Recipient intramuscular cotransfection of naked plasmid transforming growth factor β_1 and interleukin 10 ameliorates lung graft ischemia-reperfusion injury

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Objective: Multiple gene transfer might permit modulation of concurrent biochemical pathways involved in lung graft ischemia-reperfusion injury. In this study we analyzed whether recipient intramuscular naked plasmid cotransfection of transforming growth factor β_1 and interleukin 10 would result in amelioration of lung graft ischemia-reperfusion injury.

Methods: Forty-eight hours before transplantation, 6 groups (n = 6) of F344 rats received intramuscular injection of naked plasmid encoding chloramphenicol acetyltransferase, chloramphenicol acetyltransferase plus β -galactosidase, transforming growth factor β_1 , interleukin 10, or transforming growth factor β_1 plus interleukin 10 or were not treated. Donor lungs were flushed and stored for 18 hours at 4°C before transplantation. Twenty-four hours later, grafts were assessed immediately before the animals were killed. Arterial oxygenation, wet/dry ratio, myeloperoxidase, and proinflammatory cytokines (interleukin 1, tumor necrosis factor α , interferon γ , and interleukin 2) were measured, and immunohistochemistry was performed.

Results: For lung graft function, the arterial oxygenation was considerably higher in the cotransfected group receiving transforming growth factor β_1 plus interleukin 10 compared with that in all other groups ($P \leq .03$). The wet/dry ratio, reflecting lung edema, was reduced in the cotransfected group compared with that in control animals (nontreated, $P < .02$; chloramphenicol acetyltransferase, $P < .03$; chloramphenicol acetyltransferase plus β -galactosidase, $P < .01$). Myeloperoxidase, which measures neutrophil sequestration, was also reduced with cotransfection compared with that seen in control animals ($P \leq .03$). All proinflammatory cytokines were decreased in the cotransfected group compared with those in all other groups (interleukin 1 β , $P < .04$; tumor necrosis factor α , $P < .002$; interferon γ , $P < .0001$; interleukin 2, $P < .03$). These results indicate that cotransfection provides a synergistic benefit in graft function versus either cytokine alone, neutrophil sequestration, or inflammatory cytokine expression. Immunohistochemistry showed positive staining of transforming growth factor β_1 plus interleukin 10 in type I and II pneumocytes and localized edema fluid.

Conclusions: Recipient intramuscular naked plasmid cotransfection of transforming growth factor β_1 and interleukin 10 provides a synergistic effect in ameliorating lung reperfusion injury after prolonged ischemia.

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Gene therapy has evolved as a potential new alternative therapy for several pathologic conditions, such as autoimmune diseases¹ and malignant tumors.² It also represents a new modality to modulate ischemia-reperfusion (I/R) injury in lung transplantation. Severe graft dysfunction caused by I/R injury occurs in 10% to 20% of lung transplant recipients and results in progressive hypoxemia, decreased pulmonary compliance, and high permeability pulmonary edema, which often are associated with a need for prolonged intensive care.^{3,4} I/R injury leading to severe graft dysfunction is also the most common cause for mortality within the first month after lung transplantation.³

The inflammatory cascade of I/R injury is a dynamic combination of events. During the period of ischemia before reperfusion, the classical complement pathway is activated, and macrophages are attracted and release preformed cytokines, such as tumor necrosis factor α (TNF- α).⁵ These cytokines in turn increase the expression of neutrophil adhesion molecules on endothelial cells.^{5,6} Neutrophils then contribute to I/R injury by generating oxygen-derived free radicals, releasing proteases, and secreting arachidonic acid metabolites and other proinflammatory mediators.⁷

Interleukin 10 (IL-10) is a potent inhibitor of inflammatory cytokine production. It is normally produced by macrophages and T lymphocytes.⁸ Two targets of IL-10 inhibition are TNF- α and interferon γ (IFN- γ). Because macrophage cytotoxicity is induced by IFN- γ and requires TNF- α as a costimulatory signal, IL-10 modulates macrophage activity by decreasing TNF- α .⁹ Furthermore, IL-10 strongly inhibits natural killer cells that produce IFN- γ , leading to decreased IFN- γ production. Thus, by decreasing TNF- α and IFN- γ , IL-10 reduces macrophage cytotoxicity and plays a role in decreasing I/R injury.⁹

Transforming growth factor β 1 (TGF- β 1) is a pleiotropic protein with unique and potent immunoregulatory properties produced by several cells, including lymphocytes and macrophages.¹⁰ It works in concert with IL-10 to block the activation of macrophages and T helper cells.^{11,12} For example, both TGF- β 1 and IL-10 enhance production of the interleukin 1 (IL-1) receptor antagonist, a potent anti-inflammatory mediator.¹⁰

Several approaches using the delivery of gene vectors have been successful by producing transgene expression in experimental studies for lung transplantation.¹³⁻¹⁹ Transfection of viral vectors encoding heat shock protein 70, IL-10, and endothelial nitric oxide synthase has been shown to decrease lung graft I/R injury.¹⁴⁻¹⁶ Intramuscular injection of nonviral vectors encoding TGF- β 1 has also been shown to attenuate the proinflammatory response in I/R injury.¹⁷

In this study we demonstrate successful transgene expression after intramuscular naked plasmid gene transfer of

viral IL-10 (vIL-10) and TGF- β 1 and show that their cotransfection results in amelioration of lung graft I/R injury.

Material and Methods

Animals

Male F344 rats (Harlan Sprague Dawley Inc, Indianapolis, Ind) weighing between 230 and 250 g were used in all experiments. Protocols 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 (National Institutes of Health publication No. 85-23, revised 1996).

Plasmids

The plasmid viral IL-10 (pCMVieVIL-10), kindly provided by Dr Jonathan Bromberg (Mount Sinai School of Medicine, New York, NY), consists of a human cytomegalovirus immediate-early promoter and enhancer followed by the tripartite leader from Epstein-Barr virus, a hybrid intron constituted by IL-10 (BCRF1-vIL10), and a polyadenylation signal from bovine growth hormone. Endotoxin units tested were lower than 1 EU/mL (EU < 1).

The plasmid TGF- β 1 (pMP6A-TGF β 1-Active) was created in our laboratory, encoding the rodent active fragment of TGF- β 1 by mutated cysteine in serine in position 223 and 225 of the TGF- β 1 cDNA. The mutated TGF- β 1 cDNA, kindly provided by Dr Debra A. Hullett (Department of Surgery, University of Wisconsin, Madison, Wis), was amplified by means of polymerase chain reaction with Ampli-TAQ polymerase (Perkin Elmer Applied Biosystems, Foster City, Calif) from an adenovirus encoding his sequence. Primers for the amplification were constructed with *Nhe*I terminals. The amplified cDNA was then cloned in the *Nhe*I cloning site of the adeno-associated plasmid pMP6A with a cytomegalovirus initial promoter and an SV40 polyadenylation signal sequence, also kindly provided by Dr Jonathan Bromberg. Correct orientation was checked by means of *Bam*HI digestion. Adequate primers were then constructed for the sequencing of the mutated TGF- β 1 cDNA by the Protein Chemistry and Nucleic Acid Laboratory at Washington University. Purity of the plasmid after mega preps (Endo-free Mega Kit; Quiagen Inc, Valencia, Calif) was tested at Charles River Laboratories (Charleston, SC), and the endotoxin units were approximately 10 EU/mL (EU > 10).

Study Design

Nontransplant setting. Thirty-six rats divided into 4 groups (n = 3 each for 3 time points) received 1000 μ g of vIL-10, mutated TGF- β 1, or both by means of separate injections of 1 mL in each gastrocnemius muscle. Plasmids were suspended in 0.9% saline solution, and control groups were injected with 1 mL of saline solution.

All groups were killed 24, 48, or 168 hours after treatment. Muscles were immediately frozen in liquid nitrogen at -70°C . Blood was collected and centrifuged at 1000g for 30 minutes at 4°C . The plasma obtained was divided into 2 vials: one was stored at -70°C for enzyme-linked immunosorbent assay (ELISA) anal-

ysis, and the other underwent a second cycle of centrifugation (15,000g for 15 minutes) for TGF- β_1 expression measurement.²⁰

Transplant setting. Thirty-six rats divided into 6 groups (n = 6 each) received intramuscular injection in the gastrocnemius muscle of 1000 μ g of plasmid diluted in 1 mL of saline solution with chloramphenicol acetyl transferase (CAT); CAT and β -galactosidase (β -Gal); or vIL-10, TGF- β_1 , or vIL-10 and TGF- β_1 . CAT and β -Gal are nonfunctional reporter genes used as plasmid controls. One more control group was not treated with any vector. Cotransfection groups used separate intramuscular injections in contralateral muscles.

Eighteen hours before transplantation, donors were harvested as previously described.¹⁴⁻¹⁶ In brief, donors were anesthetized with intraperitoneal pentobarbital of 0.3 mL (30 mg/kg), intubated with a 14-gauge catheter by means of tracheotomy, and mechanically ventilated (model 683; Harvard Apparatus Co, South Natick, Mass) with room air. Lungs were flushed with 20 mL of cold low potassium dextran 1% glucose solution through the main pulmonary artery. The heart-lung block was removed, and the pulmonary artery, pulmonary vein, and left main bronchus were prepared with polyethylene cuffs and preserved at 4°C for 18 hours.

Forty-eight hours after intramuscular treatment, the recipients underwent transplantation. After anesthesia with subcutaneous injection of ketamine (25 mg/kg), atropine (30 mg/kg), and halothane inhalation (0.5%-1%) from orotracheal intubation, a left thoracotomy was performed. The left pulmonary vessels and main bronchus were anastomosed by the previously described cuff technique.²¹ Ventilation and perfusion were restored, and a temporary drainage chest tube was placed, which was subsequently removed in the incubator (Intensive Care System; Thermocare Inc, Incline Village, Nev) after recovery from anesthesia.

Twenty-four hours after transplantation, the recipients were killed. Animals were reanesthetized by means of intraperitoneal pentobarbital injection and their lungs were ventilated with 100% oxygen. Median laparosternotomy was performed, and the right hilum was clamped. The animals' lungs were ventilated for 5 minutes by means of a tidal volume of 1.5 mL, peak end-expiratory pressure of 1 cm H₂O, and rate of 100 breaths/min. Blood samples were then collected from the ascending aorta. The left lung was frozen in liquid nitrogen and stored in 3 separate sections: the upper section was used for ELISA; the middle section was used for myeloperoxidase (MPO) assay; and the lower section was weighed, dried at 70°C for 48 hours, and then reweighed for calculation of the wet/dry ratio (W/D). A portion of the native right lung of the donor was taken for comparison.

Six additional recipient rats were intramuscularly cotransfected with vIL-10 plus TGF- β_1 (n = 3) or not transfected (n = 3) and underwent the same transplant procedure as above for immunohistochemistry assessment. Their left lungs were flushed through the main pulmonary artery with saline solution at a pressure of 20 cm H₂O and then fixed with Histochoice (AMRESCO, Solon, Ohio). No functional or efficacy data were obtained from these animals.

Assessment

MPO. Quantitative lung homogenate MPO activity as a measure of neutrophil sequestration was determined as previously described,²² with some modification.¹⁴ Optical density was mea-

sured at 460 nm with a spectrophotometer (Genesis 5; Spectronic Instruments, Inc, Rochester, NY). For each sample, the 5-minute reading was subtracted from the 20-minute reading and standardized to the total protein present in that sample (BCA Protein Assay Kit; Pierce, Rockford, Ill). MPO enzyme activity is expressed in units on the basis of the amount of protein required to effect a Δ change of 1 optical density unit per minute at room temperature.

ELISA. All muscle and lung tissue studied was homogenized in lysis solution containing 100 mmol/L potassium phosphate (pH 7.8), 0.2% Triton X-100 with pepstatin A (5 μ g/mL; Roche Molecular Biochemicals, Mannheim, Germany), and protease inhibitor cocktail (Complete mini, Roche Molecular Biochemicals). The homogenate was incubated for 15 minutes at room temperature and then centrifuged at 15,000 rpm for 15 minutes. The supernatant was collected and analyzed for proinflammatory cytokines.¹⁸

TGF- β_1 protein activity in muscle and plasma was detected by means of ELISA according to the protocol of R&D Systems (Minneapolis, Minn) for human TGF- β_1 , which is also cross-reactive to rat and mouse. The procedure to activate the latent form of TGF- β_1 in the ELISA kit was not used for the detection of active TGF- β_1 .

The presence of vIL-10 was detected according to the BD Pharmingen (San Diego, Calif) cytokine and chemokine ELISA protocol, with few modifications. In brief, the purified rat monoclonal antibody for vIL-10 (BD Pharmingen) was diluted to 2 μ g/mL in a ligand solution (0.1 mol/L Na₂HPO₄ at pH 9.0). After overnight incubation at 4°C in a 96-well plate (MaxiSorp Surface; Nalge Nunc Int, Rochester, NY), the wells were incubated with blocking buffer (1% bovine serum in phosphate-buffered saline solution [PBS]) for 2 hours to prevent nonspecific binding. After washing with PBS-Tween, all recombinant vIL-10 standards (BD Pharmingen) and the samples were added for 3 hours. After washing with PBS-Tween-20, the wells were incubated with biotinylated anti-vIL-10 antibody (BD Pharmingen) and streptavidin-horseradish peroxidase. The colorimetric reaction was activated by the substrate solution (2,2'-azino-bis-3-etenbenzotiazolin-6-sulfonic acid) and read after 40 minutes (optical density, 405 nm).

The quantitative expression of proinflammatory cytokines (IL-1 β , IL-2, TNF- α , and IFN- γ) was performed according to the protocols of R&D Systems kits.

All samples were standardized to the total protein with the BCA kit (Pierce).

Immunohistochemistry. Immunohistochemistry, as previously described,²³ was performed according to the protocol of the Tyramide Signal Amplification kit (NEN Life Science Products, Boston, Mass). In brief, slides were incubated for 4 hours at room temperature with biotinylated rat anti-mouse/anti-human TGF- β_1 antibody (BD Pharmingen) at 1:40 dilution in TNB buffer (Blocking Reagent in 100 mmol/L Tris and 500 mmol/L NaCl, pH 7.4) and 0.1% saponin. Slides were washed in TBS (100 mmol/L Tris and 500 mmol/L NaCl/0.2% Triton X, pH 7.4) and 0.1% saponin. Slides were then incubated with streptavidin-horseradish peroxidase for 30 minutes, followed by biotinyl tyramide for 10 minutes. Finally, slides were incubated for 30 minutes with streptavidin-alkaline phosphatase. Chromogenic detection for TGF- β_1 was performed with Vector Blue Alkaline Phosphatase Substrate (Vector Laboratories, Burlingame, Calif) in 100 mmol/L Tris, pH 8.2, including 5 mmol/L levamisole and 50 μ L of Tween-20. For

Figure 1. vIL-10 expression in muscle (A) and in plasma (B) and TGF- β_1 expression in muscle (C) and in plasma (D). Muscle and plasma were collected at 24, 48, and 168 hours after intramuscular injection and were assayed by means of ELISA.

subsequent double staining to detect vIL-10, slides were washed with deionized water. The blocking step in Super Blocking Buffer was performed. Slides were incubated overnight at room temperature with biotinylated rat antiviral IL-10 antibody (BD Pharmingen) diluted 1:20 in TNB Buffer/0.1% saponin. The detection method was performed as stated above, except that streptavidin-horseradish peroxidase was substituted for streptavidin-alkaline phosphatase in the final step. Slides were developed for 5 minutes with Vector NovaRed Substrate (Vector Laboratories) and transferred to TE buffer (tris-ethylenediamine tetraacetic acid) at pH 8.0 to stop the reaction. Slides were counterstained with methyl green nuclear (Vector Laboratories) for 5 minutes and permanently mounted with Cytoseal 60 (Stephens Scientific, Kalamazoo, Mich).

The pattern of protein expression and histology using hematoxylin and eosin staining was assessed by a blinded observer (J.H.R.).

Statistics

Parametric data were analyzed by means of 1-way analysis of variance and the Fisher post hoc multiple comparison test. Values

are reported as means $\pm$ SD. Data not normally distributed were converted to a square root transformation before the analysis of variance was performed.

Results

Nontransplant Setting

vIL-10. A significant increase was detected in muscle and plasma after intramuscular administration of vIL-10 and vIL-10 plus TGF- β_1 at 48 hours after administration compared with at 24 or 168 hours after administration ($P = .002$, Fig 1, A; $P = .008$, Fig 1, B). On the basis of these results, 48 hours before transplant was chosen as the time point for intramuscular injection of vIL-10 naked plasmid in the I/R injury model.

TGF- β_1 . Muscle expression (Fig 1, C) after 24 and 48 hours was significantly increased in the cotransfected groups compared with that seen in control animals ($P < .0001$) or that seen in groups transfected with TGF- β_1 alone ($P = .0086$ to 24 hours and $P = .0027$ to 48 hours).

TABLE 1. Isolated left lung assessment

Treatment in the transplanted rodents (n = 6)	Isolated left lung graft assessments		
	Pao ₂ (mm Hg)	W/D ratio	MPO ($\Delta OD/min/mg$ protein)
18 hrs, no treatment	105.6 $\pm$ 75.4	8.7 $\pm$ 2.2	0.414 $\pm$ 0.245
CAT	76.01 $\pm$ 48.4	8.1 $\pm$ 1.9	0.435 $\pm$ 0.346
CAT + β -Gal	64.7 $\pm$ 43.5	8.6 $\pm$ 1.7	0.463 $\pm$ 0.468
TGF- β_1	127.03 $\pm$ 45.2	5.9 $\pm$ 0.6*	0.223 $\pm$ 0.111
IL-10	185.6 $\pm$ 93.3*	6.1 $\pm$ 1.1*	0.135 $\pm$ 0.05*
TGF- β_1 + IL-10	311.6 $\pm$ 137.2*	5.6 $\pm$ 0.5*	0.068 $\pm$ 0.045*

All values are compared with those of a normal, nontransplanted lung (5.54 $\pm$ 0.66). All data are expressed as means $\pm$ SD (* P < .05). $\Delta OD/min/mg$ protein, Interval change in optical density units per minute per milligram of tissue protein; 18 hr, no transfection, lung underwent 18 hours of cold ischemia, followed by transplantation into a nontreated recipient.

TABLE 2. Cytokine production in transplanted lung grafts

Treatment in the transplanted rodents (n = 6)	Cytokine production (pg/100 μ g total protein)			
	IL-1 β	TNF- α	IFN- γ	IL-2
Normal lungs	7.5 $\pm$ 2.2	ND	ND	2.21 $\pm$ 0.41
1 hr, no transfection	22.5 $\pm$ 4.9	0.33 $\pm$ 0.28	0.64 $\pm$ 0.13	3.32 $\pm$ 1.76
18 hrs, no transfection	24.02 $\pm$ 18.5	0.77 $\pm$ 0.45	0.69 $\pm$ 0.06	4.76 $\pm$ 5.52
CAT	21.9 $\pm$ 9.7	0.81 $\pm$ 0.36	1.25 $\pm$ 0.4	4.59 $\pm$ 3.42
CAT + β -Gal	24.3 $\pm$ 10.01	0.82 $\pm$ 0.35	0.77 $\pm$ 0.35	5.36 $\pm$ 5.29
TGF- β_1	24.3 $\pm$ 8.9	0.48 $\pm$ 0.13*	0.59 $\pm$ 0.27	0.37 $\pm$ 0.32*
vIL-10	19.5 $\pm$ 8.8	0.4 $\pm$ 0.15*	0.34 $\pm$ 0.13*	0.35 $\pm$ 0.16*
TGF- β_1 + IL-10	9.2 $\pm$ 5.3*	0.076 $\pm$ 0.12*	0.02 $\pm$ 0.03*	0.25 $\pm$ 0.3*

All data are expressed as means $\pm$ SD (* P < .05). 1 or 18 hrs, no transfection, Lung underwent 1 or 18 hours of cold ischemia, followed by transplantation into a nontreated recipient; ND, not detected.

Similarly, in plasma (Fig 1, D) significant differences were observed between cotransfected groups and control animals (24 and 48 hours, P < .0001) or with groups transfected with TGF- β_1 alone (24 and 48 hours, P < .0001). As with vIL-10, these results support selecting 48 hours before transplant as the optimum intramuscular injection time for TGF- β_1 in the I/R injury model. Interestingly, there were no differences in the TGF- β_1 levels between the 24- or 48-hour cotransfected groups in both muscle and plasma.

Transplant Setting

Pao₂. Lungs from the cotransfected group had superior Pao₂ levels (Table 1) compared with those of the nontreated control group (P = .0002), the CAT group (P < .0001), or the CAT plus β -Gal group (P < .0001) and those of groups treated with TGF- β_1 alone (P = .0005) or vIL-10 alone (P = .011). Cotransfection provides a synergistic benefit versus either cytokine alone in graft function as measured by Pao₂.

W/D ratio. The W/D ratio, which reflects lung edema (Table 1), was significantly lower among the TGF- β_1 , vIL-10, vIL-10 plus TGF- β_1 , and control groups (P = .03, P = .015, and P = .028, respectively). Furthermore, there was

no difference between the above study groups and normal flushed nontransplanted lungs (P = .8). This indicates that the flushing process does not affect the W/D ratio, and cotransfection provides a benefit in reducing lung edema. However, cotransfection was not superior to either cytokine alone in reducing the edema.

MPO. MPO activity, which reflects neutrophil sequestration (Table 1), was decreased significantly in the cotransfected group versus that seen in the no treatment, CAT, or CAT plus β -Gal groups (P = .0028, P = .0023, and P = .0017, respectively). Also, vIL-10 had significantly decreased MPO versus that seen in the no treatment, CAT, or CAT plus β -Gal groups (P = .03, P = .034, and P = .026, respectively). These results indicate that vIL-10 and vIL-10 plus TGF- β_1 reduce neutrophil sequestration but not TGF- β_1 alone.

Proinflammatory cytokines. For this analysis, we added 2 groups: flushed normal nontransplanted lungs and flushed transplanted lungs after 1 hour of ischemia at 4°C (1 hour, no transfection).

IL-1 β levels (Table 2 and Fig 2, A) in the cotransfected group were significantly lower compared with those in all

Figure 2. Proinflammatory cytokine expression in transplanted lungs: IL-1β (A), TNF-α (B), IFN-γ (C), and IL-2 (D). All samples were collected 24 hours after transplantation and were assayed by means of ELISA.

other control and study groups (1 hour, no transfection, $P = .0039$; 18 hours, no transfection, $P = .0063$; CAT, $P = .0073$; CAT plus β -Gal, $P = .0021$; TGF- β_1 , $P = .0021$; and vIL-10, $P = .022$). However, transfection with either cytokine alone did not significantly reduce the IL-1 β levels. Also, no difference was noted between cotransfected and normal nontransplanted lungs ($P = .688$).

TNF- α (Table 2 and Fig 2, B) was undetectable in normal nontransplanted lungs. vIL-10 significantly reduced TNF- α levels versus those in the 18 hours with no transfection, CAT, and CAT plus β -Gal groups ($P = .024$, $P = .012$, and $P = .011$, respectively), whereas TGF- β_1 significantly reduced TNF- α levels versus those seen in the CAT and CAT plus β -Gal groups ($P = .039$ and $P = .034$, respectively). Each cytokine was able to reduce the TNF- α expression, but cotransfection synergistically lowered the TNF- α expression compared with that seen in all control and study groups ($P < .0001$ for all groups).

IFN- γ (Table 2 and Fig 2, C) was also undetectable in normal nontransplanted lungs and was not significantly

different from levels in the cotransfected group ($P = .2$). Like IL-1 β , cotransfection leads to a normal cytokine balance with respect to IFN- γ . In addition, the cotransfected group had a significantly lower IFN- γ level compared with all control and study groups ($P < .0001$ for all groups). TGF- β_1 was only significantly decreased compared with that in the CAT group ($P < .0001$), and vIL-10 was significantly decreased when compared with the TGF- β_1 group ($P = .036$), as well as the flushed transplanted lungs, CAT, and CAT plus β -Gal control groups ($P = .008$, $P < .0001$, and $P < .0001$, respectively). This shows that IL-10 is effective in reducing IFN- γ , whereas TGF- β_1 is not. Yet the cotransfection results indicate that TGF- β_1 , by possibly augmenting IL-10 levels, allows for a greater reduction in IFN- γ levels.

Last, IL-2 expression (Table 2 and Fig 2, D) was very low in the 3 study groups of TGF- β_1 , vIL-10, and vIL-10 plus TGF- β_1 and was significantly reduced compared with that in the following control groups: 1 hour, no transfection; 18 hours, no transfection; CAT; and CAT + β -Gal, respec-

Figure 3. A, Hematoxylin and eosin staining for cotransfected treated lungs showing intact airways, no inflammation, no exudates, and mild edema. (Original magnification 100 $\times$.) B, Hematoxylin and eosin staining of nontreated lungs showing interstitial inflammation, few alveolar neutrophils, and fibrin exudates in alveolar ducts and spaces consistent with acute pneumonitis. (Original magnification 100 $\times$.)

tively ($P = .028$, $P = .0048$, $P = .0022$, and $P = .001$ for TGF- β_1 and vIL-10 alone; $P = .01$, $P = .001$, $P = .0007$, and $P = .0002$ for vIL-10 plus TGF- β_1). There does not appear to be any synergism exhibited with cotransfection with respect to IL-2 levels, yet either cytokine alone is extremely effective in reducing IL-2.

Histology. Hematoxylin and eosin staining in cotransfected lungs showed moderate edema and mild interstitial neutrophil infiltration when compared with that seen in normal lungs (Fig 3, A). Severe edema and moderate interstitial

infiltration of neutrophils with rare microabscesses and hyaline membranes appears in nontreated lungs (Fig 3, B).

Results of immunohistochemistry double staining for vIL-10 and TGF- β_1 was negative in normal and nontreated lungs (Fig 4, C and D). On the other hand, cotransfected lungs had a light diffuse staining of edema fluid for vIL-10 (Fig 4, B) and some localized areas of type II cells (Fig 4, B). Also, most of the type I and II alveolar epithelial cells were stained positive for TGF- β_1 (Fig 4, A, for both and Fig 4, B, for type I).

Figure 4. Positive double-staining immunohistochemistry of cotransfected lungs with TGF- β_1 positive staining, showing type I (arrow I, A and B) and type II (arrow II, A) pneumocytes in dark blue. Viral IL-10 positive staining (B) in localized edema fluid (**) in brick red and type II pneumocytes (arrow II). Superimposed positive staining for both cytokines represents edema fluid appearing purple (*) in A. The normal (C) and 18-hours ischemic nontreated lungs (D) were negative after double staining. (Original magnification 200 $\times$.)

Discussion

I/R injury involves a series of events with early and late phases. The early phase is neutrophil independent, whereas neutrophils mediate most of the injury in the late phase.⁵ During reperfusion of ischemic tissue, the classical complement pathway is activated, and the potent chemotactic factors C5a and C1q are released. These binding proteins attract macrophages. Macrophage migration is also partially mediated by activated TGF- β_1 and macrophage chemoattractant protein 1 released from injured cells.⁵ Macrophages then release the preformed cytokines TNF- α , IL-1, and IL-6, which increase the expression of neutrophil adhesion molecules, such as intercellular adhesion molecule 1, on endothelial cells.^{5,6} The chemokines IL-8 and macrophage inflammatory protein 1 also mediate neutrophil chemotaxis.

The late phase of I/R injury occurs several hours after this inflammation begins.²⁴ Normally, neutrophils are more concentrated within the pulmonary capillaries than in systemic blood.^{24,25} The inflammatory mediators, such as C5a, TNF- α , and IL-8, cause neutrophils to become less deformable and foster pulmonary capillary sequestration.²⁵ The sequestered neutrophils adhere to the capillary endothelium, migrate into the lung parenchyma and air spaces, and contribute to I/R injury by generating oxygen-derived free radicals, releasing proteases, and secreting arachidonic acid metabolites and other proinflammatory mediators.^{7,25}

In this study we evaluated the effects of IL-10 and TGF- β_1 and their anti-inflammatory role in lung transplant I/R injury. Cellular IL-10 is regularly produced during inflammatory processes by monocytes, macrophages, and T

lymphocytes.⁸ It inhibits the production of proinflammatory cytokines, such as IL-1, IL-2, IL-6, IL-12, and TNF- α , and inhibits natural killer cell production of IFN- γ . Conversely, IL-10 might have an important role in the differentiation of mastocytes.^{8,26} Human IL-10, similar to cellular IL-10, has anti-inflammatory effects.^{26,27} Viral IL-10 is structurally similar to cellular IL-10 in the sequence encoding the mature protein. It also inhibits the activity of the cytokines and downregulates the expression of major histocompatibility complex II on the surface of monocytes. Because vIL-10 does not possess the costimulatory effect of cellular IL-10 on mastocytes, it is an effective immunosuppressant agent.^{12,26,28,29}

Similar to IL-10, TGF- β_1 plays an important role in the modulation of the inflammatory process and in several other processes, such as embryonic development, tumorigenesis, wound healing, and fibrosis.³⁰ Its immunosuppressive activity is related to antagonizing inflammatory cytokines, such as IL-1, TNF- α , and IFN- γ .^{12,29} Gene transfer strategies of IL-10 and TGF- β_1 are important because of their short half-life when administered in vivo. Human studies have demonstrated a half-life of approximately 2 to 5 hours when IL-10 is given intravenously or subcutaneously.³¹ Animal studies show a half-life of 100 minutes for the latent form of TGF- β_1 compared with 2 to 3 minutes for the active form.¹⁹ Conversely, a single application of IL-10 or TGF- β_1 gene transfer might provide significant protein expression to reduce I/R injury. Furthermore, unlike other types of injury, gene transfer in experimental and clinical lung transplantation can provide cytokine expression before the onset of I/R injury.

The naked plasmid vector was chosen for gene delivery in our model for several reasons. We previously reported that muscle expression of gene transfer products using adenoviral vectors might enter the bloodstream and have beneficial effects in acute lung rejection.³² The viral vector has a higher capability to transfect cells compared with that of naked plasmid but might affect the pretransplant and post-transplant recovery secondary to the possible inflammatory side effects and the potential immunologic response of the recipient to the vector. Despite the shortcomings of lower expression, lower transfection efficiency, and high concentration necessary to have a beneficial response, the advantages of using plasmids are (1) no systemic toxicity and consequently no side effects,³³⁻³⁵ (2) the possibility of enhancing gene transfer efficiency and transgene expression with several injections,²⁹ (3) relatively lower cost, and (4) easy applicability in the clinical setting by means of simple intramuscular administration. We have shown (unpublished data), using intramuscular injection of CAT naked plasmid, that the gastrocnemius muscle was markedly transfected compared with the heart, lung, kidney, spleen, or liver. This supports that naked plasmid use produces no systemic tox-

icity or side effects. Also, we found elevated CAT protein expression in muscle for 7 days. This supports the advantage of a single intramuscular injection of naked plasmid over intravenous or pharmacologic delivery of cytokines.

In the expression phase of the study, the data show that at least 48 hours after injection are required for both anti-inflammatory cytokines to be sufficiently present in the rodent system. We also found a marked increase in TGF- β_1 expression in the cotransfected groups compared with in the groups treated with either cytokine alone. This suggests that vIL-10 augments TGF- β_1 muscle and plasma expression. We also found that TGF- β_1 expression was significantly increased after both 24 and 48 hours after injection. This implies that one could perform intramuscular injection of TGF- β_1 and vIL-10 24 hours before transplantation and possibly obtain similar results with respect to reducing I/R injury as those found when injections are performed 48 hours before transplantation. Also, this suggests that cotransfection allows for earlier recipient plasmid administration because the delivery of 2 agents will augment and expedite each other's expression compared with the delivery of a single agent.

In contrast, IL-10 expression 24 hours after injection was not significantly increased compared with that 48 hours after injection. The main factor that might decrease exogenous IL-10 expression 24 hours after injection is the low transfection efficiency in muscle fibers. Another factor is related to the short systemic half-life of IL-10. In contrast, TGF- β_1 has a very short life,³² but its plasma levels correlate with muscle levels, leading us to believe that what is being produced in muscle is remaining intact in plasma. This could be due to the large volume of plasmid used that creates damage to the muscle fibers and allows for greater transfection efficiency.^{27,36}

Immunohistochemistry results show type I and II pneumocytes are seen in transplanted lungs stained for TGF- β_1 after only intramuscular injection of plasmid. Bellocq and colleagues³⁷ found that reactive oxygen intermediates might increase TGF- β_1 release from human epithelial alveolar cells in vitro. Therefore, the reperfusion period after transplantation might provide the stimulus for such oxygen intermediates to activate alveolar cells to release and stain for TGF- β_1 in our model. One hypothesis to explain this finding is that TGF- β_1 released systemically from muscle fibers might be taken up by type I or II pneumocytes. Another possibility is that inflammatory cells, such as macrophages, infiltrate the lung graft to produce either endogenous TGF- β_1 or to stimulate alveolar cells to produce TGF- β_1 .²⁷ Wolff and colleagues³⁴ and Davis and coworkers³³ have demonstrated the efficacy of the naked plasmid for direct gene transfer into rodent skeletal muscle. However, the mechanisms for how plasmids enter muscle fibers³⁸ or how

a cytokine might augment the activity of another cytokine remain unsolved.^{35,36}

Attenuation of lung I/R injury was noted after the intramuscular cotransfection treatment of these 2 potent cytokines. When the Pao₂, MPO, and inflammatory cytokine results are examined, a synergistic benefit is seen with cotransfection. This probably reflects the fact that IL-10 and TGF- β_1 work at different points in the inflammatory pathway.^{12,29} MPO was significantly reduced in the cotransfected and vIL-10 groups compared with the reduction in the control groups. Yet the reduction in MPO is more marked with cotransfection compared with use of vIL-10 alone. This leads one to believe that TGF- β_1 augments IL-10 expression, leading to increased IL-10 levels and accounting for the observed difference in cotransfected animals. This also implies that TGF- β_1 indirectly reduces MPO activity in the cotransfection group through its effect on IL-10 expression. Arterial oxygenation is also superior with cotransfection versus use of either cytokine alone. With IL-1 β , either cytokine alone was unable to significantly reduce its expression, but cotransfection was successful in returning the level to that of normal control lungs. Similarly for IFN- γ , vIL-10 and not TGF- β_1 reduced its levels, but cotransfection markedly reduced expression compare with that seen in normal lungs. This also supports the idea that TGF- β_1 augments IL-10 expression, accounting for the greater reduction in IFN- γ levels in cotransfected animals. The IL-2 level was significantly reduced with IL-10, TGF- β_1 , and cotransfection. Interestingly, IL-2 expression was higher in normal flushed lungs compared with in the 3 study groups. This means that either the flushing process increased IL-2 or that IL-10, TGF- β_1 , and cotransfection all effectively reduced IL-2 levels to below baseline.

In conclusion, recipient intramuscular gene transfer of IL-10 and TGF- β_1 attenuates I/R injury. The plasmid-mediated gene cotransfection achieved not only gene expression but also beneficial immunologic effects by providing a synergistic effect that significantly improved lung function after prolonged cold ischemia. Multiple gene transfer of anti-inflammatory cytokines might not only reduce I/R injury but also might have an effect on preventing acute and chronic rejection in the transplant setting. These possibilities will be examined in future studies.

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Discussion

Dr W. Roy Smythe (Houston, Tex). Dr Patterson and his colleagues should be congratulated for continuing to push molecular techniques into the area of lung transplantation.

I have a couple of questions. First, for clinical applications, how are you going to apply this to patients who only know 6 to 8 hours before transplant that they are going to get a graft? Second, have you considered putting IL-10 or TGF- β into an adeno-associated virus, so that you could actually dose an experimental animal or an individual weeks or months ahead of time and still have the salutary effect when a donor organ was identified? Third, following up on that question, if a longer-term expression is desired or possible, are there any toxic effects of long-term overexpression?

Dr Daddi. Thank you Dr Smythe for your comments. In response to the first question, we agree that 48 hours before transplant for vector administration is not entirely useful for clinical application at this time. Two immediate uses for this approach are in living lobar lung transplantation and acute rejection. We are testing in our laboratory the effect of adenoviral vectors encoding TGF- β and human IL-10 in the acute rejection setting. Preliminary data confirm the anti-inflammatory effect on the transplanted graft. Ideally, this will lead us to further investigations on the molecular mechanisms involved in lung transplantation.

For the second question, we have not used or considered using adeno-associated virus for long-term use in our model. It is a very good idea worth examining further.

For the third question regarding long-term problems with overexpression of these cytokines, we have not detected any to date. We confirm that compared with the first-generation adenoviral vectors, naked plasmid elicits lower plasma expression, yet in comparison, it is nontoxic to the recipient. We also reasonably think that because of the nontoxicity of this vector, it should be applicable in clinical trials involving organ transplantation. Giving several injections of naked plasmid alone or in combination with adenoviral vector administration might be a useful therapeutic instrument. Using the plasmids 1 to 2 days or perhaps 5 days after transplantation might benefit the lung graft, and we are studying some protocols about performing this. On the other hand, you asked me whether there are any side effects of the naked plasmid on animals. We saw that there was no side effect of the naked plasmid in the postoperative period in the study groups, and we noted no postoperative complications. No preoperative or postoperative mortality occurred. Morbidity was related to the control groups. Unfortunately, our I/R injury model does not give us the possibility to investigate the long-term expression. Data from similar expression work that we conducted on day 7 with intramuscular injection of naked plasmid encoding CAT shows systemically a very mild level of protein expression and no side effects. Your comment is an interesting one and should be investigated further.