CARDIOTHORACIC TRANSPLANTATION

EX VIVO TRANSFECTION OF TRANSFORMING GROWTH FACTOR- β_1 GENE TO PULMONARY ARTERY SEGMENTS IN LUNG GRAFTS

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Objective: Proximal pulmonary artery segment transfection may provide beneficial downstream effects on the whole-lung graft. In this study, transforming growth factor- β_1 was transfected to proximal pulmonary artery segments, and the efficacy of transforming growth factor- β_1 transfection was examined in ischemia-reperfusion injury and acute rejection models of rat lung transplantation. **Methods:** In the ischemia-reperfusion injury model, orthotopic left lung transplantation was performed in F344 rats. In group I, the PPAS was isolated and injected with saline solution. In 2 other groups, lipid67:DOPE:sense (group II) or antisense transforming growth factor- β_1 pDNA construct (group III) was injected instead of saline solution. After cold preservation at 4°C for 18 hours, lung grafts were implanted. Graft function was assessed 24 hours later. In the acute rejection model, donor lung grafts were harvested. Proximal pulmonary artery segments were injected with saline solution (group I) or sense (group II) or antisense lipid gene construct (group III) and then implanted. Graft function was assessed on postoperative day 5. **Results:** In the ischemia-reperfusion injury study, there were no significant differences in oxygenation, wet-to-dry weight ratios, graft myeloperoxidase activity, or transforming growth factor- β_1 levels in platelet-poor serum or proximal pulmonary artery segment homogenates. In the acute rejection study, oxygenation was significantly improved in group II receiving transforming growth factor- β_1 (group II vs I and III, 136.0 ± 32.5 vs 54.0 ± 9.6 mm Hg and 53.8 ± 14.8 mm Hg; $P = .016$ and $.016$). There were no significant pathologic differences. Transforming growth factor- β_1 concentrations from proximal pulmonary artery segment homogenates in group II were significantly higher compared with controls. **Conclusions:** Ex vivo transfection of transforming growth factor- β_1 to proximal pulmonary artery segments did not affect reperfusion injury of lung iso-grafts. In acute rejection, however, ex vivo transfection of transforming growth factor- β_1 to proximal pulmonary artery segments improved allograft function. This suggests that transfection to proximal pulmonary artery segments exerts beneficial downstream effects on the whole-lung allograft. (J Thorac Cardiovasc Surg 1999; 117:705-13)

Ischemia-reperfusion injury and rejection remain major obstacles to successful transplantation.¹ Gene transfer to the lung graft has the potential to reduce

these major problems. Transfection of proximal pulmonary artery segments (PPAS) and subsequent gene expression offer certain advantages over more wide-

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spread gene delivery strategies. First, it is possible to confine the cationic lipid:plasmid DNA complexes to the isolated arterial segments and not expose other organs to a gene product unnecessarily. In addition, in cases of transfection with a therapeutic transgene, down-stream effects of the secreted protein may be beneficial to the whole graft. Finally, compared with whole-lung transfection, the amount of transfection complexes needed is definitely reduced. Transgene expression efficiency might be maximized by the use of a high vector to endothelial cell ratio, which is difficult to use in whole-lung transfection because of toxicity and ideal storage times.

Transforming growth factor- β_1 (TGF- β_1) is a ubiquitous molecule that exerts immunosuppressive effects on various target cells.² Prolongation of graft survival was demonstrated in rat islet xenografts.³ Transgenic TGF- β_1 prolonged cardiac allograft survival in mice.⁴ TGF- β_1 also affects ischemic protection.⁵ TGF- β_1 reduced the myocardial infarct size in rats and the amount of superoxide anions and maintained endothelial-dependent coronary relaxation.⁶ Similarly, exogenous TGF- β_1 exerted myocardial protection in felines by inhibiting circulating neutrophils from adhering to the endothelium.⁷

We hypothesized that TGF- β_1 gene transfection might have a beneficial downstream effect and limit ischemia-reperfusion injury and acute rejection of rat lung grafts.

Materials and methods

Cationic lipid:pDNA complexes. The plasmid pMP6A sense and antisense TGF- β_1 were provided by Dr Jonathan Bromberg (University of Michigan, Ann Arbor, Mich) and amplified by Genzyme Corporation (Genzyme Corporation, Framingham, Mass). The pMP6A sense TGF- β_1 encodes for the mouse TGF- β_1 driven by the cytomegalovirus promoter. Lipid 67 (GL-67; Genzyme Corporation) was provided by Genzyme Corporation and used in a 1:2 molar ratio with the neutral co-lipid, 1-dioleoyl phosphatidyl-ethanolamine (DOPE). Before use, dried lipid films were hydrated with sterile water, treated in a vortex, placed on ice for 10 minutes, and then placed in a vortex again. Equal volumes of GL67:DOPE and plasmid DNA (sense or antisense TGF- β_1) were mixed and incubated at room temperature for 30 minutes. Final concentrations were 1 mmol/L cationic lipid and 4 mmol/L plasmid DNA.

Animals. Inbred male F344 rats (Harlan Sprague-Dawley, Inc, Indianapolis, Ind) were used in the ischemia-reperfusion injury study and Brown Norway and F344 rats (Harlan Sprague-Dawley, Inc) in the acute rejection study. All animals weighed between 250 and 290 g. All animal procedures were approved by the Animal Studies Committee at Washington University. Animals received humane care in compliance

with "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).

Ex vivo gene transfer to pulmonary artery segments. Ex vivo transfection of pulmonary artery segments was performed as previously described.⁸ After 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. Heart-lung blocks were extracted, and the left pulmonary artery was isolated from the hilum to the pulmonary trunk. Saline solution or lipid:gene constructs (0.03 mL) were injected into the isolated left pulmonary artery segments with a 24-gauge polyethylene catheter. Lung grafts were then stored in low-potassium dextran-1% glucose solution at 4°C for 1 hour, then the clamps isolating the PPAS were removed from the pulmonary artery, and the lung grafts were returned to the storage solution.

In the ischemia-reperfusion injury study, donor left-lung grafts were implanted after 18 hours of cold preservation at 4°C. In the acute rejection study, lung grafts were implanted 1 hour after administration of the lipid:gene construct. Orthotopic left lung transplantation was performed by means of a modification of the previously described "cuff technique."⁹

Experimental design and groups

Ischemia-reperfusion injury study. Animals were divided into 3 groups: saline solution control (group I; n = 6), sense (group II; n = 6), and antisense (group III; n = 6) TGF- β_1 transfection groups. All animals were reanesthetized 24 hours after reperfusion. The isolated function of the left lung iso-graft was assessed by arterial blood gas analysis during mechanical ventilation with 100% oxygen (tidal volume, 1.5 mL; respiratory rate, 100/min; positive end-expiratory pressure, 1.0 cm H₂O). Blood samples were obtained from the ascending aorta for arterial blood gas analysis and quantitation of TGF- β_1 in platelet-poor serum with an enzyme-linked immunosorbent assay (ELISA).¹⁰ After the animals were killed, the lung graft was flushed with cold saline solution and removed from the thoracic cavity. This was then cut into 3 equal pieces perpendicular to the longitudinal axis. The middle piece was frozen immediately in liquid nitrogen for myeloperoxidase assay. The lower piece was weighed for immediate determination of wet-to-dry weight ratio (W/D ratio) and again after drying at 80°C for 48 hours. The remaining upper piece was discarded. PPAS was put into 0.4 mL of cold acid-ethanol solution (93% ethanol, 2% concentrated hydrochloric acid [0.24 mol/L], 85 µg/mL phenyl-methylsulfonyl fluoride, 5 µg/mL pepstatin A) and immediately homogenized for 1 to 2 minutes on ice with a tissue tearer (Model 985-370 type II; Biospec Products Inc, Racine, Wis).¹⁰

Acute rejection study. Three groups were created in the same manner as in the ischemia-reperfusion injury study: saline solution control (group I; n = 6), sense (group II; n = 6), and antisense (group III; n = 6) TGF- β_1 transfection

groups. Before the animals were killed on postoperative day 5, blood samples were obtained for arterial blood gas analysis and quantitation of TGF- β_1 in platelet-poor serum. The lung graft was flushed with cold saline solution and fixed with 10% neutral buffered formalin. Histopathologic specimens of lung graft were stained with hematoxylin and eosin. The lung grafts were examined by a blinded pathologist (J.M.R.) experienced in the grading of rat lung acute rejection. The grafts were graded separately for acute vascular rejection and airway inflammation with the revised working formulation as described elsewhere.¹¹ PPAS were placed into 0.4 mL of cold acid-ethanol solution and immediately homogenized for 1 to 2 minutes on ice with a tissue tearer.

Myeloperoxidase assay. Quantitative myeloperoxidase activity was determined as described previously.¹² Optical density (OD) was measured at 460 nm with a spectrophotometer (model PMQ II; Carl Zeiss, Oberkochen/Wuett, Germany). The color development between these time points had previously been determined to be linear for rat lung tissue. 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.

ELISA for TGF- β_1 . Blood samples (3 mL) were collected into ethylenediaminetetraacetic acid-containing tubes with phenylmethylsulfonyl fluoride (85 μ g/mL) and pepstatin A (5 μ g/mL).¹⁰ Serum was obtained by centrifugation at 3500g for 15 minutes and clarified by centrifugation at 10000g for 10 minutes. Then platelet-poor serum was stored at -80°C until ELISA assessment. The PPAS homogenate was stored at 4°C . After overnight extraction, the extract was clarified by centrifugation at 10000g for 10 minutes and stored at -80°C . The ELISA kit that is cross-active between human, mouse, and rat was provided by Genzyme Corporation and used for quantitation of TGF- β_1 levels. OD was measured at 450 nm with a microplate reader (MR 600; Dynatech Laboratories Inc, Alexandria, Va).

Statistical analysis. All values are presented as the mean $\pm$ standard error of the mean. In the pathologic rejection score, Kruskal-Wallis rank test was used to compare groups. In the other assessments, 1-way analysis of variance with pairwise comparison by the Fischer method was used after the Bartlett test.

Results

Ischemia-reperfusion injury study

Arterial blood gas analysis. Graft function was analyzed with the mean PaO_2 . The PaO_2 in group II was 115.8 ± 30.0 . There were no significant differences between the 3 groups (PaO_2 for groups I and III were 142.6 ± 62.2 and 139.9 ± 49.1 , respectively; $P = .704$ and $.733$).

W/D weight ratio. As an assessment of graft injury, W/D ratios were measured to evaluate graft edema formation. The W/D ratio in group II was 7.23 ± 0.58 . There were no significant differences between the 3

groups (W/D ratio for groups I and III were 6.24 ± 0.67 and 6.62 ± 0.49 , respectively; $p = .251$ and $.469$).

Myeloperoxidase assay. As another assessment of graft injury, myeloperoxidase activity was measured to evaluate the amount of neutrophils that attached to the endothelial surface or migrated to interstitial spaces across the endothelium and played an important role in ischemia-reperfusion injury (Fig 1, A). Myeloperoxidase activity in group II was 0.095 ± 0.01 . There were no significant differences between the 3 groups (myeloperoxidase activity for groups I and III were 0.085 ± 0.013 and 0.109 ± 0.015 , respectively; $P = .589$ and $.475$).

Acute rejection study

Arterial blood gas analysis. Allograft function was assessed on the basis of mean arterial oxygen tension values (Fig 1, B). The PaO_2 in group II was significantly higher than in control groups I and III (136.0 ± 32.5 versus 54.0 ± 9.6 mm Hg and 53.8 ± 14.8 mm Hg, for groups II, I, and III, respectively; $P = .016$ and $.016$).

Histologic findings. The acute rejection scores are shown in Fig 1, B. Grafts were graded separately for acute vascular rejection and airway inflammation. The cases that were difficult to classify were scored between 2 grades. Most grafts were classified 2 (mild acute rejection/airway inflammation) or 3 (moderate acute rejection/airway inflammation). Acute vascular rejection scores for groups II, I, and III were 2.42 ± 0.38 , 2.67 ± 0.61 , and 3.08 ± 0.30 , respectively; $P = .476$ and $.070$. Airway inflammation scores for groups II, I, and III were 2.17 ± 0.21 , 2.58 ± 0.30 , and 2.67 ± 0.21 , respectively; $P = .300$ and $.211$. There were no significant differences between the groups.

ELISA for TGF- β_1 . The quantitation of TGF- β_1 in platelet-poor serum and PPAS homogenate is shown in Fig 2. TGF- β_1 in platelet-poor serum in group II were 0.075 ± 0.029 ng/mL in the ischemia-reperfusion injury study and 0.183 ± 0.056 ng/mL in the acute rejection study. There were no significant differences in platelet-poor serum between the groups in either the ischemia-reperfusion injury study (TGF- β_1 in platelet-poor serum for groups I and III were 0.066 ± 0.032 ng/mL and 0.085 ± 0.026 ng/mL, respectively; $P = .826$ and $.807$) or the acute rejection study (TGF- β_1 in platelet-poor serum for groups I and III were 0.137 ± 0.057 ng/mL and 0.085 ± 0.004 ng/mL, respectively; $P = .494$ and $.154$; Fig 2, A). In the ischemia-reperfusion injury study, TGF- β_1 levels from group II PPAS homogenates were significantly higher than group III (1.00 ± 0.18 vs 0.42 ± 0.08 ng/pulmonary artery, respectively; $P = .005$; Fig 2, B). In the acute rejection study, TGF- β_1 levels from group II PPAS homogenates

Fig 1. Acute rejection study. **(A)** The Pao₂ in group II was significantly higher than groups I and III. **(B)** Rejection scores. There were no significant differences between groups.

were significantly higher than controls (group II vs I and III, 1.36 ± 0.23 ng/pulmonary artery vs 0.47 ± 0.05 and 0.40 ± 0.02 ng/pulmonary artery, respectively; $P = .003$ and $.001$; Fig 2, B).

Ex vivo PPAS transfection and ex vivo whole-left lung transfection. The comparison oxygenation data of the degree of gene transfection between ex vivo PPAS transfection and ex vivo whole-left lung transfection is shown in Fig 3. Ex vivo whole-left lung transfection was performed with 0.5 mL of the same lipid:gene construct and the same exposure and ischemic times. In the acute rejection model, oxygenation in whole-lung transfection was more improved than in PPAS transfection (234.2 ± 61.3 mm Hg vs 136.0 ± 32.5 mm Hg, respectively). However the improvement was not significant ($P = .187$, unpaired *t* test comparison). In the reperfusion injury model, lung function of PPAS transfection group was higher than in the whole-lung transfection group, although the differ-

ence was again not significant (152.1 ± 35.7 mm Hg vs 64.6 ± 13.5 mm Hg, respectively; $P = .1314$).

Discussion

Early lung graft dysfunction including ischemia-reperfusion injury is not unusual during the first few days after lung transplantation.¹³ Ischemia-reperfusion injury is an acute lung injury with increased vascular permeability. Pathophysiologic mechanisms of reperfusion injury are still unclear, but neutrophil migration from the vasculature to the interstitial spaces plays a major role in tissue injury.^{14,15}

Acute lung allograft rejection occurs to a variable degree in virtually all transplant recipients within the first month after transplantation. Although acute rejection is infrequently fatal, it has been repeatedly identified as the principal risk factor for chronic rejection.¹³ Acute rejection is orchestrated primarily by helper T lymphocytes that recognize donor major histocompati-

Fig 2. Quantitation of TGF- β_1 with ELISA. (A) TGF- β_1 in platelet-poor serum. There were no significant differences in platelet-poor serum between groups in either the ischemia-reperfusion injury study or the acute rejection study. (B) TGF- β_1 in homogenates of PPAS. TGF- β_1 levels in the PPAS homogenates from group II were significantly higher than in group III in the ischemia-reperfusion injury study. In the acute rejection study, TGF- β_1 levels in PPAS homogenates from group II were significantly higher than in the other groups.

bility complex and that secrete cytokines that stimulate the proliferation of cytotoxic T lymphocytes, which effect graft injury.¹⁶

Numerous investigations into the function of TGF- β_1 have been performed. TGF- β_1 is 1 of a number of multifunctional molecules that exert global effects on cell growth and differentiation, including embryonic development, tumorigenesis, wound healing, and fibrosis. In addition to these growth regulatory activities, TGF- β_1 has potent immunoregulatory effects. The immunosuppressive properties of TGF- β_1 include inhibition of thymocyte proliferation,¹⁷⁻¹⁹ T- and B-cell proliferation,²⁰⁻²² cytokine production,²³ natural killer cell activity,²⁴ cytotoxic T-lymphocyte development,²⁵⁻²⁷ and lymphokine-activated killer cell activity.²⁸ In the field of reperfusion injury, TGF- β_1 inhibits leukocyte adhesion to the endothelium and neutrophil migration

across the endothelium by diminishing E-selectin expression^{29,30} and decreasing IL-8 synthesis.³¹ These particular properties of TGF- β_1 were expected to be sufficiently potent to ameliorate 2 major obstacles in lung transplantation, namely ischemia-reperfusion injury and rejection. We examined the efficacy of TGF- β_1 transfection in rat ischemia-reperfusion injury and acute rejection models.

In the reperfusion injury study, we expected endothelial protection by TGF- β_1 . However, no such protection was noted after TGF- β_1 transfection. In the previous studies on optimization of cationic lipid-mediated gene transfer, reperfusion was an essential factor for ex vivo transfection under the usual storage conditions. Although transgene expression was apparent 3 hours after reperfusion, maximal transgene expression was obtained 24 hours after reperfusion (submitted for pub-

Fig 3. Comparison between ex vivo PPAS transfection and ex vivo whole-left lung transfection with PaO₂. In acute rejection, whole-lung transfection resulted in improved oxygenation compared with PPAS transfection, although this was not statistically significant. In ischemia-reperfusion injury, the PaO₂ after PPAS transfection was higher after whole-lung transfection although the difference was not significant.

lication). Ischemia-reperfusion injury begins immediately after reperfusion and is dramatically amplified during the first 60 minutes of reperfusion.^{32,33} Furthermore, a significant increase in myeloperoxidase activity, 1 of the parameters of neutrophil migration, has been observed 120 minutes after reperfusion in occluded rabbit lungs.³⁴ Ischemia-reperfusion injury may have already occurred before the expression of TGF- β_1 after TGF- β_1 transfection. TGF- β_1 transfection did not result in a significant increase in TGF- β_1 levels obtained from the sense TGF- β_1 transfection group compared with the saline solution control group in PPAS homogenates. The quantitation of TGF- β_1 in the PPAS homogenate in the reperfusion injury model is lower than in the acute rejection model. We speculate that the viability of the PPAS endothelium was decreased during 18 hours of cold preservation because transgene expression was maximized during 24 to 72 hours after reperfusion in the previous study and the integrity of the transfected PPAS endothelium was maintained after 18 hours cold preservation and 24 hours after transplantation (submitted for publication). Many factors including acute shear stress and cytokines (IL-1 β , TNF- α) induce endothelial cell TGF- β_1 synthesis. This may be also a possible reason for no differences in the quantity of TGF- β_1 between groups. A sig-

nificant increase in TGF- β_1 was not apparent in the platelet-poor serum in either ischemia-reperfusion injury or acute rejection studies. This may mean that PPAS transfection has an effect only in the lung, namely local immunosuppression. The half-life of the TGF- β_1 molecule in vivo is very short. Most TGF- β_1 molecules in serum are immediately combined with α_2 -macroglobulin, changed to a latent form, almost completely trapped by the liver, and scavenged.³⁵ This is the reason that it is difficult to maintain high concentrations of TGF- β_1 in the serum. Also this is the reason that local TGF- β_1 transfection is expected to be more effective than systemic TGF- β_1 administration.

In the acute rejection study, TGF- β_1 transfection was manifested by significant increases in TGF- β_1 levels in PPAS homogenates. Graft function, as assessed by arterial oxygenation, showed significant improvements after sense TGF- β_1 transfection. This improvement may support our hypothesis of beneficial downstream effects of PPAS transfection. As mentioned earlier in this article, the TGF- β_1 molecules are immediately combined with α_2 -macroglobulin and changed to a latent form. However, latent TGF- β_1 is activated on the surface of endothelial cells.³⁶ The presence of huge pulmonary microvascular endothelia distal to the PPAS is an advantage for activation of latent TGF- β_1 . In the present study, the improvement in graft function was not so great. Likewise, in the pathologic assessment, differences between the groups were not significant. There was no clear evidence of ischemic damage in the allografts, although this would be difficult to ascertain histologically in the presence of acute rejection.

The absence of pathologic improvements in the grading of acute rejection is likely multifactorial. First, it is difficult to accurately grade acute pulmonary rejection in the rat. The situation is not akin to the clinical condition, where acute rejection may be mild. Second, many of the grafts had acute rejection scores between 2 and 3, making it harder to observe a truly statistical benefit in such a narrow margin. Third, it was difficult to ascertain the exact number of transfected cells. Studies using in situ hybridization and immunohistochemical analysis are ongoing to determine the percentage of transfected cells. Thus potentially, a smaller number of cells was transfected than predicted, resulting in decreased effectiveness of gene expression.

The duration of the immunosuppressive effects of TGF- β_1 transfection is not absolutely clear. Acute rejection is a multifactorial process, with several overlapping and redundant pathways that, without treatment, lead to graft failure. Although TGF- β_1 has many diverse immunosuppressive actions, its ability to sup-

press acute rejection for the long-term is limited indeed. Further, even if TGF- β_1 gene therapy were able to completely abolish acute rejection, its effect would certainly be limited by the duration of transgene expression, which is finite. Thus gene therapy for the treatment of acute rejection likely represents a transient immunosuppressive phenomenon, potentially on the order of several days.

TGF- β_1 transfection may not be effective in the reperfusion injury model. The effect of TGF- β_1 on endothelial cell-derived vasoactive mediator release is complex, and it is not evident whether vasodilator or vasoconstrictors dominate.³² Likewise, although TGF- β_1 has some anti-ischemic effects, its main effects are immunosuppressive. The absence of a positive effect on ischemia-reperfusion lung injury therefore may be related to lack of a potent TGF- β_1 anti-ischemic effect. Alternatively, the percentage of PPAS transfected with TGF- β_1 may have been too low to effect a change on ischemia-reperfusion injury. In addition, the effectiveness of TGF- β_1 may have been quenched because it was released upstream from the tissue that needs to be affected, allowing the possibility that it may have been scavenged in the bloodstream before being able to exert an effect. Further, the 18-hour preservation time may have resulted in severe reperfusion injury to the lung that may not have been reversible or treatable. This would then negate any potential beneficial effects from TGF- β_1 therapy. Finally, lung models of reperfusion injury are poor and may not be analogous to the human situation.

In conclusion, ex vivo transfection of TGF- β_1 to PPAS does not affect ischemia-reperfusion injury of lung isografts after 18 hours of cold preservation. However, in the setting of acute rejection, ex vivo transfection of TGF- β_1 to PPAS improves graft oxygenation. This suggests that PPAS transfection provides beneficial downstream effects on the whole-lung graft.

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Discussion

Dr Robert C. Robbins (*Stanford, Calif*). You have developed an excellent concept for trying to improve the function of autograft tissue. The use of ex vivo gene therapy has the advantage that the allograft during the time of ischemia and transport can be modified with certain genetic techniques that

have been reported today. It allows the modification of the graft to occur with a more concentrated and effective method than the use of systemic modification. It also avoids the treatment of other organs. In selecting the target or the molecule in genetic therapy, one must decide whether the target should be blocked or increased because of the beneficial effects.

You have selected TGF-β because of its immunosuppressive effects and its effects on ischemia and reperfusion, and I think that is an excellent target.

You have selected a liposome as the vector for delivery of genetic material. There are many different vectors that can be used to deliver the DNA into the nucleus: either unmodified DNA in a plasmid form, the use of liposomes as we have seen reported here today, or the use of viral vectors. Each has its own potential set of problems; however, the use of liposomes seems to be an effective means for delivering DNA in this study.

For any gene therapy study, there are 3 concepts that remain important: the transfection efficiency, the efficacy of the transfection, and the observed biologic effect.

The transfection efficiency simply states how effective one is in getting the DNA construct localized into the nucleus. The efficacy deals with measurement of mRNA protein levels of the target to measure the effectiveness. And then finally, obviously, the biologic effect is the end clinical result that we see.

In the current study there were 3 groups; the control saline-treated group, the sense-treated group, which tried to achieve increased production of TGF-β, and the antisense treatment, which I would have thought would have blocked TGF-β. I think the results were particularly disappointing in that you saw no effect on reperfusion injury and even though you saw better oxygenation in the acute rejection model, pathologically there were no differences.

Have you looked at the transfection efficiency? Particularly, have you used epitope tag labeling of your vector and construct to see if you are getting the construct into the nucleus?

Dr Yano. Actually we did not look at the efficacy or efficiency of transfection in these studies.

Dr Robbins. It would be helpful in any future studies if you do that because we could learn a lot from finding out whether the problem with not seeing any difference in reperfusion injury was that you are not transfecting the cell. Another option is to look at the transfection efficacy by looking at mRNA levels. That also would give us some indication as to whether you selected a poor target or whether your method is not good.

You selected 18 hours for your ischemia-reperfusion study, and you pointed out in your paper that that might be a problem in trying to achieve good transfection efficiency and efficacy. Have you performed any studies that look at the clinically relevant 4- to 6-hour ischemic period. If you have, can you comment on that?

Dr Yano. We have not looked at shorter ischemic times in these studies.

Dr Robbins. You used oxygenation from the aorta as your end point for assessing graft function. There are other ways to look more specifically at the transplanted lung function, such as compliance studies, flow probes around the pulmonary artery, and looking at the difference in pulmonary artery and left atrial oxygenation. Have you any comment on that or have you done any studies that would tell us more specifically about the function of the lung?

Dr Yano. We have not investigated these other measures of lung function.

Dr Robbins. Finally, have you combined this with other immunosuppressants? We have tried blocking intercellular adhesion molecules with antisense oligonucleotides and have seen that alone it had no effect on acute rejection. Have you tried to combine this strategy with other immunosuppressants, and if you have done that whether you had seen any difference in acute rejection?

Dr Yano. We have not done this.

Dr Mora. As a coauthor of the paper, I wanted to clarify some of the points that Dr Robbins had raised. First, in terms of the TGF- β_1 antisense cDNA that we used in the study, this was simply the normal TGF- β_1 gene inserted in an inverted manner in the original plasmid. This is different from antisense oligonucleotides that are typically designed to block a particular gene from forming functional protein products. The antisense TGF- β_1 plasmid produced no functional TGF- β_1 protein; therefore it served as a control in these experiments.

In terms of the efficiency of gene transfection, we have not performed any epitope-tagging studies to determine how much of the cDNA construct entered the nucleus after injection

into the pulmonary artery. However, this is a great suggestion.

The second question concerned the efficacy of gene transfection. In a separate unrelated study, our laboratory has recently demonstrated increased whole-lung TGF- β_1 mRNA levels after intratracheal transfection of TGF- β_1 liposome-cDNA complexes, using reverse-transcriptase polymerase chain reaction. We have not performed that assay in this study, however.

The third question asked about shorter lung graft ischemic times of 4 to 6 hours, as opposed to the 18 hours used in these experiments. We have not studied shorter ischemic times extensively because our rodent model is not very sensitive in terms of arterial oxygenation measurements at short graft ischemic times. In the past, we had tried various ischemic times and had decided to use 18 hours of ischemia as a model of ischemia-reperfusion lung injury. I think that it would be good, however, to reproduce the experiments using shorter ischemic times to ascertain this.

With regard to the question whether other assays of lung function were used, such as compliance testing of the grafted lung, we have not attempted this because it would be quite technically challenging in the rodent.

The last question asked whether systemic immunosuppression was attempted in any of these experiments. We have not used standard immunosuppression in our experiments simply because there are no data that this is beneficial in the setting of transfection of active genes with liposomal complexes, as in our study.

I hope this clarifies some of the issues raised by Dr Robbins.