CARDIOTHORACIC TRANSPLANTATION

IN VIVO ADENOVIRUS-MEDIATED ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE TRANSFER AMELIORATES LUNG ALLOGRAFT ISCHEMIA-REPERFUSION INJURY

Takashi Suda, MD^a
Bassem N. Mora, MD^a
Franco D'Ovidio, MD^a
Jonas A. Cooper, BS^a
Masafumi Hiratsuka, MD^a
Wanjiang Zhang, MD^b
T. Mohanakumar, PhD^b
G. Alexander Patterson, MD, FRCS(C)^a

Objective: Nitric oxide regulates vascular tone, inhibits platelet aggregation, and inhibits leukocyte adhesion, all of which are important modulators of ischemia-reperfusion injury. This study aimed to determine the effects of endothelial constitutive nitric oxide synthase gene transfer on ischemia-reperfusion injury in a rat lung transplant model. **Methods:** In group I, donor animals were injected intravenously with 5×10^9 pfu of adenovirus-encoding endothelial constitutive nitric oxide synthase. Groups II and III served as controls, whereby donor animals were injected with either 5×10^9 pfu of adenovirus encoding β -galactosidase or saline solution, respectively. Twenty-four hours after injection, left lungs were harvested and preserved for 18 hours at 4°C, then implanted into isogeneic recipients, which were put to death 24 hours later. Recombinant endothelial constitutive nitric oxide synthase gene expression was evaluated by Western blotting and immunohistochemistry. Lung grafts were assessed by measuring arterial oxygenation, myeloperoxidase activity, and wet/dry weight ratios. **Results:** Western blotting confirmed the overexpression of endothelial constitutive nitric oxide synthase in lungs so transfected compared with controls. Twenty-four hours after reperfusion, mean arterial oxygenation was significantly improved in group I compared with group II and III controls (189.4 ± 47.1 mm Hg vs 71.7 ± 8.9 mm Hg and 67.8 ± 12.2 mm Hg, $P = .02$, $P = .01$, respectively). Myeloperoxidase activity, a reflection of tissue neutrophil sequestration, was also significantly reduced in group I compared with groups II and III (0.136 ± 0.038 Δ OD/mg/min vs 0.587 ± 0.077 and 0.489 ± 0.126 Δ OD/mg/min, $P = .001$, $P = .01$, respectively). **Conclusion:** Adenovirus-mediated gene transfer with endothelial constitutive nitric oxide synthase ameliorates ischemia-reperfusion injury as manifested by significantly improved oxygenation and decreased neutrophil sequestration in transplanted lung isografts. Endothelial constitutive nitric oxide synthase gene transfer may reduce acute lung dysfunction after lung transplantation. (J Thorac Cardiovasc Surg 2000; 119:297-304)

Advances in techniques to introduce exogenous genetic material into mammalian somatic cells have laid the foundation to treat inherited and acquired dis-

ease at a genetic level. In the TMC Worldwide Gene Therapy Enrollment Report,¹ more than 2100 patients received gene therapy in 1996 (more than 1700 of these

From the Division of Cardiothoracic Surgery,^a the Department of Surgery,^b Washington University School of Medicine, Barnes-Jewish Hospital, St Louis, Mo.

Supported by National Institutes of Health grant 1 R01 HL41281.

Read at the Twenty-fifth Annual Meeting of The Western Thoracic Surgical Association, Olympic Valley (Lake Tahoe), Calif, June 23-26, 1999.

Received for publication June 29, 1999; revisions requested Aug 19, 1999; revisions received Sept 17, 1999; accepted for publication Sept 21, 1999.

Address for reprints: G. Alexander Patterson, MD, Division of Cardiothoracic Surgery, Washington University School of Medicine, One Barnes-Jewish Hospital Plaza, 3108 Queeny Tower, St Louis, MO 63110.

Copyright © 2000 by Mosby, Inc.

0022-5223/2000 \$12.00 + 0 12/6/103294

in the United States), in comparison with 1062 in December 1995. This increase of more than 1000 patients in a year reflects the worldwide enthusiasm for using gene therapy to treat various human diseases.^{1,2} Gene transfer into the lung has the potential to be a powerful treatment for pulmonary disease. In lung transplantation, gene transfer into the donor lung graft is feasible and may be useful in reducing ischemia-reperfusion injury and rejection. Recently, our laboratory has demonstrated the beneficial effects of gene transfer in ischemia-reperfusion injury and acute rejection in a rat lung graft model using adenovirus vectors, cationic lipid vectors, or naked plasmid.³⁻⁵

The transplanted lung has been shown to express transgene products when the vector was administered either endobronchially or intravenously.^{3,5} With an adenoviral vector delivered intravenously in vivo, no evidence of recombinant β -galactosidase reporter gene expression was observed by either gross or microscopic examination of the lungs.⁶ However, we³ recently demonstrated that β -galactosidase reporter gene expression was detectable, albeit in a small percentage of cells, in the endothelium of pulmonary vessels after intravenous adenovirus transfection.

Nitric oxide (NO), a potent vasodilator, is a free radical gas synthesized from L-arginine by NO synthases. NO regulates vascular tone, inhibits smooth muscle cell proliferation and migration, inhibits platelet aggregation, inhibits leukocyte adhesion, and scavenges superoxide anions.⁷ In experimental lung transplantation, we have previously demonstrated that inhaled NO reduces lung graft reperfusion injury when administered to the donor before harvest⁸ or to the recipient after transplant.⁹ We have also demonstrated the beneficial effect of inhaled NO in human allograft reperfusion injury.¹⁰ Therefore overexpression of endothelial constitutive NO synthase (ecNOS) in transplanted lung grafts might reduce subsequent lung graft ischemia-reperfusion injury. The aims of this study were to use an in vivo adenoviral ecNOS gene transfection strategy to accomplish ecNOS overexpression in rat lung isografts and to determine the effect of ecNOS on subsequent ischemia-reperfusion injury in a rat lung isograft model.

Materials and methods

The first group of experiments (nontransplant setting, groups E-I, E-II, and E-III) aimed to demonstrate in vivo intravenous gene delivery and to confirm gene expression. The second group of experiments (transplant setting, groups I, II, and III) aimed to evaluate the effect of ecNOS gene transfection in a rat lung isograft model after 18 hours of cold preservation.

Animals. F344 rats (Harlan Sprague-Dawley Inc, Indianapolis, Ind), weighing 250 to 270 g, were used in all experiments. All animal procedures were approved by the Animal Studies Committee at Washington University. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 85-23, revised 1985).

Adenoviral vectors. AdCMVNOS is a replication-deficient adenoviral vector encoding the ecNOS gene, driven by the cytomegalovirus immediate early promoter, as described in detail elsewhere.¹¹ It was provided as a gift by Dr Timothy O'Brien (Divisions of Endocrinology and Metabolism Medicine, Mayo Clinic, Rochester, Minn).

First-generation replication-deficient adenovirus serotype 5 carrying the *Escherichia coli* LacZ gene encoding for β -galactosidase and driven by the constitutive cytomegalovirus promoter (AdCMV β -gal) served as controls. It was provided as a gift from the Gene Therapy Center at the University of North Carolina Chapel Hill, North Carolina.

Adenoviral amplification was done by propagation in 293 cells to obtain high titer stocks, as determined by the plaque assay (courtesy of Dr R. Jude Samulski and Dr Douglas McCarty, Gene Therapy Center Vector Core Facility, University of North Carolina, Chapel Hill, North Carolina). Purified viral aliquots were stored at -80°C in 10% glycerol buffered with 10 mmol/L Tris, 140 mmol/L NaCl, and 1 mmol/L MgCl_2 . Immediately before use, these stocks were thawed and diluted in 1 mL of sterile normal saline solution.

Experimental groups

Experiment 1 (nontransplant setting)

EXPRESSION OF ECNOS GENE (GROUPS E-I, E-II, AND E-III). These experiments were performed to demonstrate in vivo gene delivery and to confirm gene expression. The rats were divided into 3 groups (3 per group). In groups E-I and E-II, animals received by jugular injection 5×10^9 plaque-forming units (pfu) of adenovirus encoding ecNOS (group E-I) or β -galactosidase (group E-II). In group E-III, rats were injected with normal saline solution. Animals were killed 24 hours after injection and lung grafts were harvested. The expression of recombinant ecNOS gene was determined by immunohistochemistry and Western blot analysis as described below.

Experiment 2 (transplant setting)

EFFECTS OF ECNOS GENE TRANSFECTION (GROUPS I, II, AND III). Animals were randomly divided into 3 groups. Donor animals received 5×10^9 pfu of adenovirus encoding ecNOS (group I, $n = 6$), 5×10^9 pfu of adenovirus encoding β -galactosidase as adenoviral controls (group II, $n = 6$), or normal saline solution without adenovirus (group III, $n = 6$). Twenty-four hours after injection, organs were harvested by means of a standard technique described earlier³⁻⁵ and were preserved for 18 hours at 4°C . Orthotopic left lung transplants were then performed in isogeneic recipients. Recipients were killed to assess the lung function 24 hours later.

Rat lung transplantation. Animals were anesthetized by

a subcutaneous injection of ketamine chloride (25 mg/kg) and atropine sulfate (0.25 mg/kg). After endotracheal intubation with a 14-gauge catheter, the animals' lungs were mechanically ventilated with a small-animal Harvard ventilator (Harvard Apparatus Co, Inc, S Natick, Mass) (tidal volume: 2.5 mL; respiratory rate: 60 breaths/min) with 0.5% halothane and 99.5% oxygen. A small incision was made in the left lower part of the neck and the left external jugular vein was mobilized. Normal saline solution (1 mL), either alone (groups E-III, III) or containing 5×10^9 pfu of adenovirus encoding ecNOS (groups E-I, I) or β -galactosidase (groups E-II, II), was injected intravenously over a period of 20 minutes. The incision was then closed and the animals were allowed to recover from anesthesia.

Twenty-four hours after gene transfection, donor lungs were harvested as described below. In brief, after general anesthesia, mechanical ventilation, and systemic heparinization, donor rat lungs were flushed through the main pulmonary artery with 20 mL of cold (4°C) low-potassium dextran-1% glucose solution at 20 cm H₂O pressure. The heart-lung block was then removed with the lungs inflated at end-tidal volume. The left lung was stored for 18 hours at 4°C in low-potassium dextran-1% glucose solution until implantation. Recipient animals were anesthetized, intubated, and subjected to a left thoracotomy. The pulmonary vessels and bronchus were anastomosed by a modification of the previously described "cuff technique."¹² Ventilation and perfusion were restored and a temporary chest tube was placed, which was removed after recovery from anesthesia.

Immunohistochemistry. The labeled streptavidin biotin technique was used. Lungs were perfused through the pulmonary arterial trunk with 20 mL of normal saline solution and 20 mL of HistoChoice solution (Amresco, Solon, Ohio). The specimens were fixed in HistoChoice fixative for 24 hours at 4°C and embedded in paraffin wax. Tissue sections 7 μ m thick were cut on a microtome and mounted on slides. After deparaffinization, the sections were pretreated with Dako Target Retrieval Solution (Dako, Carpinteria, Calif) applied with steam at 95°C for 30 minutes and were preincubated with SuperBlock Blocking Buffer in TBS (Pierce Chemical Company, Rockford, Ill) for 30 minutes at room temperature. The sections were incubated with a mouse anti-ecNOS immunoglobulin G1 monoclonal antibody (Transduction Laboratories, Lexington, Ky) at 1:50 dilution in blocking solution (consisting of 10% SuperBlock, 10% normal goat serum in TBS/0.2% Triton X [Union Carbide, Danbury, Conn]) or without primary antibody in blocking solution at 4°C overnight. After unbound primary antibodies were washed off with TBS/0.2% Triton X solution, the sections were incubated with biotinylated goat anti-mouse immunoglobulin G antibody for 30 minutes, followed by incubation with alkaline phosphatase-conjugated streptavidin (Dako) for 30 minutes at room temperature. After washing with TBS and immerse detection buffer (consisting of 100 mmol/L Tris, 500 mmol/L NaCl, 50 mmol/L MgCl₂, pH 9.5), specific binding was detected by means of the BCIP/NBT substrate working solution (Vector Laboratories,

Burlingame, Calif) containing 5 mmol/L levamisole (Vector Laboratories) at room temperature for 10 minutes. The slides were then counterstained with nuclear fast red dye, dehydrated with graded alcohol and xylene, mounted, and covered with a coverslip.

Western blot analysis of ecNOS. The presence of ecNOS was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and Western immunoblotting as previously described.^{3,13} In brief, frozen specimens were homogenized in cold buffer A (consisting of 10 mmol/L Tris/HCl, pH 7.5; 250 mmol/L sucrose; and 0.1 mmol/L phenylmethylsulfonyl fluoride) using a ratio of 2 mL buffer A per gram of tissue. The homogenate was centrifuged at 30,000g for 10 minutes at 4°C. The supernatant was removed, rapidly frozen, and stored at -80°C until analysis. Protein content of the supernatant was measured by ultraviolet spectrophotometry. Thereafter, 20 μ g total protein was loaded in each lane. Proteins were separated by SDS/PAGE on 0.75-mm thick, 12.5% acrylamide gels. The proteins on the gel were immediately transferred to nitrocellulose membranes (Hybond C, Amersham, Bucks, United Kingdom) for Western blotting. The membrane was then incubated at room temperature for 1 hour with a mouse anti-ecNOS immunoglobulin G1 monoclonal antibody at 1:1500 dilution. After repeated washings, the membrane was incubated with peroxidase-labeled anti-mouse second antibody (at 1:1000 dilution at room temperature for 1 hour) and then with enhanced chemiluminescence detection reagent (Amersham International Ltd, Little Chalfont, Bucks, United Kingdom). The nitrocellulose paper was then exposed to Fujifilm Medical X-Ray film (Fuji Photo Film Co, Tokyo, Japan). Human endothelial lysate served as a positive control (Transduction Laboratories). The degree of ecNOS expression was semi-quantitatively evaluated with computed densitometry with the use of NIH image software (National Institutes of Health, Bethesda, Md).

Effects of ecNOS gene transfection. After 24 hours of reperfusion, recipient animals were reanesthetized by means of the donor technique described above, and their lungs then were mechanically ventilated with 100% oxygen. Median laparotomy-sternotomy was performed and the contralateral right hilum was clamped. The animals' lungs were then ventilated for 5 minutes with a tidal volume of 1.5 mL, respiratory rate of 100 breaths/min, and positive end-expiratory pressure of 1.0 H₂O to assess the function of the lung isograft by arterial blood gas analysis using blood samples obtained from the ascending aorta. After the animal was put to death, each left lung graft was divided into two specimens: the lower half was snap-frozen in liquid nitrogen for myeloperoxidase activity measurements, and the remaining upper half was weighed, dried at 80°C for 48 hours, then weighed again for calculation of the wet/dry weight ratio.

Myeloperoxidase activity. Quantitative myeloperoxidase activity was determined as previously described.^{3,14} Optical density (OD) was measured at 460 nm with a spectrophotometer (model PMQ II, Carl Zeiss, Oberkochen/Wuett,

Fig 1. Immunohistochemistry for eNOS gene expression in rat lungs. Strongly positive eNOS staining was observed in the vascular endothelium of eNOS-transfected lungs (**A**, 100 $\times$ magnification; **B**, 400 $\times$), but not in β -galactosidase-transfected lungs (**C**, 100 $\times$) or normal lungs (**D**, 100 $\times$).

Germany). Color development was linear from 5 to 20 minutes. One unit of enzyme activity was defined as the amount of 1.0 OD units per minute per milligram of tissue protein at room temperature.

Statistical analysis. Values are reported as mean $\pm$ standard error of the mean. Data were analyzed after logarithmic correction. One-way analysis of variance with pairwise comparison by the Fisher protected least significant difference method was used to compare overall differences among groups.

Results

Experiment 1 (nontransplant setting)

Immunohistochemistry of eNOS. Positive immunohistochemical staining for eNOS was observed in the endothelium of vessels in eNOS transfected lungs (Fig 1, A and B). This was not seen in lungs transfected with either β -galactosidase (Fig 1, C) or flushed with saline solution alone (Fig 1, D). Weak and patchy staining in airway epithelial cells was present in all 3 groups. No staining of smooth muscle, macrophages, or alveolar parenchyma was observed in any of the groups.

Western blot analysis of eNOS. Western blotting

identified a single band of protein from the detergent-extracted homogenate with an estimated molecular mass of 140 kDa for eNOS in all groups, corresponding to the endogenous eNOS present in rat lungs (Fig 2, top). At least 2-fold higher expression of eNOS protein was present in animals that had been transfected with eNOS (group E-I) than in the other 2 groups (Fig 2, bottom).

Experiment 2 (transplant setting)

Arterial blood gas analysis. Left lungs from group I, which had been transfected with eNOS, had better arterial oxygen (PaO_2) levels than groups II or III. A mean PaO_2 of 166.48 ± 38.3 mm Hg was obtained from group I animals compared with 71.7 ± 8.9 mm Hg for group II and 67.8 ± 12.2 mm Hg for group III. This difference between group I versus groups II or III was statistically significant with $P = .01$ for group I versus II and $P = .03$ for group I versus III.

Mean arterial carbon dioxide tensions were not significantly different in any of the groups (23.4 ± 5.3 , 29.8 ± 3.4 , and 23.3 ± 3.9 mm Hg for groups I, II, and III, respectively, $P > .5$).

Myeloperoxidase activity. Myeloperoxidase activity,

Fig 2. Western blot analysis of ecNOS in lung tissue. Lane 1 represents a positive control, lanes 2 to 4 represent group I (ecNOS-transfected lungs), lanes 5 to 7 represent group II (β -galactosidase-transfected lungs), and lanes 8 to 10 represent group III (saline controls). The histogram shows semiquantitative comparisons between these groups after measurement of relative densitometry. Each bar represents mean $\pm$ standard error of spectrophotometric analysis of blots with 3 samples within each group.

a reflection of tissue neutrophil sequestration, in lung homogenates was significantly reduced in group I compared with groups II and III. Myeloperoxidase activity in group I was $0.136 \pm 0.038 \Delta\text{OD}/\text{mg}/\text{min}$ versus $0.587 \pm 0.077 \Delta\text{OD}/\text{mg}/\text{min}$ for group II and $0.489 \pm 0.126 \Delta\text{OD}/\text{mg}/\text{min}$ for group III. This corresponded to $P = .001$ for group I versus II and $P = .01$ for group I versus III.

Wet/dry weight ratio. Mean wet/dry weight ratios in all groups were not significantly different. For group I, an average wet/dry weight ratio of 6.0 ± 0.6 was obtained, versus 6.1 ± 0.4 for group II and 6.2 ± 0.3 for group III ($P > .9$ for group I vs II and group I vs III).

Discussion

In clinical lung transplantation, significant graft dysfunction has been reported in 15% to 20% of cases.^{15,16} Early graft dysfunction is an acute lung injury accompanied by vascular permeability that is presumably related to ischemia-reperfusion injury. The exact pathophysiologic mechanisms of ischemia-reperfusion injury remain unclear. A variety of factors have been implicated in its pathogenesis, including activation of increased production of neutrophils and platelets, cytokines, and oxygen-derived free radicals.¹⁷⁻¹⁹

In normal situations, NO synthesized by constitutive NOS in endothelial cells inhibits leukocyte adhesion and platelet aggregation. However, after ischemia-reperfusion, ecNOS and NO production levels decrease, leading to increased leukocyte adherence to the endothelium. Various cytokines produced by these inflammatory cells induce inducible NOS in endothelial cells, smooth muscle cells, leukocytes, and macrophages, resulting in large quantities of NO.²⁰ This is known to react with superoxides produced after ischemia-reperfusion by endothelial cells, leukocytes, and macrophages and to react rapidly to form the stable peroxynitrate anion (ONOO^-). The cytotoxicity caused by ONOO^- is known to be one of the most important factors modulating ischemia-reperfusion injury.²¹

Several strategies have been shown to reduce ischemia-reperfusion injury of organs, such as the use of prostaglandins, nicorandil, and free radical scavengers.²²⁻²⁴ Recently, the use of inhaled NO has been reported to significantly reduce lung allograft ischemia-reperfusion injury presumably by altering vascular tone, inhibiting platelet aggregation, inhibiting leukocyte adhesion, and scavenging superoxide anions.^{8-10,25} In the present study, myeloperoxidase levels were significantly reduced in ecNOS-transfected

lungs. As myeloperoxidase activity reflects tissue neutrophil sequestration, ecNOS gene transfection may have supplemented the reduced NO production seen after ischemia-reperfusion, inhibited neutrophil adhesion, and thereby decreased ischemia-reperfusion injury. However, the improvement seen in lung oxygenation varied widely. In addition, there were no significant differences between groups in wet/dry lung ratio. This observation is perplexing and may be due to less than optimal gene transfection efficiency accomplished by this *in vivo* intravenous application. If the optimal degree of gene expression can be identified and accomplished by this *in vivo* approach, more efficient ecNOS gene transfection may produce more uniform reduction in reperfusion injury. The ischemia-reperfusion model we used in this experiment has been used by our group to investigate a variety of therapeutic strategies.^{3,4} It produces a uniformly severe injury in control animals. Perhaps a lesser degree of ischemia-reperfusion injury might have had a more consistent response to this transfection strategy.

We have previously reported that the *ex vivo* route (administered to the graft after harvest) is a more efficient means of lung graft transfection.²⁶ However, when this strategy is used, transgene expression is not observed for 1 to 2 hours after reperfusion. To have an impact on the events of reperfusion, we thought that the transgene must be present at the time of reperfusion. With presently available technology, that requirement mandates an *in vivo* approach. Western blot analysis confirmed the overexpression of ecNOS in ecNOS-transfected lungs. These results point to the feasibility of a donor-directed intravenous approach of gene transfection into pulmonary vascular cells. We have not studied the long-term effects of adenovirus encoding ecNOS or how long these effects will be present, because the transgene expression does not need to last a long period to prevent reperfusion injury. However, Champion and associates²⁷ used an *in vivo* study of the long-term effects and expression of AdCMVecNOS delivered in lungs.²⁷ In mice treated with AdCMVecNOS, the maximum augmentation of pulmonary vasodepressor response to bradykinin was observed 1 day after transfection, and the responses to bradykinin were significantly different from control responses 7 days after transfection. The augmented decrease in pulmonary arterial pressure in response to bradykinin returned to the baseline response level 7 to 14 days after transfection with AdCMVecNOS. Adenovirus-mediated cytotoxicity or inflammation has been discussed as a potential problem by other investigators.^{28,29} In our study, no inflammatory response was

seen in histologic sections stained with hematoxylin and eosin. However, we did not specifically investigate host cytotoxicity or immune responses to adenoviral administration.

In conclusion, systemic administration of a recombinant adenoviral vector encoding ecNOS gene resulted in increased ecNOS protein in lung grafts, which reduced lung isograft ischemia-reperfusion injury, as manifested by significantly improved oxygenation and decreased neutrophil sequestration. We believe that ecNOS gene transfer may reduce acute lung dysfunction after lung transplantation.

We thank Dr Timothy O'Brien (Divisions of Endocrinology and Metabolism Medicine, Mayo Clinic, Rochester, Minn) for kindly providing adenovirus encoding ecNOS. We also thank Kathleen Grapperhaus for technical assistance, Jill Manchester for assisting with the myeloperoxidase assay, Mary Ann Kelly and Dawn Schuessler for secretarial support, and Dr Hiroshi Sugimura for his assistance. Statistical advice was obtained from Richard B. Schuessler, PhD.

REFERENCES

1. Marcel T, Gausz JD. The TMC Worldwide Gene Therapy Enrollment Report, end 1996. *Hum Gene Ther* 1997;8:775-800.
2. Crystal RG. Transfer of genes to humans: early lessons and obstacles to success. *Science* 1995;270:404-10.
3. Hiratsuka M, Mora BN, Yano M, Mohanakumar T, Patterson GA. *In vivo* adenovirus-mediated gene transfer of heat shock protein 70 protects lung grafts from ischemia-reperfusion injury. *Ann Thorac Surg* 1999;67:1421-7.
4. Yano M, Mora BN, Ritter JM, Scheule RK, Yew NS, Mohanakumar T, et al. *Ex vivo* transfection of transforming growth factor β 1 gene to pulmonary artery segments in lung grafts. *J Thorac Cardiovasc Surg* 1999;117:705-13.
5. D'Ovidio F, Yano M, Ritter JH, Mohanakumar T, Patterson GA. Endobronchial transfection of naked TGF- β 1 cDNA attenuates acute lung rejection. *Ann Thorac Surg* 1999;68:1008-13.
6. Schachtner SK, Rome JJ, Hoyt RF Jr, Newman KD, Virmani R, Dichek DA. *In vivo* adenovirus-mediated gene transfer via the pulmonary artery of rats. *Circ Res* 1995;76:701-9.
7. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 1993;329:2002-12.
8. Fujino S, Nagahiro I, Triantafillou AN, Boasquevisque CH, Yano M, Cooper JD, et al. Inhaled nitric oxide at the time of harvest improves early lung allograft function. *Ann Thorac Surg* 1997;63:1383-9.
9. Okabayashi K, Triantafillou AN, Yamashita M, Aoe M, Cooper JD, Patterson GA. Inhaled nitric oxide reduces lung allograft reperfusion injury. *Surg Forum* 1994;155:276-8.
10. Date H, Triantafillou AN, Trulock EP, Pohl MS, Cooper JD, Patterson GA. Inhaled nitric oxide reduces human lung allograft dysfunction. *J Thorac Cardiovasc Surg* 1996;111:913-9.
11. Chen AFY, O'Brien T, Tsutsui M, Kinoshita H, Pompili VJ, Crotty TB, et al. Expression and function of recombinant endothelial nitric oxide synthase gene in canine basilar artery. *Circ Res* 1997;80:327-35.

12. Mizuta T, Kawaguchi A, Nakahara K, Kawashima Y. Simplified rat lung transplantation using cuff technique. *J Thorac Cardiovasc Surg* 1989;97:578-81.
13. Janssens SP, Bloch KD, Nong Z, Gerard RD, Zoldhelyi P, Collen D. Adenoviral-mediated transfer of the human endothelial nitric oxide synthase gene reduces acute hypoxic pulmonary vasoconstriction in rats. *J Clin Invest* 1996;98:317-24.
14. Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity: assessment of inflammation in rat and hamster models. *Gastroenterology* 1984;87:1344-50.
15. Haydock DA, Trulock EP, Kaiser LR, Ettingen NA, Triantifillou AN, Ochoa LL, et al. Lung transplantation: analysis of thirty-six consecutive procedures performed over a twelve-month period. *J Thorac Cardiovasc Surg* 1992;103:329-40.
16. Davis RD, Trulock EP, Manley J, Pasque MK, Sundaresan S, Cooper JD, et al. Differences in early results after single-lung transplantation. *Ann Thorac Surg* 1994;58:1327-35.
17. Mandfil G. ARDS, neutrophils, and pentoxifylline. *Am Rev Respir Dis* 1988;138:1344-50.
18. McCord JM. Oxygen-derived free radicals in post ischemic tissue injury. *N Engl J Med* 1985;312:159-63.
19. Quayumi AK, Jamieson WRE, Poostizadeh A. Effect of platelet-activating factor antagonist CV-3988 in preservation of heart and lung for transplantation. *Ann Thorac Surg* 1991;52:1026-32.
20. Ma XL, Weyrich AS, Lefer DJ, Lefer AM. Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circ Res* 1993;72:403-12.
21. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implication for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 1990;87:1620-4.
22. Aoe M, Trachiotis GD, Okabayashi K, Manchester JK, Lowry OH, Cooper JD, et al. Administration of prostaglandin E₁ after lung transplantation improves early graft function. *Ann Thorac Surg* 1994;58:655-61.
23. Yamashita M, Schmid RA, Fujino S, Cooper JD, Patterson GA. Nicorandil, a potent adenosine triphosphate-sensitive potassium-channel opener, ameliorates lung allograft reperfusion injury. *J Thorac Cardiovasc Surg* 1996;112:1307-14.
24. Lambert CJ, Eagan TM. Optimal timing of administration of a free radical scavenger in lung preservation. *Transplantation* 1992;54:205-9.
25. Adatia I, Lillehei C, Arnold JH, Thompson JE, Palazzo R, Fackler JC, et al. Inhaled nitric oxide in the treatment of postoperative graft dysfunction after lung transplantation. *Ann Thorac Surg* 1994;57:1311-8.
26. Boasquevisque CH, Mora BN, Schmid RA, Lee TC, Nagahiro I, Cooper JD, et al. Ex vivo adenoviral-mediated gene transfer to lung isografts during cold preservation. *Ann Thorac Surg* 1997;63:1556-61.
27. Champion HC, Bivalacqua TJ, D'Souza FM, Ortiz LA, Jeter JR, Toyoda K, et al. Gene transfer of endothelial nitric oxide synthase to the lung of the mouse in vivo. *Circ Res* 1999;84:1422-32.
28. Crystal RG, McElvaney NG, Rosenfeld MA, Chu CS, Mastrangeli A, Hay JG, et al. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nat Genet* 1994;8:42-51.
29. Knowles MR, Hohneker KW, Zhou Z, Olsen JC, Noah TL, Hu

PC, et al. A controlled study of adenoviral-vector-mediated gene transfer in the nasal epithelium of patients with cystic fibrosis. *N Engl J Med* 1995;333:823-31.

Discussion

Dr Vaughn Starnes (*Los Angeles, Calif*). Dr Suda and associates are to be congratulated on a very fine experimental model. I would like to summarize it a bit for the association. Basically, he had 3 experimental groups. One received the ecNOS adenovirus vector to produce endogenous NO to ameliorate the effects of ischemic reperfusion. The second group was designed to look at the reporter gene to assess the transfection efficiency. A third group was basically saline control. The hypothesis was that administration of the ecNOS gene could increase endogenous synthesis of NO, therefore limiting the injury that can occur after reperfusion ischemic injury. Dr Suda, if that is the hypothesis, did you actually measure endogenous ecNOS before ischemia such that you have a baseline?

Dr Suda. The Western blot and immunohistochemistry studies were performed before reperfusion. Therefore the results in the control groups show endogenous ecNOS.

Dr Bryan F. Meyers (*St Louis, Mo*). I am one of Alec Patterson's colleagues and am backing up Dr Suda in Dr Patterson's absence. The Western blot and the immunohistochemical results that were shown were obtained 24 hours after transfection but before cold storage and reimplantation. I think the missing information you are looking for is actually the immunohistochemical and the Western blotting results after the cold storage and insertion to show that there is a diminished ecNOS in the nontreated animals. The Western blot and immunohistochemistry that he showed were in the nonischemic animals. The studies were done 24 hours after transfection, just before those lungs became graft lungs.

Dr Starnes. As you mentioned, the oxygenation and myeloperoxidase data that would suggest decreased leukocyte sequestration in the lung were quite variable. They seem to suggest benefit from the adenoviral transfection of ecNOS into the graft. Was this variability due to a transfection efficiency, or was it due to effects of cold storage on the vector itself? In other words, did you think the number of endothelial cells were 30% transfected, 20% reliably, 100%, or could it have been that they were variably transfected?

Dr Suda. The variability of results may be due to less than optimal gene transfection efficiency accomplished by this in vivo intravenous application. I do not know the number of endothelial cells that were transfected.

Dr Starnes. If you had a group that had the reporter gene in it, β -galactosidase marker, why did you not give us that information as staining data to provide some idea of the transfection efficiency after cold storage?

Dr Suda. Our previous study, reported by Dr Hiratsuka, demonstrated that β -galactosidase reporter gene expression was detectable in the endothelium in pulmonary vessels, so we did not perform that in this study.

Dr Meyers. Just to amplify, one of Dr Suda's coauthors on this paper, Dr Hiratsuka, is publishing an article in *The*

Annals of Thoracic Surgery, which evaluates a very similar laboratory model looking at heat shock protein. He also used β -galactosidase as a control and used the blue-gal assay after ischemia and reperfusion to show the transfection of the pulmonary endothelial cells with β -galactosidase. There was diffuse staining throughout the pulmonary vasculature, although it is fairly spotty, and I cannot recall the actual percent of cells that had the blue-gal assay.

Dr Starnes. Given the concern about systemic effects of intravenously administered adenoviral vectors, did you consider administering this by the endobronchial route, that is, by aerosolizing vector into the lung grafted cell?

Dr Suda. Because it is difficult to transfect the pulmonary endothelium through airway administration, I think that is not a suitable method for ischemia-reperfusion injury. I have never heard of a successful functional study in lung transplantation for ischemia-reperfusion injury in which the airway was used, so we chose intravenous administration to transfect the endothelium of the pulmonary vessels.

Dr Starnes. These adenoviral vectors are not like antibiotics. They can transfect and they can infect all sorts of organs as they are administered, so the fact that you were able to record it and transfect the lung tissue also means you probably were able to transfect the kidney and the heart and anywhere else you had endothelium. As we use these vectors, they are going to have to be targeted in some way to the specific organ of interest. That is the reason I ask the question.

Dr Meyers. Dr Hiratsuka's article also showed that in the animals that were pretreated 24 hours in advance of lung donation, there was staining in the kidneys and in the liver. This sort of treatment would be unacceptable for multiorgan donors in a human situation. One of the limitations of gene therapy in organs that are about to be put in cold storage is that you need to have them being expressed at the time of cold storage for there to be an effect immediately on reperfusion. Given the constraints that Dr Suda and his group had regard-

ing the available vectors and genes to transfect, this was their best compromise.

Dr Michael Grosso (*Browns Mills, NJ*). I want to congratulate you but also the Association for continuing to support basic science in this age of managed care. Given Dr Starnes' comments, since it does require 24 hours before harvest to transfect these, where do you see us heading in terms of being able to overcome these obstacles in the clinical setting?

Dr Suda. In previous studies, gene expression was present as early as 6 hours after intravenous injection, and it significantly increased after 24 hours; however, clinical extended exposure time may not be suitable in the setting of organ transplantation. Therefore we chose this time frame to demonstrate the effectiveness of gene transfection for eNOS within the setting of ischemia-reperfusion injury. We did not test shorter time frames. More efficient vectors and systems for gene therapy will possibly permit shorter time frames to accomplish significant levels of transgene protein expression.

Dr Grosso. The literature is beginning to suggest, at least at the laboratory level, that lung ischemia reperfusion may be more responsible for bronchiolitis obliterans and graft failure over the long term in these transplants than actual chronic rejection. How do you feel about that?

Dr Meyers. I do not think he can answer that question based on the work he has done for this paper.

Dr Michael Jaklitsch (*Boston, Mass*). β -Galactosidase, although very useful in in vitro models, has numerous pitfalls in vivo. In particular, mast cells will artificially express the gene product for β -galactosidase. Many laboratories that are working in this field have turned to the luciferase gene to try to avoid these problems in the in vivo model. I would refer you to an article in *Circulation* in 1993, which shows the problem of β -galactosidase in an in vivo rabbit model.

Dr Meyers. Dr Suda was somewhat handcuffed by what was available in the laboratory at the time. I think that on the basis of today's experience he has the next few months of experiments lined up.