulation, and the lung sections were examined by confocal microscopy 1 week after gene transfer. These sections were immunostained with FITC-labeled anti-Ang-1 (top left, green). The larger panel on the right shows the merged image. Cells positive for both CMTMR and Ang-1 (closed arrows) appear yellow in the merged image and represent engrafted cells that are overexpressing the Ang-1 transgene. In contrast, a proportion of CMTMR-positive cells do not exhibit Ang-1 immunofluorescence (open arrows). Representative Western blots for Tie-2 (B, top) and β-actin (B, bottom) of protein extracts from lungs of normal rats or rats treated with MCT with or without Ang-1 gene transfer. Immunoblots of lung protein extracts precipitated with anti-Tie-2 and developed with phosphotyrosine (C, top) or Tie-2 antibodies (C, bottom).

Figure 4. Immunohistochemistry for caspase-3 in lung sections from rats treated with MCT alone (A, panels a and c) and MCT together with Ang-1 (A, panels b and d) at 2 weeks (a and b) and 4 weeks (c and d) after MCT. In the MCT animals at 2 weeks, immunostaining for caspase-3 was localized mainly to the endothelium of small pulmonary arterioles (a). At 4 weeks (c), other cell types, including SMC and inflammatory cells, exhibited increased caspase-3 activity. In animals treated with Ang-1 together with MCT, markedly less caspase-3 staining was apparent at both 2 (b) and 4 (d) weeks. Caspase-3 activity (B) and RVSP (C) at various time intervals after injection of MCT. Caspase-3 activity was increased in the MCT alone group (closed bars, n=3 to 5 per each group), whereas Ang-1 gene transfer prevented the increase in caspase-3 activation in response to MCT treatment (open bars). In the same animals, RVSP was progressively increased in the MCT-alone groups, and this was markedly blunted in the pAng-1 group. n=3 to 6 per each group; **P<0.01.
Recent reports have confirmed the role of the Ang-1/Tie-2 pathway is active in the adult under physiological conditions. Data provided evidence that the Ang-1/Tie-2 receptor pathway in regulating vascular permeability in adult mice with targeted dermal overexpression of Ang-1. However, the functional importance of the Ang-1/Tie-2 system in the pulmonary vasculature remains to be elucidated.

In cell culture models, Ang-2 expression has been reported to be increased by exposure to hypoxic conditions, whereas Ang-1 and Tie-2 were not much changed. In the lung, Ang-1 and Tie-2 were both decreased by systemic hypoxia, but the effect of longer periods of hypoxia that are required to produce chronic PH has not been studied. However, in patients with pulmonary arterial hypertension undergoing embolectomy, increased Ang-1 expression was reported in the lung parenchyma, which was proportional to the increase in preoperative pulmonary vascular resistance and medial wall hypertrophy. Although this finding might suggest a causal relationship between activation of the angiopoietin system and development of PH, it is also possible that increased Ang-1 production represents a compensatory response to vascular damage in this disease. Indeed, increased pulmonary VEGF expression has also been reported in patients with PPH, particularly in association with plexiform lesions, yet overexpression of this angiogenic factor in experimental models of PH seems to be protective. In the present study, MCT-induced PH was associated with a profound decrease in Tie-2 expression, both at the mRNA and protein levels, with no significant changes in expression of the Ang-1 or Ang-2. Reduction in pulmonary Tie-2 could have resulted from either a decrease in expression on a cellular basis or a reduction in the overall number of cells expressing Tie-2. In support of the latter mechanism is the observation in the present report that MCT induced widespread EC apoptosis as well as prior evidence for significant loss of pulmonary microvasculature in MCT-induced PH. However, it is possible that MCT also reduced the Tie-2 expression in the remaining microvascular endothelial cells in a manner similar to hypoxia.

The present study directly tested the role of the Tie-2 pathway in the development of experimental PH by overexpressing the Ang-1 gene, targeted to the pulmonary microvasculature. Rats treated with Ang-1 together with MCT showed significant attenuation of PH, blunted RV remodeling, and reduced muscularization of distal arterioles. Moreover, Ang-1 gene transfer was associated with a dramatic survival benefit in this model of experimental PH that is normally associated with near-universal fatality. Therefore, these data are consistent with a central role of the Ang-1/Tie-2 pathway in protecting the pulmonary circulation from the consequences of MCT-induced endothelial injury.

The present report also explored the potential mechanisms by which Ang-1 prevented the onset of MCT-induced PH. Ang-1 has been shown to induce capillary-like tube formation in vitro models of angiogenesis, although unlike other angiogenic growth factors, Ang-1 does not induce EC proliferation. We and others have previously reported that gene transfer of VEGF resulted in marked improvement in models of PH, suggesting that angiogenesis may play a protective role in the adaptation of the pulmonary microcirculation to the vascular changes during the onset of PH. Activation of the Tie-2 pathway by Ang-1 has also been
implicated in promoting EC survival\textsuperscript{3,30,31}; at least in part through activation of the phosphatidylinositol-3'-kinase/Akt pathway.\textsuperscript{31} In a previous report, the administration of MCT was shown to be associated with widespread EC apoptosis, particularly localized to the distal (precapillary) arterioles, which are critical for the regulation of pulmonary vascular resistance.\textsuperscript{20} Reduction in RV systolic pressure and RV hypertrophy in response to VEGF gene transfer in the MCT model of PH was reported to be associated with reduced EC apoptosis, suggesting that this may be a mechanism by which angiogenic factors may protect the pulmonary circulation.

In contradistinction to a role of EC apoptosis in the pathogenesis of PH are several studies that support increased EC proliferation in experimental models\textsuperscript{32} and human primary PH,\textsuperscript{33} possibly by contributing to arteriolar occlusion and plexiform lesion formation. However, EC proliferation may be a fairly late phenomenon, for example, associated the development of plexiform lesions, which are often found in the lungs of patients with end-stage pulmonary arterial hypertension\textsuperscript{33} and not in most experimental models of PH. In addition, attempts to inhibit hypoxia-induced PH by reducing EC proliferation using a VEGF receptor antagonist\textsuperscript{3} have resulted in a paradoxical worsening of PH and pulmonary arterial remodeling, which could be prevented by the coadministration of an inhibitor of apoptosis.

In the present study, apoptosis in the lung vasculature was determined by measuring caspase-3 activity at various time points after MCT administration. Caspases represent a family of cysteine proteases that act as principal apoptotic mediators. In agreement with our earlier report,\textsuperscript{20} apoptosis was increased early in the course of PH, with an initial peak at 2 weeks, a time point that precedes the development of significant increases in pulmonary pressures. At this time, apoptosis was observed mainly in the vascular endothelium of the pulmonary microcirculation. Interestingly, the increase in caspase activity seemed to be biphasic, with a second wave occurring at 4 weeks, which was associated with apoptosis of a variety of cell types, including vascular smooth muscle cells and inflammatory cells. Overexpression of Ang-1 significantly prevented the increases in activated caspase-3, consistent with its potent action in promoting EC survival.\textsuperscript{3} This was associated with significant reductions in RVSP, suggesting that a decrease in EC apoptosis may have prevented the development of PH, possibly by reducing the loss of fragile precapillary arterioles. Additional investigation will be required to fully define the relationship between EC apoptosis, microvascular occlusion, and PH; however, the present data add to the growing body of literature pointing to an important contribution of this process in the pulmonary vascular response to injury.

It is also possible that increased Tie-2 activity may have resulted in improved endothelial function and the restoration of the critical balance between production of vasoconstrictor and vasodilator factors. Ang-1 has been implicated in the maintenance of EC homeostasis in the adult vasculature.\textsuperscript{5} In the present study, Ang-1 gene transfer led to a marked upregulation of eNOS gene expression in MCT-treated rats. NO may protect against the development of PH by several effects, including vasodilation, antiproliferative effects on pulmonary vascular smooth muscle, reduced endothelial adhesion molecule expression and inhibition of platelet activation, and in situ thrombosis.\textsuperscript{24,35} In addition, NO has been reported to inhibit caspases by S-nitrosylation\textsuperscript{36,37} and thus may also contribute to enhanced EC survival in response to Ang-1 gene transfer.

In conclusion, we have shown that cell-based gene transfer of Ang-1 to the pulmonary microvasculature effectively inhibits MCT-induced PH, while at the same time reducing the downregulation of Tie-2 expression and MCT-induced EC apoptosis. The results suggest that the Ang-1/Tie-2 system is important in the protection of the pulmonary endothelium against MCT-mediated injury and that Ang-1 gene therapy may be useful in the treatment of PH.

Note Added in Proof
Since the final stages of the review process, the authors have become aware of another report suggesting that Ang-1 may play a causal role in the pathogenesis of human pulmonary arterial hypertension (PAH),\textsuperscript{38} which increases the controversy regarding the role of Ang-1 in PAH. This report underscores the need for carefully controlled experimental studies directly evaluating the in vivo effects of the angiopoietin pathway in this disease in order to better interpret observational data derived from human tissue samples.

Acknowledgments
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References


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RT-PCR: Amplification was performed for 32 cycles with an annealing temperature of 60°C.

“endogenous” Ang-1 primers:

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<th>Type</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Sense</td>
<td>5' GCCACTTGAGAATTACATTGTGG3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'CGCGGATTTTATGCTCTAATGAACGTG3'</td>
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“exogenous” (plasmid-derived) Ang-1 primers:

<table>
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<tr>
<th>Type</th>
<th>Primer Sequence</th>
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</thead>
<tbody>
<tr>
<td>Sense</td>
<td>5'CAGTTGCTGACTACAAAGACGATG3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'CCCATTTTETATCCCTCCAGCCT3'</td>
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</table>

For the determination of “exogenous” pAng-1 expression, the upstream primer was located within the T7 priming site of the pFLAG-CMV-1 vector whereas the downstream primer was located within the Ang-1 coding region. To control for RNA quantity and quality, a second aliquot of the same reverse transcription reaction was amplified with the following primers for the constitutively-expressed gene GAPDH:

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<tr>
<th>Type</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Sense</td>
<td>5'CTCTAAGGCTGTGGGCAAGGTCAT3'</td>
</tr>
<tr>
<td>Antisense</td>
<td>5'GAGATCCACCACCCTGTTAGCTGTA3'</td>
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This reaction was carried out for 25 cycles with an annealing temperature of 58°C. PCR products were then resolved and run on a 1.5% agarose gels. The same reverse transcription reaction was amplified with the following primers for the constitutively-expressed gene Tie-2:

<table>
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<th>Type</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Sense</td>
<td>5'CAGGACCTTCACAACAGCTCTATCGG3'</td>
</tr>
</tbody>
</table>
Antisense 5'CTGTCGAAGAATGTCACTAAGGGTCC3'

This reaction was carried out for 30 cycles with an annealing temperature of 64°C. PCR products were then resolved and run on a 1.5% agarose gels.

**Western analysis:** Proteins were transferred to nitrocellulose membranes (Hybond ECL; Invitrogen). The membrane was blocked with 4% non-fat dry milk in TBS-Tween buffer (20 mM Tris-HCl, pH 7.4, 135 mM NaCl, 0.1% Tween) for 30 min, and then incubated with a primary polyclonal antibody against Tie-2 (1:3000, Santa Cruz Biotechnology, Inc.), monoclonal antibody against eNOS (1:2000, Transduction Laboratories), or monoclonal antibody against actin (1:4000, Santa Cruz Biotechnology, Inc.). Membranes were then washed in TBS-Tween, and the immobilized proteins were incubated with HRP secondary antibodies (Amersham Pharmacia Biotech). The proteins were detected using an enhanced chemiluminescence detection kit (New England Biolabs, Beverly, MA). Densitometric analysis of Western blots was carried out using the Molecular Analysis system (Bio-Rad Laboratories).

**Caspase-3 activity:** A colorimetric assay kit (Promega, CA) was used for the detection of activated caspase-3, a cysteine aspartic acid specific protease. Lungs were also harvested from rats treated with MCT alone or together with Ang-1 transfected cells for immunocytochemistry. Paraformaldehyde-fixed tissue was cut into 5 micron sections and mounted onto Superfrost slides. Immunohistochemistry was performed using an antibody for caspase-3 (1:50; Promega, CA). The sections were deparaffinized by xylene and
rehydrated. After quenching endogenous peroxidase with 3% H₂O₂ for 10 min, the sections were incubated with the polyclonal anti-caspase-3 antibody (1:250 dilution) overnight at 4°C. The sections were then washed with PBS for 15 minutes and incubated with biotinylated anti-rabbit IgG, and immunodetection was performed using peroxidase-conjugated Avidin-biotin (Vector Laboratories) for 60 min in room temperature, and visualized using diaminobenzidine chromogen as the substrate. For negative controls, an identical procedure was used except for the omission of the primary antibody.

**Imunofluorescent microscopy:** Lung sections were incubated for 1 hour with a goat-derived polyclonal anti-angiopoietin antibody (Santa Cruz, USA), diluted 1:100 in a PBS solution containing 5% rabbit serum, 5% fetal calf serum, and 0.1% Triton X-100. The sections were washed with PBS, and then incubated for 1 hour with rabbit anti-goat FITC-conjugated IgG (Vector Laboratories, Mississauga, Ontario) diluted 1:100 in the above buffer. Negative control slides were incubated with the same solutions omitting the primary antibody. The sections were again washed with PBS, mounted on glass slides with Vectashield (Vector Laboratories, Mississauga, Ontario) and examined with a scanning confocal microscope (MRC-600, BioRad, Hercules, California).