Regulation of Endothelin-1 by Angiopoietin-1: Implications for Inflammation

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Endothelin-1 (ET-1) is increasingly recognized as a proinflammatory mediator in various diseases, such as atherosclerosis and acute respiratory distress syndrome (ARDS). Angiopoietin-1 (Ang-1), a ligand of the endothelial receptor Tie2, inhibits endothelial apoptosis, reduces vascular leakage, and suppresses the induction of inflammatory markers, indicating that it has diverse vasoprotective, anti-inflammatory actions. Thus, we examined the effects of Ang-1 on ET-1 production *in vitro* and *in vivo* and investigated cell-based gene transfer of Ang-1 in a rat model of lipopolysaccharide (LPS)-induced ARDS. Cultured human endothelial cells were treated with recombinant Ang-1 with or without tumor necrosis factor-α (TNF-α) (100 U/ml). ET-1 release into the culture medium after 24 hrs was determined by enzyme-linked immunosorbent assay. Levels of preproendothelin-1 (ppET-1) mRNA were measured by quantitative reverse transcription–polymerase chain reaction. Fisher344 rats were subjected to cell-based gene transfer to the lung circulation by injecting syngeneic fibroblasts transfected with Ang-1 cDNA or a null plasmid vector. After 24 hrs, LPS (100 μg/kg body wt) was instilled intratracheally to induce pulmonary inflammation. Bronchoalveolar lavage was performed 6 hrs later, and lungs were harvested for histologic and molecular analyses. ET-1 release from cultured endothelial cells was dose-dependently reduced by Ang-1, which also prevented induction of ET-1 release by TNF-α ($P < 0.05$). RNA expression of ppET-1 was similarly reduced. In LPS-challenged lungs, ppET-1 RNA was induced 3.4-fold, and ET-1 protein in lavage fluid was increased 5.6-fold ($P < 0.05$). Ang-1 gene transfer attenuated the LPS-induced increases in ppET-1 RNA and lavage ET-1 protein by 34% and 33%, respectively ($P < 0.05$). The downregulation of ET-1 correlated with the amelioration of pulmonary inflammation, as indicated by reductions in leukocyte infiltration (by 43%) and intra-alveolar septal thickening (by 40%). These results show that ET-1 transcript and protein levels are downregulated by Ang-1 in both *in vitro* and *in vivo* systems and that cell-based Ang-1 gene transfer markedly ameliorated inflammation *in vivo* in an experimental model of ARDS. Thus, cell-based gene transfer of Ang-1 may provide a novel treatment strategy for ARDS by attenuating vascular inflammation *via* suppression of ET-1. Exp Biol Med 231:985–991, 2006

Key words: endothelin-1; angiopoietin-1; pulmonary inflammation; acute respiratory distress syndrome; acute lung injury; cell-based gene therapy

Introduction

Endothelin-1 (ET-1) is a mediator of vascular inflammation, cell proliferation, and fibrosis in addition to being a potent vasoconstrictor (1–3). In various experimental models of vascular and inflammatory diseases, treatment with ET-1 antagonists has resulted in marked beneficial effects. In particular, treatment with ET-1 antagonists has been shown to reduce pulmonary vascular leak and inflammation in several models of lung injuries and in experimental acute respiratory distress syndrome (ARDS) (4–7). Moreover, patients who died from ARDS were shown to have high plasma and lung ET-1 expression compared to control patients (2, 8, 9).

Angiopoietin-1 (Ang-1) is a ligand of the endothelial-specific tyrosine-kinase receptor Tie2 and is an essential mediator of angiogenesis (10–12). Ang-1 is also an endothelial survival factor (13) and was recently shown to protect blood vessels against plasma leakage *in vivo* and to inhibit endothelial permeability *in vitro* (14–16). As well, Ang-1 inhibits leukocyte adhesion to vascular endothelium and reduces the expression of tissue factor and various adhesion molecules in endothelial cells stimulated by inflammatory cytokines (16–19). These findings strongly support a crucial anti-inflammatory role for this pro-angiogenic and antiapoptotic factor, perhaps with the

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capacity to maintain endothelial function and prevent pathologic changes in endothelial gene expression.

In this study, we hypothesized that the downregulation of ET-1 expression by Ang-1 is an important mechanism of its homeostatic effects on the vascular endothelium. We further explored whether delivery of Ang-1 to the lungs by cell-based gene transfer (20) can suppress cytokine induction of ET-1, thereby reducing vascular inflammation in a rat model of lipopolysaccharide (LPS)-induced ARDS.

Materials and Methods

Cell Culture. HMEC-1 human dermal microvascular endothelial cells (21) were cultured in MCDB131 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 m*M* glutamine (all from Invitrogen, Burlington, Canada), 1 μg/ml hydrocortisone (Sigma Chemical, Oakville, Canada), and 10 μg/ml epidermal growth factor (BD Collaborative Biomedical, Mississauga, Canada). Prior to treatments, cells were treated overnight with medium containing 1% serum and no supplements of growth factor or hormones. Cells were then treated with Ang-1 (R&D, Minneapolis, MN) and/or tumor necrosis factor α (TNF-α; R&D or Roche, Laval, Canada) prepared in the same medium with 1% serum.

Animal Model. 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. Syngeneic male Fisher344 rats (Charles River Co., St. Constant, Canada) were anesthetized (200 mg/kg ketamine, 10 mg/kg xylazine). Fibroblast cells were cultured using an explant technique from skin biopsy. Cells between the fifth and eighth passages were transfected with the null plasmid vector, pFLAG-CMV-1 (pFLAG), or the same plasmid vector containing the full-length cDNA for human Ang-1 (pAng-1) using Superfect (Qiagen, Mississauga, Canada). After 24 hrs, cells were trypsinized, washed, and suspended in Dulbecco's phosphate-buffered saline (PBS) for injection into the pulmonary circulation.

Male Fisher344 rats (250 ± 30 g) were anesthetized and randomly assigned to one of four experimental groups. The left exterior jugular vein was isolated by blunt dissection and cannulated with PE50 polyethylene tubing. Rats were injected with transfected fibroblasts (1.5×10^6 cells in 1 ml of Dulbecco's PBS) and allowed to recover in an incubator. Twenty-four hours after cell administration, rats were reanesthetized, orally intubated using a 14-gauge intravenous catheter, and subsequently received intratracheal instillation of LPS (from *Escherichia coli* 026:B6; 100 μg/kg body wt; 1 mg/ml) or an equivalent volume of saline. Six hours after LPS challenge, rats were reanesthetized and tracheotomized, and bronchoalveolar lavage was performed by gently inflating and deflating lungs with 1 ml of saline three times. Lavage total cells were counted using a hemocytometer, and the percentages of neutrophils, monocytes, and alveolar macrophages were determined by counting hematoxylin and eosin-stained cells on a smear. Lung tissue was then harvested and flash-frozen in liquid nitrogen for later reverse transcription–polymerase chain reaction (RT-PCR) analysis. In separate rats, lung tissue was then harvested by paraformaldehyde-inflation for paraffin embedding and subsequent histologic analysis. The average intra-alveolar septal thickness was quantified in hematoxylin and eosin-stained sections using ImageJ software (National Institutes of Health).

RNA Extraction and Quantitative RT-PCR (qRT-PCR). Total RNA was extracted from confluent cultures using the GenElute RNA Kit (Sigma Chemical), following the manufacturer's protocols. Total RNA was extracted from whole rat lung using Trizol extraction (Invitrogen) following the manufacturer's protocols. Fifty nanograms of RNA extracted from cell cultures or 2 μg of RNA extracted from rat lung and Omniscript Reverse Transcriptase (Qiagen) were used in RT reactions, which were terminated by heating at 85°C for 10 mins; samples were then diluted 1:10 in PCR-grade water.

Each diluted RT was then used in PCRs in duplicate to assess the RNA abundance of human ppET-1 (sense primer: 5′-GCT CGT CCC TGA TGG ATA AA-3′; antisense primer: 5′-CTG TTG CCT TTG TGG GAA GT-3′) and β-actin (sense primer: 5′-AGC CTC GCC TTT GCC GA-3′; antisense primer: 5′-CTG GTG CCT GGG GCG-3′ [22]) for cell culture experiments and rat ppET-1 (sense primer: 5′-GCT TCT ACA GTT TCT TGT TCA GAC-3′; antisense primer: 5′-GGA TGC AAA CGA AGA CAG GTT AGG-3′), rat and human Ang-1 (sense primer: 5′-GAG CTC CTT GAG AAT TAC ATT GTG G-3′; antisense primer: 5′-CGA GTT GAT TTA GTA CCT GGG TCT C-3′), and rat 18S (sense primer: 5′-GAC GAT CAG ATA CCG TCG TAG TTC-3′; antisense primer: 5′-GTT TCA GCT TTG CAA CCA TAC TCC-3′) for rat experiments, using the SYBR Green I PCR Master Mix and the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA). RTs and PCRs with various dilutions of total RNA were done in parallel to determine the amplification efficiencies (E) according to the equation:

$$E = 10^{[-1/\text{slope}]} \quad (23, 24),$$

where the slope is that of the standard curve plot (threshold cycle or crossing point [CP] vs. RNA input) generated by the ABI PRISM system. Relative ppET-1 RNA expression was then calculated in comparison to β-actin RNA expression for cell culture experiments using Pfaffl's formula:

$$\text{ratio of relative expression} = \frac{(E_{\text{ppET-1}})^{[\text{CPppET-1(untreated control)} - \text{CPppET-1(TNF-treated)}]}}{(E_{\beta\text{-actin}})^{[\text{CP}\beta\text{-actin(untreated control)} - \text{CP}\beta\text{-actin(TNF-treated)}]}} \quad (24).$$

The relative abundance of ppET-1 RNA in untreated control cells was expressed as 100% for comparative

purposes. Similarly, rat ppET-1 RNA expression and rat and human Ang-1 RNA expression were calculated in comparison to 18S for rat experiments. The relative abundance of rat ppET-1 and rat and human Ang-1 RNA in rats that received pFLAG-transfected fibroblast cells and intratracheal instillation of saline (pFLAG+Saline) was expressed as 100% for comparative purposes.

ET-1 Enzyme-Linked Immunosorbent Assay (ELISA). HMEC-1 cells were seeded in twelve-well plates and grown to confluence in 3–4 days. After overnight serum-starvation, medium in each well was changed to include Ang-1 and/or TNF-α. In each set of experiments, there was also one well with no cells and medium with no Ang-1 (blank). Medium in each well was collected after 6 or 24 hrs of incubation and was briefly centrifuged to remove floating cellular debris that was present. Supernatants were transferred to fresh Eppendorf tubes and stored frozen at –70°C. ET-1 ELISA was conducted using a kit from ALPCO Diagnostics (Salem, NH) following the manufacturer's instructions. For normalization purposes, cells in each well were lysed in 0.2 *N* NaOH and quantitated for protein content using a modified Lowry's Method (BioRad, Mississauga, Canada). ET-1 ELISA was also performed on precipitated bronchoalveolar lavage fluid collected from rat lung and spun at 3000 *g* for 20 mins at 4°C to remove any cellular debris.

Statistics. Data are represented as mean ± standard error of the mean. Differences between groups were assessed using analysis of variance (with *post hoc* comparisons using Student-Newman-Keuls test). A value of $P < 0.05$ was considered statistically significant.

Results

Effects of Ang-1 on ET-1 Expression in Cultured Endothelial Cells. ET-1 release from HMEC-1 cells, as determined by ELISA, was only marginally reduced by 250 ng/ml of Ang-1 after 6 hrs, but a marked reduction was observed after 24 hrs, displaying a dose-dependent suppression by as much as 30% (Fig. 1). Co-treatment with 100 U/ml of TNF-α for 24 hrs resulted in an increase in ET-1 release, which was also dose-dependently reduced by Ang-1 (Fig. 2). The expression of ppET-1 mRNA in cells treated with various doses of Ang-1 and TNF for 6 hrs was assessed by quantitative RT-PCR. Although ppET-1 RNA was not reduced by Ang-1 under basal conditions, Ang-1 dose-dependently attenuated the ET-1 induction by TNF-α. (Fig. 3).

Effect of Ang-1 Cell-Based Gene Therapy on Whole Lung Ang-1 mRNA. Ang-1 RNA expression was assessed in whole rat lung by quantitative RT-PCR analysis (Fig. 4). In rats treated with pFLAG null-transfected cells, intratracheal instillation of LPS produced a 50% decrease in total Ang-1 mRNA expression compared with saline controls ($P < 0.05$). In contrast, pretreatment with pAng-1–transfected cells restored Ang-1 expression to sham levels.

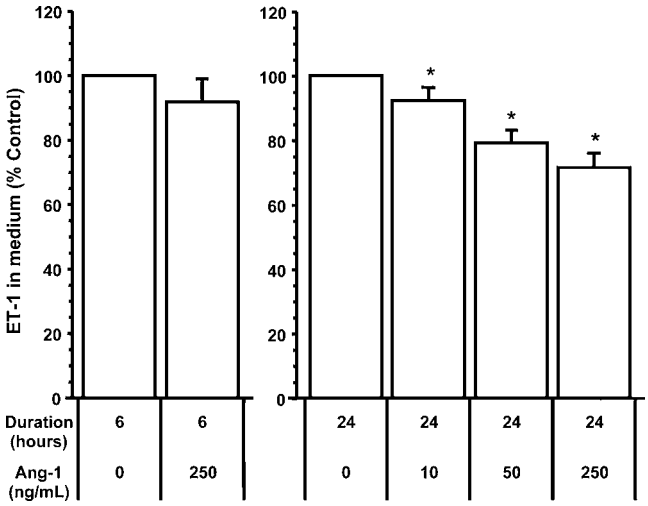

Figure 1. ET-1 release by HMEC-1 cells in the presence of Ang-1. HMEC-1 cells were treated with 10, 50, or 250 ng/ml of Ang-1 for 6 ($n = 5$) or 24 ($n = 11$) hrs, and culture medium samples were collected for ELISA. ET-1 contents in the medium were normalized to protein content of cell lysates collected from each well and were expressed as a percentage of that in control samples from untreated cells. (Means ± SEM. *, $P < 0.05$ vs. untreated control at the same time point.)

Effect of Ang-1 Cell-Based Gene Therapy on ET-1 Expression. ET-1 expression was assessed both by quantitative RT-PCR analysis on whole lung RNA and by ELISA on bronchoalveolar lavage fluid (Figs. 5 and 6). PreproET-1 RNA was increased 3-fold following LPS challenge, compared to saline in rats pretreated with

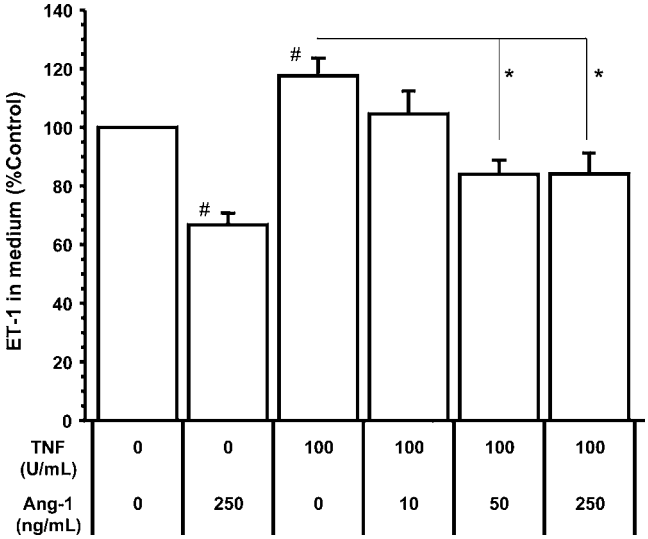

Figure 2. ET-1 release by HMEC-1 cells in the presence of Ang-1 and TNF-α. HMEC-1 cells were treated with 10, 50, or 250 ng/ml of Ang-1 with or without 100 U/ml of TNF-α for 24 hrs, and ET-1 contents in culture medium were assessed by ELISA. (Means ± SEM, $n = 6$. #, $P < 0.05$ vs. untreated control. *, $P < 0.05$ vs. samples treated with 100 U/ml TNF-α without Ang-1.)

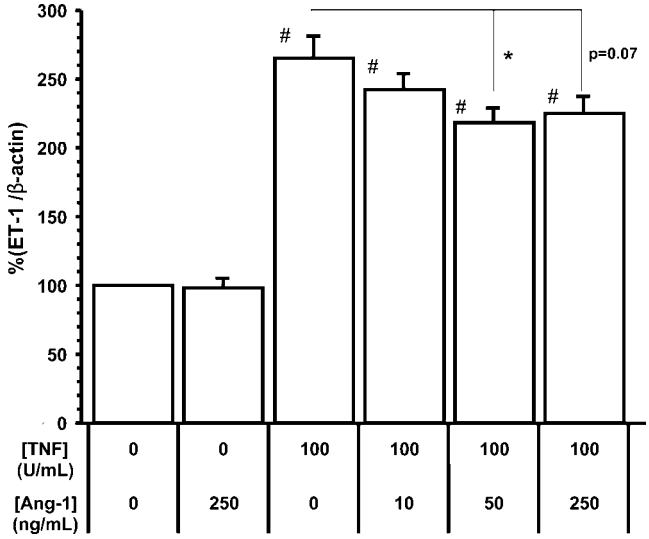

Figure 3. ppET-1 RNA levels in HMEC-1 cells treated with Ang-1 and TNF-α. HMEC-1 cells were treated with 10, 50, or 250 ng/ml of Ang-1 with or without 100 U/ml of TNF-α for 6 hrs, and ppET-1 RNA levels were determined by quantitative RT-PCR. (Means ± SEM, $n = 7$. #, $P < 0.05$ vs. untreated control; *, $P < 0.05$ vs. samples treated with 100 U/ml TNF-α without Ang-1.)

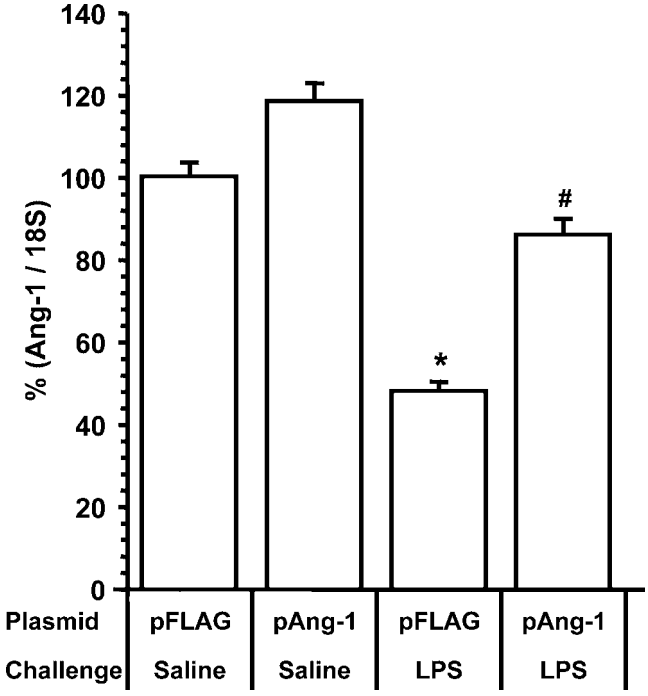

Figure 4. Ang-1 expression after cell-based gene transfer. Whole lung Ang-1 expression, as measured by RT-PCR and expressed relative to 18S, was significantly reduced following LPS challenge in rats that received pFLAG null-transfected cells. Ang-1 gene transfer restored Ang-1 RNA expression to levels consistent with control. (Mean ± SEM from $n = 8$ experiments per group; *, significantly different from pFLAG+Saline; #, significantly different from pFLAG+LPS).

pFLAG-transfected cells. Ang-1 gene transfer resulted in a 34% reduction in ppET-1 RNA compared with pFLAG in LPS-challenged rats ($P < 0.05$). ET-1 protein in lavage fluid was increased 5.6-fold following LPS challenge in rats pretreated with pFLAG-transfected cells. Lavage ET-1 protein was attenuated by 33% with pretreatment with pAng-1–transfected cells compared with pFLAG in LPS-challenged rats ($P < 0.05$).

Effect of Ang-1 Therapy on Lung Morphology Following LPS. Paraffin-embedded lung tissue samples were stained with hematoxylin and eosin and analyzed for morphologic differences. Representative histology is shown in Figure 7. Administration of pAng-1–transfected cells did not significantly alter lung morphology compared to pFLAG in rats that received intratracheal instillation of normal saline. The intra-alveolar septal thickness was 7.0 ± 0.2 μm in animals pretreated with pFLAG null-transfected cells, compared to 6.9 ± 0.2 μm in animals pretreated with pAng-1–transfected cells after intratracheal instillation of normal saline. Following LPS challenge, significant intra-alveolar septal thickening was observed as a result of edema formation and inflammatory cell infiltration. The average intra-alveolar septal thickness in rats pretreated with pFLAG-transfected cells and challenged with LPS was 16.8 ± 1.5 μm ($P < 0.05$). Pretreatment with pAng-1–transfected cells significantly reduced the intra-alveolar septal thickness compared with pFLAG in LPS-challenged rats to 10.1 ± 0.7 μm.

Effect of Ang-1 Therapy on Airspace Inflammation Following LPS. Airspace inflammation was assessed by cell fraction analysis of bronchoalveolar lavage (Table 1). Total cells in lavage were increased 4-fold following LPS challenge, compared with saline in rats that were pretreated with pFLAG-transfected cells ($P < 0.05$). These cells were predominantly neutrophils, but monocytes and alveolar macrophages were also present. Pretreatment with pAng-1–transfected cells resulted in a 43% reduction in total cells compared with pFLAG ($P < 0.05$). This decrease was attributed to a 45% reduction in neutrophils and a 64% reduction in monocytes ($P < 0.05$).

Discussion

Ang-1 clearly reduced the amount of ET-1 released into the culture medium from cultured human microvascular endothelial cells in a dose-dependent manner. The reduction in both protein and RNA levels indicates that Ang-1 actively downregulates the expression of the ET-1 gene. Moreover, the increase in ET-1 expression induced by the proinflammatory cytokine TNF-α (25) was suppressed by Ang-1, a result that is consistent with an important anti-inflammatory activity.

In a rat model of LPS-induced ARDS, rats were pretreated with the injection of pAng-1–transfected fibroblast cells into the pulmonary microcirculation. This cell-based angiopoietin-1 gene therapy restored whole lung Ang-1 RNA expression to levels consistent with controls and resulted in the attenuation of ET-1 expression in the rat lung

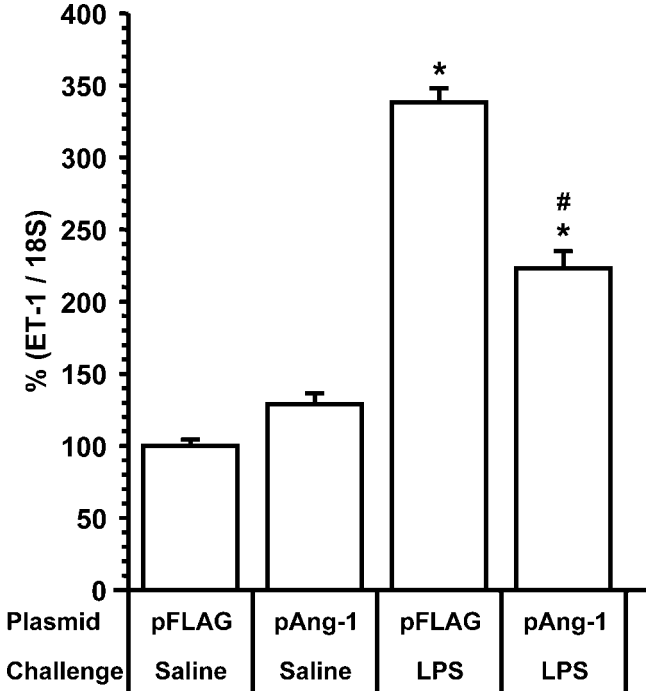

Figure 5. ET-1 expression after cell-based Ang-1 gene transfer. Whole lung ET-1 expression, as measured by qRT-PCR and expressed relative to 18S, was significantly increased following LPS challenge in rats that received pFLAG null-transfected cells. Cell-based Ang-1 gene transfer restored attenuated RNA expression by 34%. (Mean ± SEM from $n = 8$ experiments per group; *, significantly different from pFLAG+Saline; #, significantly different from pFLAG+LPS.)

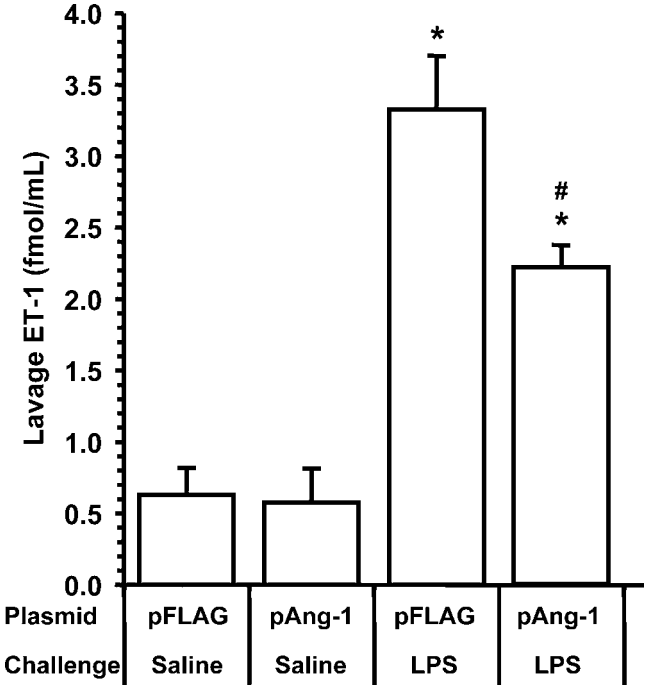

Figure 6. ET-1 secretion after cell-based Ang-1 gene transfer. ET-1 protein in bronchoalveolar lavage fluid, as measured by ELISA, was significantly increased following LPS challenge in rats that received pFLAG null-transfected cells. Cell-based Ang-1 gene therapy restored attenuated lavage ET-1 protein by 33%. (Mean ± SEM from $n = 8$ experiments per group; *, significantly different from pFLAG+Saline; #, significantly different from pFLAG+LPS.)

and lavage fluid. As a result, there was decreased airspace inflammation, as evidenced by a decrease in total cells, neutrophils, and monocytes in bronchoalveolar lavage and by a decrease in the intra-alveolar septal thickness 6 hrs after LPS challenge. These results are in contrast to those of previous reports utilizing the dual ET-1 receptor antagonist bosentan or selective ET_B receptor antagonists in animal models of ARDS. While ET-1 receptor antagonists improve pulmonary artery pressure (6) and pulmonary vascular leak (4, 26), the effects on airspace inflammation were not explored in these reports. In addition, it was reported that bosentan and a selective ET_B receptor antagonist reduced airspace neutrophil accumulation but increased airspace mononuclear cells, for no net change in total lavage cells (5).

It remains to be determined to what extent the decrease in airspace inflammation induced by Ang-1 cell-based gene therapy was attributable to decreased ET-1 expression or whether the decrease in ET-1 is secondary to reduced lung inflammation by Ang-1. Moreover, the mechanisms by which ET-1 is reduced by Ang-1 remain to be elucidated. The *in vitro* data showed that Ang-1 directly downregulated endothelial ET-1 expression and release; however, the

Table 1. Cells in Bronchoalveolar Lavage Following Ang-1 Therapy[a]

	Total Cells (10^6 cells/ml)	Neutrophils (10^6 cells/ml)	Monocytes (10^4 cells/ml)	Macrophages (10^5 cells/ml)
pFLAG+Saline	2.5 ± 0.6	1.9 ± 0.4	5.5 ± 2.9	5.6 ± 1.9
pAng-1+Saline	2.4 ± 0.3	2.0 ± 0.3	4.0 ± 1.0	3.4 ± 0.7
pFLAG+LPS	10.5 ± 1.7 [b]	10.1 ± 1.6 [b]	27.5 ± 4.7 [b]	1.6 ± 0.3
pAng-1+LPS	6.0 ± 0.7 [c]	5.6 ± 0.8 [c]	9.5 ± 2.0 [c]	2.5 ± 1.6

[a] The total number of cells in lavage, including neutrophils and monocytes, was increased following LPS challenge in rats that received pFLAG null-transfected cells. Ang-1 gene therapy reduced the total number of cells, neutrophils and monocytes, compared with pFLAG in LPS-challenged rats. The number of macrophages in lavage was not significantly different between all experimental groups. (Mean ± SEM from $n=$ 8 experiments per group.)
[b] Significantly different from pFLAG+Saline.
[c] Significantly different from pFLAG+LPS.

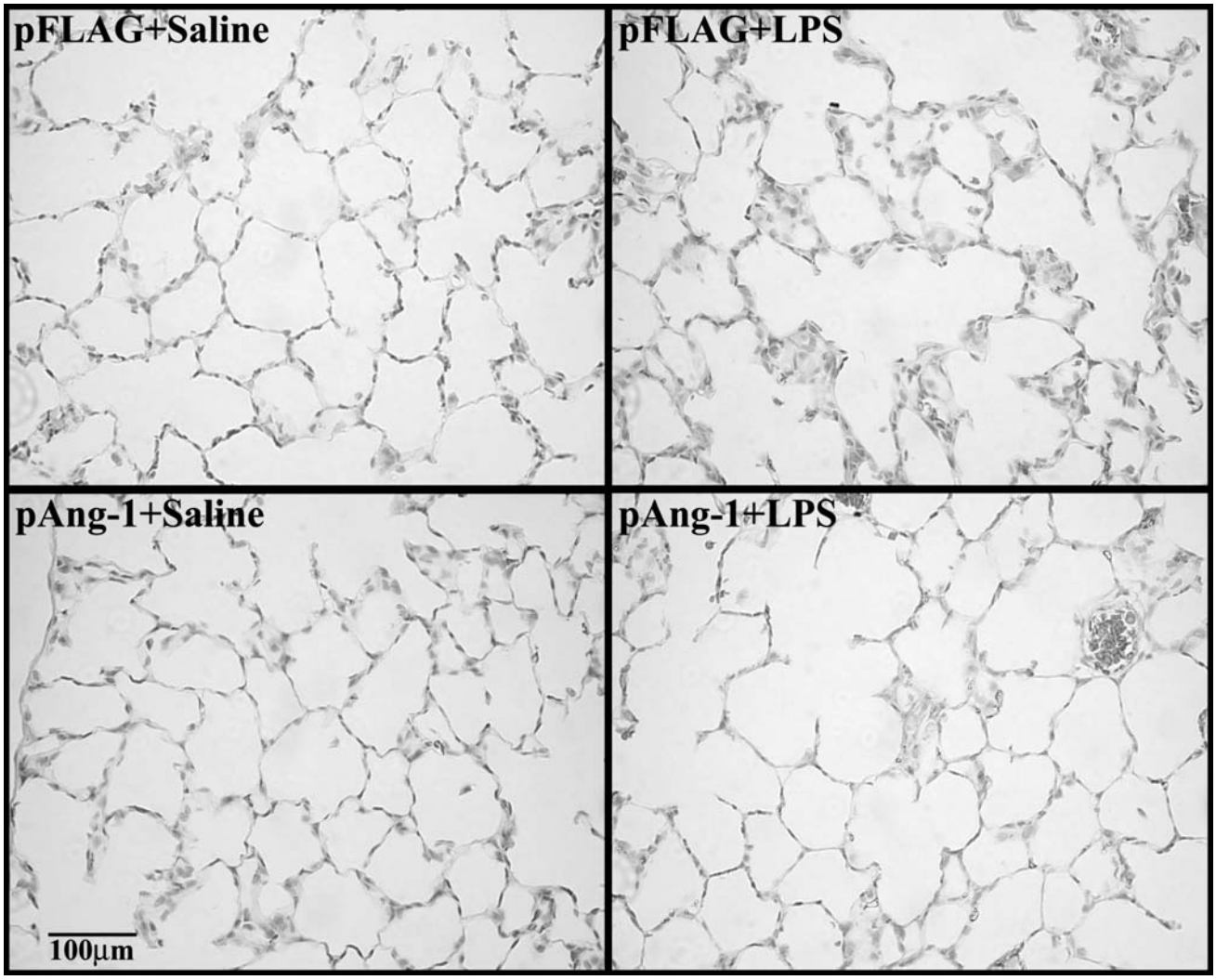

Figure 7. Representative lung histology following Ang-1 therapy. Paraffin-embedded tissue sections were stained with hematoxylin and eosin. There was a significant increase in intra-alveolar septal thickness following LPS challenge in rats that received pFLAG null-transfected cells. Pretreatment with pAng-1–transfected cells reduced the intra-alveolar septal thickness compared with pFLAG in LPS-challenged rats.

decrease in ET-1 observed *in vivo* may also be due to a reduction in leukocyte infiltration, since inflammatory cells are an important source of ET-1. Importantly, ET-1 has been implicated in the pathophysiology of ARDS (27), and both bosentan and ET_B receptor antagonists were unable to reduce airspace cell accumulation in ARDS (5). It may be postulated that cell-based gene transfer of Ang-1 provides protection to the lung over and above that obtained by blocking ET-1 alone, or it may be postulated that the reduction in ET-1 expression from cell-based gene transfer of Ang-1 is more effective in countering inflammation than currently available ET-1 receptor antagonists. These questions are being addressed in ongoing studies.

The data presented here are consistent with an important anti-inflammatory role for Ang-1 in both cell culture conditions and in experimental ARDS *in vivo*, and they indicate that the anti-inflammatory properties of Ang-1 may be attributable to downregulation of ET-1. Importantly, cell-based gene transfer of Ang-1 may provide a novel treatment strategy for ARDS by attenuating vascular inflammation *via* a reduction in ET-1.

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